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Characterization of the functional domains of the herpes simplex virus (HSV-2) strain HG52 RL1 gene

by

SALWA BDOUR

A thesis presented for the Degree of Doctor of Philosophy

in

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Unless otherwise stated, all the results were obtained by the author's own efforts.

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Addendum

Chapter 5- Discussion

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SUMMARY

The long repeat region of HSV-1 contains the diploid RL1 gene which encodes the protein ICP34.5, a 43K polypeptide of 263 amino acids in HSV-1 strain F and a 37K polypeptide of 248 amino acids in HSV-1 strain 17^+ infected cells (Ackermann *et al.*, 1986; Chou and Roizman, 1986, 1990; McKay *et al.*, 1993). The role of RL1 in virulence of HSV-1 has been studied by Chou *et al* (1990), MacLean, A *et al* (1991) and McKie *et al* (1994). The discovery of the RL1 gene in HSV-1 was followed by sequencing data of the corresponding region of HSV-2 strain HG52 which indicated that HG52 possesses a counterpart of the HSV-1 RL1 gene (McGeoch *et al.*, 1991). The organization of HG52 RL1 differs from that of HSV-1 although both initiation codons are in equivalent positions. The coding sequence of HG52 RL1 lacks the $(PAT)_{10}$ repeat element and is disrupted by a reiterated sequence proposed to be an intron. Removal of this intron results in a second exon containing a 63-amino acids HSV-1/HSV-2 conserved region, being in frame. To characterise the functional domains of the HSV-2 RL1 gene, three mutant viruses have been constructed and characterized: (1) 2620, with a stop codon within the conserved region, 46 amino acids upstream of the 3' end of RL1 (2) 2621, with an in frame stop codon 9bp downstream of the initiating ATG and (3) 2622, in which the proposed intron has been apparently deleted. The mutant viruses 2620, 2621 and 2622 were characterized *in vivo* following intracerebral inoculation of Balb/c mice and *in vitro* using BHK21/C13 and 3T6 mouse embryo fibroblast cells. The mutant 2621 was avirulent with a $LD_{50} > 10^7$ p.f.u./mouse compared to 3.16×10^6 p.f.u./mouse for 2604 and 10^2 p.f.u./mouse for HG52. The 2620 mutant was also avirulent with a LD_{50} of 2.37×10^6 p.f.u./mouse. The 2622 mutant was intermediate with a LD_{50} of 10^5 p.f.u./mouse. The growth of the mutants 2620 and 2621 was not impaired in BHK21/C13 but was impaired in 3T6 cells. The growth of 2622 was comparable to that of the wild-type HG52 in BHK21/C13 cells and intermediate between HG52 and the avirulent deletion mutant 2604 in 3T6 cells.

Rescuants of the mutants (R2620, R2621, R2622) were generated by *in vitro* and *in vivo* marker rescue. Those generated by *in vitro* marker rescue demonstrated an intermediate

virulence phenotype. Rescuants, generated by *in vivo* marker rescue, except for R2620 were restored to the full virulence phenotype of the wild-type HG52 pl.17. A secondary mutation in the genome of 2620 and its rescuants generated by *in vitro* marker rescue could explain failure of the R2620 rescuants to return to wild-type virulence. The secondary mutation could be due to passage of the virus during purification and/or virulence heterogeneity within the HG52 pl.17 stock. Individual plaques of HG52 pl.17 demonstrated variable virulence phenotypes. The rescuants generated *in vitro* and individual plaques of HG52 pl.17 were characterised by growth in 3T6 cells which differentiates between ICP34.5 positive and negative virus. The 3T6 growth and plaque morphology strongly discount a possible mutation in RL1 which should be confirmed when an antiserum against ICP34.5 of HG52 becomes available. It seems that the growth phenotype of HG52 variants *in vitro* does not always mimic the *in vivo* phenotype thus emphasising the necessity of the animal model to determine virulence. The demonstrated heterogeneity of the HG52 pl.17 stock emphasises the necessity of *in vivo* marker rescue experiments to confirm the phenotype of constructed mutants.

Abbreviations

A	adenine
ACV	acyclovir
AMV	avian myeloblastosis virus
APS	ammonium persulphate
ATP	adenosine triphosphate
BHI	brain heart infusion
BHK21/C13	baby hamster kidney cells batch 21 clone 13
bp	base pairs
C	cytosine
^oC	degrees centigrade
CAT	chloramphenicol acetlytransferase
cDNA	complementary DNA
CHO	chinese hamster ovary
Ci	curie
CIP	calf intestinal alkaline phosphatase
c.p.e.	cytopathic effect
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
ddATP	2'3'-dideoxyadenosine-triphosphate
ddCTP	2'3'-dideoxycytidine-triphosphate
ddGTP	2'3'-dideoxyguanosine-triphosphate
ddTTP	2'3'-dideoxythymidine-triphosphate
dNTPs	2'-deoxynucleotide-5'-triphosphate
ddNTPs	2',3'-dideoxynucleotide-triphosphate
DECP	diethylpyrocarbonate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DRG	dorsal root ganglia
DTT	dithiothreitol
dut	dUTPase
dUTPase	deoxyuridine triphosphate nucleotidohydrolase
E.coli	*Eschericia coli*
EDTA	ethylenediaminetetra acetic acid

G	guanine
g	gravity
h	hour
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid
HSV-1	herpes simplex virus 1
HSV-2	herpes simplex virus 2
ICP	infected cell polypeptide
IE	immediate early
Ig	immunoglobulin
IPTG	isopropyl-β-D-thiogalactoside
IR_L	internal long repeat
IR_S	internal short repeat
k	kilo (ie. 10^3)
kbp	kilobase pairs
L	long segment
l	litre
LAT	latency associated transcript
MOPS	3-(N-morpholino) propane sulfonic acid
MuLV	moloney murine leukemia virus
mg	milligrams
μl	microlitre
μM	micromolar
μCi	microcurie
ml	millilitres
mM	millimolar
m.o.i.	multiplicity of infection
M_r	molecular weight
mRNA	messenger RNA
m.u.	map units
ng	nanogram
No.	number
np	nucleotide position
NP40	nonidet P40
OBP	origion-binding protein
OBPC	C terminus of OBP
OD	optical density
ORF(s)	open reading frame(s)
Ori_L	origin of replication in U_L
Ori_S	origin of replication in U_S

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
p.f.u.	plaque forming units
p.i.	post-infection
pl.	plaque
p.mole	picomole
R1	large subunit of ribonucleotide reductase
R2	small subunit of ribonucleotide reductase
RE	restriction enzyme
RNA	ribonucleic acid
RNaseA	ribonuclease A
RNasin	ribonuclease inhibitor
r.p.m.	revolutions per minute
RR	ribonucleotide reductase
RT	room temperature
RT	reverse transcriptase
S	short segment
SDS	sodium dodecyl sulphate
T	thymidine
TEMED	N, N, N', N', -tetramethlyethylene diamine
TIF	transinducing factor
Tris	tris (hydroxymethyl) aminomethane
TK	thymidine kinase
TR_L	long terminal repeat
TR_S	short terminal repeat
ts	temperature sensitive
U	unit
U_L	long unique
ung	uracil N-glycosylase
U_S	short unique
U.V.	ultraviolet
V	volts
Vmw	molecular weight in kilodaltons of HSV induced polypeptides
VP	virion protein
v/v	volume/volume (ratio)
W	watts
w/v	weight/volume (ratio)
w/w	weight/weight (ratio)

X-gal 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside ($C14H15BrClN)6$

ONE AND THREE LETTER ABBREVIATIONS FOR AMINO ACIDS

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

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CHAPTER 1

INTRODUCTION

CHAPTER 1: INTRODUCTION

Herpes simplex virus (HSV) is a member of the family Herpesviridae which includes many viruses of medical and veterinary importance. Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) which belong to the subfamily Alphaherpesvirinae are among eight human herpes viruses characterised to date. HSV is a neurotropic virus responsible for a broad spectrum of clinical diseases ranging from relatively benign recurrent cutaneous lesions to fatal encephalitis. The life cycle of HSV includes acute, latent and recurrent phases. In acute infection, the genes of HSV are expressed and the extent of disease is controlled by the cellular and humoral responses of the host. In latent infection, gene expression is repressed except for a limited region of the HSV genome encoding latency-associated transcripts (LATs). Latency in HSV represents a challenging problem in understanding how a virus which can kill most cell types remains mostly asymptomatically in humans for life without destroying the sensory neurons in which it resides. HSV can emerge intermittently from sensory neurons to cause a recurrent peripheral infection. The mechanism by which the productive pattern of gene expression in acutely-infected neurons switches to a latent state and how HSV reactivates remain unanswered questions in HSV biology.

In this chapter, HSV infections and the structure of HSV are discussed in sections 1.1-1.4. Sections 1.5 and 1.6 are devoted to the lytic and latent phases of HSV infection. Special attention is given to genes coding for proteins or glycoproteins involved in virulence of HSV [section 1.7]. The aims of the project are outlined in section 1.8.

1.1. Herpes simplex virus infections

Two herpes simplex virus (HSV) serotypes have been identified: HSV type 1 (HSV-1) and HSV type 2 (HSV-2). Differentiation of HSV-1 and HSV-2 has been based upon pock size studies on chorioallantoic membranes (Rodgers, 1973), neutralisation (Nahmias and Dowdle, 1968), monoclonal antibody-based assay and restriction enzyme analysis (Lonsdale *et al.*, 1979; Buchman *et al.*, 1981). Serological studies have

demonstrated that HSV-1 infections are generally acquired during childhood and by 60 years of age, up to 90% of the population have antibodies to HSV-1 (Nahmias *et al.*, 1970). HSV-2 infections are usually not acquired until puberty and are correlated with sexual activity. Recent studies (Johnson *et al.*, 1989) have shown that the seroprevalence of HSV-2 antibody rises with increasing age in the post-adolescent years, an epidemiological pattern typical of a sexually transmitted disease (Kinghorn, 1993).

HSV causes genital infections, gingivostomatitis, conjunctivitis, herpetic whitlow and keratitis. HSV ocular infections affect approximately 500 000 people in the U.S.A. each year (Liesengang, 1988; Binder, 1977). HSV encephalitis is the most commonly reported viral infection of the CNS, accounting for 10%-20% of all viral encephalitis in the U.S.A. (Wiedbrauk and Johnson, 1993). Patients compromised by either immune therapy, underlying disease or malnutrition are at increased risk from HSV recurrent infection (Whitley, 1990).

Historically, HSV-1 has been associated with oral infections, while HSV-2 has been associated with genital infections. This distinction is no longer valid as today, 30-50% of genital herpes infections are caused by HSV-1 (Kalinyak *et al.*, 1977; Smith *et al.*, 1977; Kinghorn, 1993) and 5-20% of oral infections are caused by HSV-2 (Wiedbrauk and Johnston, 1993). The increased incidence of genital herpes has caused an increase in the incidence of neonatal herpes (Sullivan-Bolyai *et al.*, 1983; Whitley *et al.*, 1991). Neonatal HSV infection remains life-threatening for the newborn in all countries. Genital HSV infection results in 1600 cases of neonatal herpes yearly in the U.S.A. (Whitley, 1990).

1.2. Virion structure

HSV virions consist of three morphological structures: an icosahedral nucleocapsid, an amorphous tegument surrounding the capsid and an outer envelope which exhibits glycoprotein spikes (reviewed by Dargan, 1986 and Rixon, 1993). The HSV capsid is approximately 125nm in diameter, exhibiting 5:3:2 axial symmetry and is composed of 162 capsomeres, of which 150 are hexameric (hexons) and 12 pentameric (pentons) and

320 triplexes that provide intercapsomeric connections (Wildy *et al.*, 1960; Schrage *et al.*, 1989; Zhou *et al.*, 1994). Three capsid forms have been observed and purified from wild type virus-infected cells: (i) empty capsids (type A) which lack viral DNA, (ii) intermediate capsids (type B) which lack viral DNA and possess 2 proteins not found in A capsids, VP21(UL26 C-terminus) and VP22a(UL26.5) which occupy the inner capsid space and are thought to act as scaffolding proteins that are released when DNA packaging occurs (Rixon *et al.*, 1988) and (iii) full capsids (type C) which contain viral genomes and are the immediate precursors of mature virions that undergo tegumentation and envelopment before being released from the cell (Gibson and Roizman, 1972; Atkinson *et al.*, 1978; Furlong *et al.*, 1972; Rixon, 1993). The capsid is composed of seven proteins: VP5 (UL19), VP19C (UL38), VP23 (UL18), and VP26 (UL35), VP22a (UL26.5), VP21 (UL26 C-terminus), VP24 (UL26 N-terminus), (Braun *et al.*, 1984a,b; Costa *et al.*, 1984; Newcomb and Brown, 1989, 1991; Rixon, 1993) and is surrounded by an amorphous layer, the tegument (Roizman and Furlong, 1974). The tegument contains proteins which function in very early and in late events in the replication cycle. Some of the viral proteins which are contained within the tegument include Vmw65(αTIF) (Batterson and Roizman, 1983; Campbell *et al.*, 1984), the virion host shutoff (*vhs*) protein (Fenwick, 1984; Smibert *et al.*, 1992), VP1/2 (UL36) (Chou and Roizman, 1989; McNabb and Courtney, 1992), VP13/14(UL47) (McGeoch *et al.*, 1993; McKnight *et al.*, 1987; Zhang *et al.*, 1991), VP22(UL49) (McGeoch *et al.*, 1993; Elliot and Meredith, 1992) and the UL37 product which is a γ1 phosphoprotein (Schmitz *et al.*, 1995). Vmw175 has been localised to the tegument of light particles (Yao and Courtney, 1989; McLauchlan and Rixon, 1992). Other proteins have been identified as components of the virus particle and are assumed to be located within the tegument. These include proteins encoded by UL11 (MacLean *et al.*, 1989; Baines and Roizman, 1992), UL13 (Rubenstein *et al.*, 1972; Lemaster and Roizman, 1980; Overton *et al.*, 1992); US9 (Frame *et al.*, 1986) and US11 (Roller and Roizman, 1992).

The tegument is completely enclosed in a trilaminar membrane envelope (Wildy *et al.*, 1960; Darlington and Moss, 1968) of mean diameter 180nm which has surface spikes 8-

10nm long spaced 5nm apart (Wildy *et al.*, 1960). Use of monoclonal antibodies coupled to immunogold labelling allowed identification of some viral glycoproteins (gB, gC and gD) projecting from the virion envelope (Stannard *et al.*, 1987). To date, 10 HSV-1 glycoproteins have been identified: gB(UL27), gC(UL44), gD(US6), gE(US8), gG(US4), gH(UL22), gI(US7), gK(UL53), gL(UL1) and gM(UL10) (Spear, 1976; Frame *et al.*, 1986, Buckmaster *et al.*, 1984; Sullivan and Smith, 1988; Longnecker *et al.*, 1987; Hutchinson *et al.*, 1992a,b; Ramasway and Holland, 1992; Baines and Roizman, 1993). An 11th gene, US5 is predicted to encode the glycoprotein gJ (McGeoch *et al.*, 1993). In addition to the glycoproteins, the envelope contains cell lipids (Asher *et al.*, 1969).

1.3. Structure and organisation of the HSV genome

HSV DNA is linear, double-stranded (Becker *et al.*, 1968) and contains 152 kilobase pairs (McGeoch *et al.*, 1986; Perry and McGeoch, 1988). The genome of HSV-2 has not been completely sequenced, but DNA hybridisation and comparison of DNA sequences have demonstrated that it is highly related to HSV-1 with a slightly higher G+C content of 68.3% compared to 67% (Kieff *et al.*, 1972; Davison and Wilkie, 1983; McGeoch *et al.*, 1988a). To date, the coding sequences of corresponding genes show in general 70-80% identity with one major exception: the coding sequence of HSV-2 US4 (gG2) contains an extra sequence of about 1460bp (McGeoch *et al.*, 1987). Moreover, the coding sequence of the HSV-2(HG52) RL1 gene is predicted to be interrupted by an intron which is absent in that of HSV-1 (McGeoch *et al.*, 1991). The non-coding regions, in particular the major repeats, typically show greater differences and in some cases have become highly diverged (Davison and Wilkie, 1981; Whitton and Clements, 1984).

The HSV-1 and HSV-2 genomes consist of two covalently joined segments, designated long (L) and short (S). Each component is composed of a unique sequence (U_L, U_S) flanked by terminal and inverted repeat sequences (TR, IR). The repeats of the L-component, TR_L and IR_L are respectively designated *a b* and *a' b'*, while those of the S

component, TRs and IRs, are respectively designated *a' c'* and *c a* (Wadsworth *et al.*, 1975). The organisation of the HSV genome is illustrated in Fig. 1.1. The genome of HSV-1 encodes at least 77 genes, 59 map in U_L, 13 map in U_S and two copies of 4 genes map in the repeated sequences (McGeoch *et al.*, 1985, 1986, 1988a, 1993; Liu and Roizman, 1991a, Barker and Roizman, 1992; Barnett *et al.*, 1992, Georgopoulou *et al.*, 1993). The genes identified to date in HSV-1 are listed in Table 1.1. The most recent 77th gene designated ORF P, has been identified by Lagunoff and Roizman (1994).

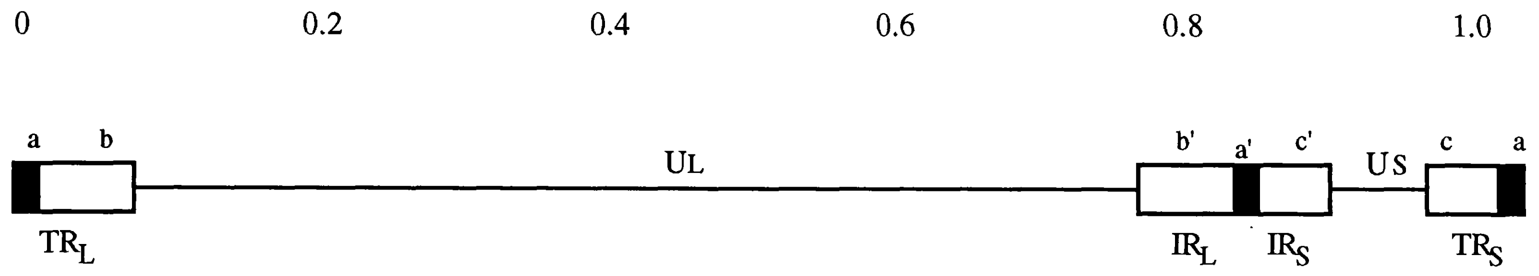
The L and S genome components invert relative to one another giving four equimolar isomers (Fig. 1.1) termed P (prototype), I_L (inversion of the L component), I_S (inversion of the S component) and I_{SL} (inversion of both S and L components) (Sheldrick and Berthelot, 1974, Hayward *et al.*, 1975; Clements *et al.*, 1976; Wilkie and Cortini, 1976; Roizman, 1979). Due to isomerisation, restriction enzyme analysis of DNA yields three classes of fragments (Clements *et al.*, 1976; Skare and Summers, 1977) which occur at different frequencies. Fragments derived entirely from the U_S and U_L regions appear in 1M quantities relative to the molarity of intact viral DNA. As each terminus is present in only 2 of the 4 isomers, terminal sequence fragments are present in 0.5M quantities while fragments which consist of the joint sequences are present in only 1 of the 4 isomers so are in 0.25M quantities (Wilkie and Cortini, 1976; Skare and Summers, 1977).

1.4. The '*a*' sequence

The '*a*' sequence which varies in size from 250-550bp is a sequence found as a direct repeat at the termini and an inverted repeat at the L/S junction of the HSV genome. Tandem reiterations (1-5 copies) of the '*a*' sequence are found at the L terminus and the L/S junction, but only a single copy is found at the S terminus (Wilkie, 1976; Wagner and Summers, 1978). The structure of the '*a*' sequence is highly conserved consisting of both unique (U) and direct repeat (DR) sequences and is represented as: DR1-Ub-DR2n-DR4m-Uc-DR1 (Fig. 1.2). Ub and Uc are named by virtue of their proximity to the *b'* and *c'* repeated regions, respectively. Adjacent '*a*' sequences share the intervening DR1

Figure 1.1. Structure of the HSV genome

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


A.
0
0.2
0.4
0.6
0.8
1.0
a
b
TR$_L$
UL
b'
a'
c'
IR$_L$
IR$_S$
US
c
a
TR$_S$

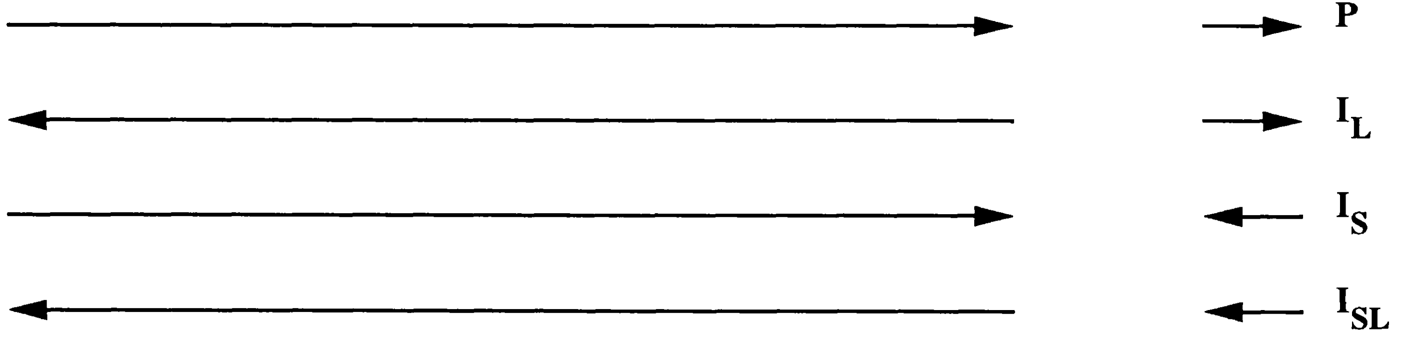

B.
P
I$_L$
I$_S$
I$_{SL}$

Table 1.1. The HSV genes identified to date

Gene	Protein/ function	Status
RL1	Neurovirulence factor (ICP34.5)	ne
ORF P	Encodes a protein of 248 amino acids, function unknown	ne
RL2	IE protein; transcriptional regulator (Vmw110)	ne
UL1	Glycoprotein L, role in cell entry	e?
UL2	Uracil-DNA glycosylase	ne
UL3	Function unknown	ne
UL4	Function unknown	ne
UL5	Component of DNA helicase-primase	e
UL6	Associated with virion capsid and involved in packaging of nascent DNA	e
UL7	Function unknown	--
UL8	Component of DNA helicase primase, DNA replication	e
UL8.5	Encodes a protein of 486 amino acids (OBPC), potential role in HSV DNA replication	--
UL9	*Ori*-binding protein essential for DNA replication	e
UL10	Glycoprotein M (gM)	ne
UL11	myristylated tegument protein, role in envelopment and egress of virions	ne
UL12	Deoxyribonuclease, role in maturation/packaging of nascent DNA	e
UL13	Tegument protein, protein kinase, host shutoff	ne
UL14	Function unknown	--
UL15	Role in cleavage of concatemeric DNA	e?
UL16	Function unknown	ne
UL17	Function unknown	e
UL18	Capsid protein triplexes (VP23), involved in cleavage of concatemeric DNA	e
UL19	Major capsid protein (VP5); constitutes the hexons and pentons, involved in cleavage of concatemeric DNA	e
UL20	Integral membrane protein, role in egress of virions	e/ne
UL21	Function unknown	--
UL22	Virion surface glycoprotein H, role in virion entry	e
UL23	Thymidine kinase, DNA replication	ne
UL24	Function unknown	ne

UL25	Virion protein; role in capsid maturation and DNA packaging	e
UL26	Proteinase, the N-terminal portion is capsid protein VP24, C-terminal is VP21, involved in capsid assembly	e
UL26.5	Scaffolding protein of B capsids (VP22a), processed by UL26 proteinase, involved in DNA packaging	e
UL27	Virion surface glycoprotein B, role in cell entry	e
UL28	Role in capsid maturation/DNA packaging	e
UL29	ssDNA binding protein (ICP8), essential for DNA replication	e
UL30	Catalytic subunit of replicative DNA polymerase	e
UL31	Nuclear phosphoprotein, function unknown	--
UL32	Role in capsid maturation/DNA packaging	--
UL33	Role in capsid maturation/DNA packaging	e
UL34	Membrane associated phosphoprotein	--
UL35	Capsid protein (VP26), present on tips of hexons	e
UL36	Very large tegument protein (ICP1/2; VP1/2), involved in cleavage of concatemeric DNA and virion assembly	e
UL37	Tegument γl phosphoprotein, function unknown, may have DNA-binding role	--
UL38	Capsid protein (VP19C), component of triplexes	e
UL39	Ribonucleotide reductase large subunit (R1)	e/ne
UL40	Ribonucleotide reductase small subunit (R2)	e/ne
UL41	Virion protein, host shutoff	ne
UL42	Subunit of replicative DNA polymerase, increases processivity	e
UL43	Function unknown, probable integral membrane protein	ne
UL44	Virion surface glycoprotein C, role in cell entry	ne
UL45	Envelope protein, mediates cell fusion	ne
UL46	Modulates IE gene transactivation by Vmw65	ne
UL47	Tegument protein (VP13/14), modulates IE gene transactivation by Vmw65	ne
UL48	Major tegument protein (VP16,Vmw65, αTIF), transactivate IE genes	e
UL49	Tegument protein (VP22)	--
UL49A	Possible membrane protein	ne
UL50	Deoxyuridine triphosphatase	ne
UL51	Function unknown	e/ne
UL52	Component of DNA helicase-primase	e

UL53	Glycoprotein K, role in cell fusion	e?
UL54	IE protein, post-translational regulator of gene expression (ICP27, Vmw63)	e
UL55	Function unknown	ne
UL56	Function unknown, role in intraperitoneal virulence	ne
LAT	Family of transcripts expressed during latency, function unknown, protein-coding capacity uncertain	ne
RS1	IE protein (ICP4, Vmw175), transcriptional regulator	e
US1	IE protein (ICP22, Vmw68), transcriptional regulator	e/ne
US2	Function unknown	ne
US3	Protein kinase	ne
US4	Glycoprotein G	ne
US5	Glycoprotein J	ne
US6	Glycoprotein D, role in entry	e
US7	Glycoprotein I, complex together with gE, role in cell-to-cell spread and is a receptor for Fc of IgG	ne
US8	Virion surface glycoprotein E, complex together with gI, role in cell-to-cell spread and is a receptor for Fc of IgG	ne
US8.5	Function unknown	ne
US9	Tegument protein	ne
US10	Virion protein	ne
US11	Virion protein, ribosome associated in infected cells	ne
US12	IE protein, function unknown (ICP47, Vmw12)	ne

e: essential for virus growth in culture cells

ne: non-essential

e/ne: necessity depends on culture conditions or temperature

e?: the data regarding status is not conclusive

--: absence of information regarding the status

Adapted from: McGeoch *et al.*, 1985, 1986, 1988a, 1993; Addison *et al.*, 1984, 1990; Al-Kobaissi *et al.*, 1991; Liu and Roizman, 1991a; Rosen-Wolff and Darai, 1991; Barker and Roizman, 1992; Barnett *et al.*, 1992; Georgopoulou *et al.*, 1993, Chang and Roizman, 1993; Purves *et al.*, 1993; Baines and Roizman, 1993; Baradaran *et al.*, 1994; Haanes *et al.*, 1994; Lagunoff and Roizman, 1994; Overton *et al.*, 1994; Baines *et al.*, 1995; Schmitz *et al.*, 1995; Patel and MacLean, 1995.

Figure 1.2. Structure of the HSV-1 '*a*' sequence

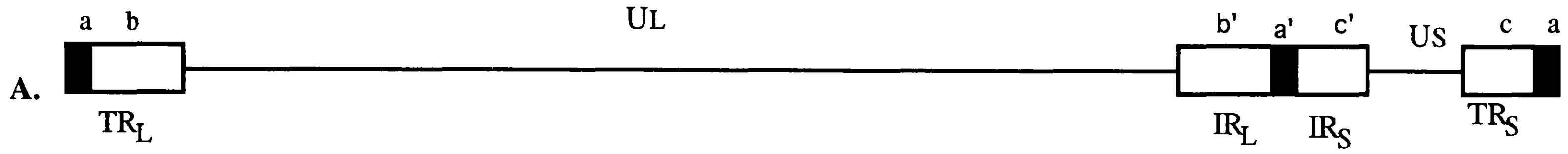
The HSV genome is shown (A). An expansion of the IR_L/IR_S junction showing the structure of the '*a*' sequence (B). The '*a*' sequence consists of unique and directly repeated elements. In HSV-1 strain F (Mocarski and Roizman, 1981, 1982):

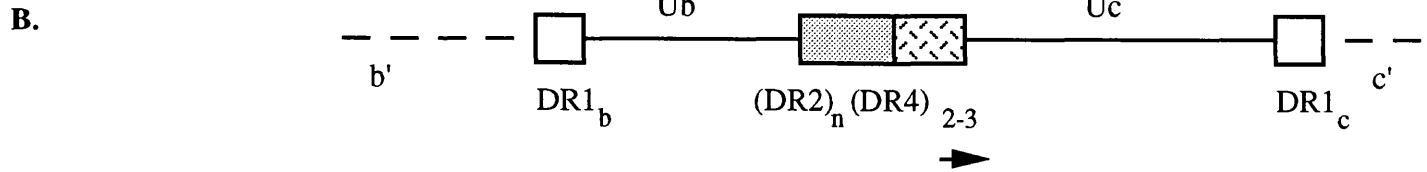
Ub- a unique region located towards the b' region of the genome.

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

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

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


A.
a
b
UL
b'
a'
c'
US
c
a
TR$_L$
IR$_L$
IR$_S$
TR$_S$


B.
Ub
Uc
b'
DR1$_b$
(DR2)$_n$ (DR4) $_{2-3}$
DR1$_c$
c'

(Mocarski and Roizman, 1981). The size of the '*a*' sequence varies from strain to strain due to variation in the number of copies of DR2 and DR4. In HSV-1(F), the '*a*' sequence consists of 20bp of DR1, 65bp of Ub, 12bp of DR2 present in 19-22 copies and 37bp of DR4 present in 3 copies, 58bp of Uc and a final copy of DR1 (Mocarski and Roizman, 1981, 1982). The '*a*' sequence appears to be a *cis*-acting site for inversion as insertion of the '*a*' sequence elsewhere in the genome (Mocarski *et al.*, 1980) or deletion of the entire internal inverted repeat sequences (*a' b' c'*) leads to additional inversions or the loss of the ability of the L and S components to invert respectively (Poffenberger *et al.*, 1983). Also Chou and Roizman (1985) demonstrated that deletion of DR4 drastically reduces inversion while deletion of both DR2 and DR4 completely abolishes inversion. Deletion of the Ub and Uc domains does not affect the ability of the '*a*' sequence to mediate inversion. The '*a*' sequence was also shown to contain the *cis*-acting sites for the circularisation of the genome after infection, for cleavage of the HSV genome into unit length concatamers and for encapsidation of DNA (Mocarski and Roizman, 1982; Vlazny *et al.*, 1982; Varmuza and Smiley, 1985).

In the course of studies on the function of the '*a*' sequence, Chou and Roizman (1986) reported that the '*a*' sequence in HSV-1(F) contains the promoter-regulatory domain and transcription initiation site of a diploid gene located in the *b* sequences of the inverted repeats of the L component. A chimeric construct consisting of the '*a*' sequence fused to a promoterless thymidine kinase gene (tk) was inducible in transfected cells and regulated as a γ1 gene when inserted into the viral genome. The expression of thymidine kinase occurred only when the '*a*' sequence was oriented with Ub adjacent to tk while no expression occurred when Uc was adjacent. Nucleotide sequence analysis revealed the presence of an ORF which could potentially encode a protein designated ICP34.5 [see section 1.7.3.3].

1.5. HSV lytic infection

1.5.1. Adsorption

The entry of HSV virions into cells is preceded by adsorption and binding of the virions

to a cell surface component. Heparan sulphate acts as the initial receptor for both HSV-1 and HSV-2 (WuDunn and Spear, 1989; Lycke *et al.*, 1991; Shieh *et al.*, 1992; Shieh and Spear, 1994). Enzymatic removal of heparan sulfate from the cell surface significantly reduces the binding of virus to cells and renders them resistant to infection (WuDunn and Spear, 1989). Binding of HSV is also severely impaired in Chinese hamster ovary (CHO) mutant cells defective in biosynthesis of either heparan sulphate alone or in all glycosaminoglycans or defective in sulphation of heparan sulphate (Shieh *et al.*, 1992). The interaction of virions with heparan sulphate is mediated by the viral structural glycoprotein gC of which at least two regions form a functional domain involved in binding (Trybala *et al.*, 1994). HSV-1 mutants lacking gC exhibit significant impairments in adsorption and penetration (Herold *et al.*, 1991). Neutralising antibodies specific for HSV-1 gC are able to block the binding of virus to cells (Svennerholm *et al.*, 1991; Fuller and Spear, 1985). The glycoproteins gD and gB are essential for secondary interactions at the cell surface which lead to virus entry into cells (Fuller and Spear, 1985; Cai *et al.*, 1988; Ligas and Johnson, 1988; Kuhn *et al.*, 1990; Herold *et al.*, 1994).

1.5.2. Penetration

Herpes simplex virus can enter cells by fusion with plasma membranes (Morgan *et al.*, 1968; Fuller and Spear, 1987). A number of glycoproteins present in the virion envelope are required for penetration into cells after initial attachment. These include gB, gD, gH, gK and gL (Fuller and Spear, 1987; Cai *et al.*, 1988; Highlander *et al.*, 1988; Ligas and Johnson, 1988; Forrester *et al.*, 1992; Roop *et al.*, 1993). gB-null viruses can bind to cell surfaces but are unable to enter cells unless treated with polyethylene glycol, a membrane fusion agent (Cai *et al.*, 1988). Neutralising monoclonal antibodies specific for gB, gD or gH can block viral penetration with little or no effect on adsorption (Fuller and Spear, 1987; Fuller *et al*, 1989; Highlander *et al.*, 1988; Navarro *et al.*, 1992). Virions lacking gD can adsorb to cells but fail to penetrate (Ligase and Johnson, 1988). Truncated soluble forms of both HSV-1 and HSV-2 gD can inhibit HSV entry but not adsorption (Johnson *et al.*, 1990) by interacting with gD receptors thus blocking viral

penetration but not adsorption. Binding of soluble gD is abolished by treatment of cells with proteases but not by enzymes which remove heparan sulphate (Johnson *et al.*, 1990) indicating binding to protein receptors.

The glycoproteins gH and gL form a heterodimer which acts during entry of virus into cells (Hutchinson *et al.*, 1992a). gL-null mutants fail to incorporate gH into virion envelopes (Roop *et al.*, 1993). Mutant viruses lacking gH and gL (Forrester *et al.*, 1992; Roop *et al.*, 1993) are able to adsorb onto cells but fail to penetrate and initiate infection. The precise role of glycoproteins in HSV penetration is not known. Fuller and Lee (1992) proposed a model which involves a cascade of virus-cell interactions in which gH and gD play a role. The model proposes that gD performs at least two functions (i) it contributes to stable virion attachment *via* interaction with a cellular component or receptor, and (ii) it interacts with a viral component which is directly involved in initiation of fusion of viron envelopes and cell membranes. For this reason, antibodies to gD that block attachment of virus do not bind to the same site as antibodies that block penetration. In contrast, gH seems to participate in fusion initiation cooperatively with gD and perhaps other viral proteins.

1.5.3. Release and replication of viral DNA

Following fusion of the virion envelope with the cell membrane, viral capsids are transported to the nuclear pore (Tognon *et al.*, 1981; Batterson *et al.*, 1983) and DNA is released into the nucleoplasm to allow gene expression [see section 1.5.8]. The cellular cytoskeleton probably mediates transport to nuclear pores (Kristensson *et al.*, 1986). In the nucleus, HSV-DNA replication is detected about 3 h post-infection and continues for at least 9-14h (Roizman *et al.*, 1963; Roizman and Roane, 1964). Head to tail viral DNA concatamers accumulate in the nuclei (Jacob and Roizman, 1977; Jacob *et al.*, 1979). HSV-DNA replicates by a rolling circle mechanism (Ben-Porat and Tokazewski, 1977; Jacob *et al.*, 1979; Roizman, 1979) with synthesis initially restricted to a few well-defined sites within the nucleus called replication compartments or inclusions (Quinlan *et al.*, 1984; Kops and Knipe, 1994) which are organised in the nuclear interior rather

than at the periphery (Kops and Knipe, 1994). The number and size of these compartments increases throughout infection until the entire nucleus is filled with replicating viral DNA (Rixon *et al.*, 1983).

HSV encodes a number of DNA replication proteins which are located in the replication compartment of infected cells (Quinlan *et al.*, 1984; Kops and Knipe, 1988; Goodrich *et al.*, 1990). Some of these proteins interact with specific viral DNA sequences which serve as origins of DNA replication. Three origins of replication have been identified, two designated Ori_S have the same sequence and map in the *c* inverted repeat sequences of the S component (Vlazny and Frenkel, 1981; Spaete and Frenkel, 1982; Stow, 1982; Stow and McMonagle, 1983; Deb and Doelberg, 1988). The third, Ori_L is located in the middle of the long unique segment (Weller *et al.*, 1985; Gray and Kaerner, 1984). Sequence analysis revealed palindromic organisation of Ori_S and Ori_L. Ori_L exhibits a single perfect palindrome of 144bp while Ori_S exhibits a 45bp palindrome (Weller *et al.*, 1985; Knopf *et al.*, 1986; Lockshon and Galloway, 1986).

Genes involved in DNA synthesis were identified by transfection of cells with a plasmid containing an origin of HSV replication and various fragments of the HSV genome (Challberg, 1986). Seven L component genes were identified whose products are essential and sufficient for viral DNA synthesis (Wu *et al.*, 1988). These are: UL30 and UL42 which encode the subunits of the viral DNA polymerase in which UL30 is the catalytic subunit and UL42 acts to increase the processivity of the enzyme (Powell and Purifoy, 1977; Coen *et al.*, 1984; Parris *et al.*, 1988; Gallo *et al.*, 1988; Crute and Lehman, 1989), UL29 specifies a single-strand specific-DNA binding protein (ICP8) (Purifoy and Powell, 1976; Ruyechan and Weir, 1984; Quinlan *et al.*, 1984; Kops and Knipe, 1988; Wu *et al.*, 1988), UL9 specifies a protein which binds to a specific sequence within the origins of viral DNA synthesis (Elias *et al.*, 1986; Elias and Lehman, 1988; Koff and Tegtmeyer, 1988; Olivo *et al.*, 1989; Elias *et al.*, 1990). The products of UL5, UL8 and UL52 form a complex in which each protein is present in equimolar ratios and functions as a primase and a helicase (McGeoch *et al.*, 1988b; Crute *et al.*, 1989; Calder and Stow, 1990; Dodson and Lehman, 1991; Calder *et al.*,

1992; Klinedinst and Challberg, 1994).

HSV enzymes involved in nucleic acid metabolism include thymidine kinase (Kit and Dubbs, 1963; Dubbs and Kit, 1964; Tenser and Dunstan, 1979), ribonucleotide reductase (Bacchetti *et al.*, 1986; Ingemarson and Lankinen, 1987; McGeoch *et al.*, 1988a), uracil-DNA glyosylase (Caradonna and Cheung, 1981; McGeoch *et al.*, 1988a; Worrad and Caradonna, 1988) and dUTPase (Wohlrab and Francke 1980; Caradonna andCheung,1981; Williams and Parris, 1987). Some of these enzymes are discussed in section 1.7 in relation to their role in HSV pathogenicity.

1.5.4. Cleavage and packaging of HSV DNA

Following viral DNA replication, newly synthesised concatameric DNA molecules are processed into unit length genomic molecules and packaged into capsids. There is evidence that cleavage and packaging are linked processes. Variants with mutations in capsid genes UL19, UL38, UL18 and UL26 encoding the protease fail to process concatamers to full-length molecules (Weller *et al.*, 1987;Pertuiset *et al.*, 1989; Desai *et al.*, 1993; Gao *et al.*, 1994). Cleavage of concatamers occurs within the terminally reiterated '*a*' sequences at the junctions between adjacent viral genomes (Vlazny and Frenkel, 1981; Mocarski and Roizman, 1982; Deiss *et al.*, 1986; Deiss and Frenkel, 1986). The process of DNA cleavage (Mocarski and Roizman, 1982) generates genomes with (i) a free L-component terminus consisting of one to several directly repeated '*a*' sequences and ending in a DR1 containing 18bp and one 3' nucleotide extension (ii) a free S-component terminus consisting of one '*a*' sequence with a terminal DR1 sequence containing only a single base pair and one 3' nucleotide extension. Joining of the two partial DR1 sequences forms a complete DR1 which results in circularisation of the DNA. Cleavage within this shared DR1 results in linearisation for packaging (Mocarski and Roizman, 1982). Two conserved domains designated *pac*1 and *pac*2 were identified in Ub and Uc, respectively and appear to be essential for the cleavage and packaging process (Varmuza and Smiley, 1985; Deiss *et al.*, 1986). Viral proteins responsible for cleavage and packaging have not been identified. Two viral proteins

(VP1/2 and an uncharacterised 140-KDa protein) play a role in recognition of the DR1-Uc region by forming a complex which binds specifically to the Uc sequence containing *pac*2 (Chou and Roizman, 1989; McNabb and Courtney, 1992) . Recently, Baines *et al* (1994) reported that the UL15 gene encodes two polypeptides which share sequences from the second exon and which are required for cleavage of viral DNA from concatamers and packaging into capsids.

1.5.5. Virion assembly

Capsids are assembled in the nucleus and viral DNA is packaged into pre-formed capsids (Preston *et al.*, 1983). Little is known about how virus particles are formed but the available evidence suggests that VP22a (ICP35) is required for encapsidation of viral DNA (Preston *et al*, 1983; Rixon *et al.*, 1988; Sherman and Bachenheimer, 1988). VP22a is an abundant protein in B capsids and was initially identified as the product of HSV-1 gene UL26 from the genomic location of the lesion in the mutant *ts*1201. This mutant is defective in processing of the VP22a protein at the non-permissive temperature (NPT). When cells infected with *ts*1201 were shifted from NPT to the permissive temperature in the presence of cycloheximidine, both processing of VP22a and packaging of viral DNA took place indicating that VP22a is involved in the packaging process (Preston *et al.*, 1983). Nucleotide sequencing studies assigned the coding sequence of VP22a to the open reading frame UL26 (McGeoch *et al.*, 1988a). In a subsequent study, Liu and Roizman (1991b) showed that the domain of UL26 contains two transcriptional units which coterminate at the 3' end but have different 5' ends. VP22a is encoded by the 3' domain of UL26, designated UL26.5.

1.5.6. Envelopment and egress

Assembled capsids leave the nucleus and gain tegument and envelope before exiting from cells. These events are poorly understood. It is apparent that capsids leave the nucleus by budding through the inner nuclear membrane into the space between the inner and outer nuclear membranes (Darlington and Moss, 1968; Nii *et al.*, 1968;

Johnson and Spear, 1982). Opinions differ concerning the route by which virions leave the perinuclear cisternae. Two pathways have been suggested (Fig. 1.3). In the first, virions enveloped at the inner nuclear membrane fuse with the outer nuclear membrane to release capsids into the cytoplasm. In the second pathway, the envelope gained at the inner nuclear membrane is retained and virions leave the perinuclear cisternae in a vacuole formed from the outer nuclear membrane. Both models propose that maturation of virions takes place in the Golgi complex and that mature virions leave the cell by exocytosis from the Golgi-derived vesicle (Rixon, 1993).

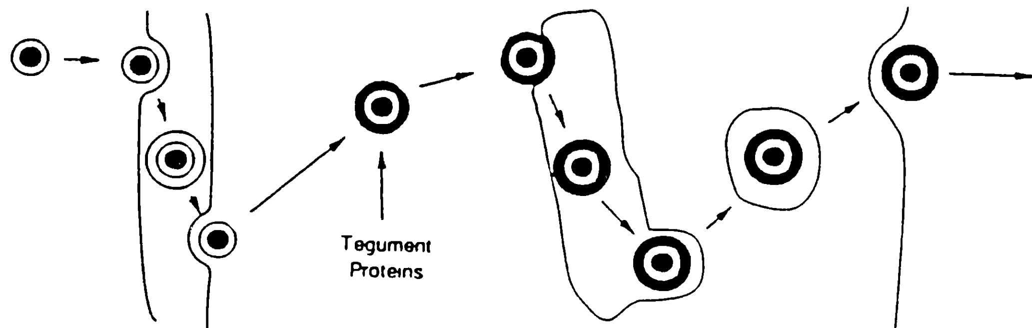
The first evidence that an HSV gene is required for transit of virions to the perinuclear space was provided by Baines *et al* (1991). Deletion of 53% of the UL20 gene, encoding an integral membrane protein, resulted in accumulation of enveloped and unenveloped capsids in the space between the inner and outer nuclear lamellae. The mechanism by which the product of UL20 enables the transport of virions is unclear. Another gene, UL11 encoding a myristylated protein might also play role in virus envelopment and release (MacLean *et al.*, 1989, 1992; Baines and Roizman, 1992; Baines *et al.*, 1995).

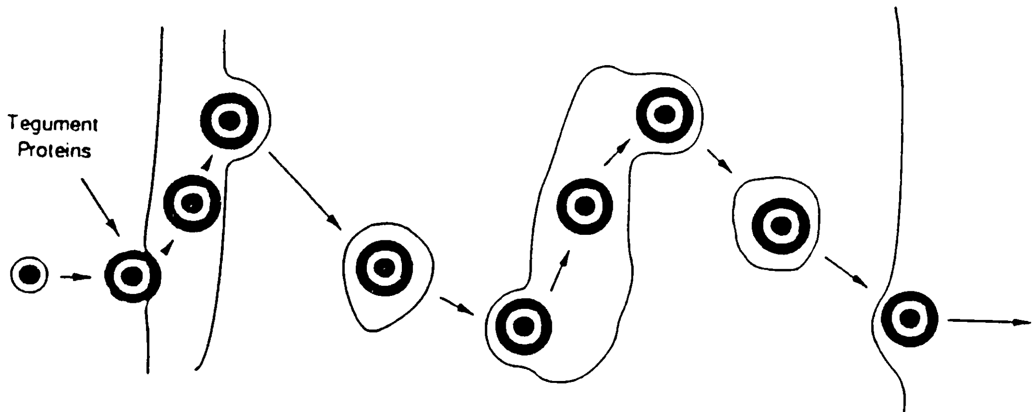
1.5.7. Shutoff of host macromolecular metabolism

Infection of cells with HSV leads to early shutoff of host gene expression due to the activity of the virion host shutoff (*vhs*) protein encoded by the UL41 gene (McGeoch *et al.*, 1988a; Kwong *et al.*, 1988; Fenwick and Everett, 1990b; Smibert *et al.*, 1992). Host DNA synthesis is shutoff (Roizman and Roane, 1964), host protein synthesis declines very rapidly (Read and Frenkel, 1983; Sydiskis and Roizman, 1966), host ribosomal RNA synthesis is reduced (Wagner and Roizman, 1969) and glycosylation of host proteins ceases (Spear *et al.*, 1970). The effect of the *vhs* protein is not limited to preexisting cellular mRNAs, but also induces destabilisation and degradation of viral IE, E and L mRNAs (Fenwick and Everett, 1990a; Strom and Frenkel, 1987; Kwong and Frenkel, 1987). Thus virus mutants that encode a defective *vhs* protein are defective in virion host shutoff and produce viral mRNAs with longer half-lives than those mRNAs produced by the wild-type virus (Strom and Frenkel, 1987; Kwong and Frenkel, 1987;

Figure 1.3. Proposed pathways for virion egress.

In the first pathway (A), tegumentation takes place in the cytoplasm and the virion envelope is derived from the cytoplasmic membrane. In the second pathway (B), tegumentation takes place in the nucleus and the virion envelope is derived from the inner nuclear membrane. In both cases, maturation of virions takes place in the Golgi complex and mature virions leave the cell by exocytosis. (Adapted from Rixon, 1993)


NUCLEUS
CYTOPLASM
(A)
Tegument Proteins


(B)
Tegument Proteins

Oroskar and Read 1987, 1989). The level of *vhs* activity varies markedly between different HSV strains and is particularly weak in HSV-1 strain 17+ (Fenwick and Everett, 1990a). Recent studies (Smibert *et al.*, 1994) have shown that the *vhs* protein forms a complex with VP16(α-TIF) and blocks the ability of VP16 to enter a multiprotein complex on the immediate-early TAATGARATTC element.

Other HSV proteins can also serve to suppress host protein synthesis. Overton *et al* (1994) demonstrated that the product of the UL13 gene is also involved. Cells infected with UL13-negative mutants are able to synthesise actin at the same level as mock-infected cells whereas actin synthesis is considerably reduced in cells infected with wild-type virus. The amounts of UL41 protein present in wild-type and UL13-negative virions are approximately equivalent. The level of UL41 protein expressed in cells infected with UL13-negative mutants is drastically reduced compared to wild-type infected cells, but the kinetics of synthesis are not affected. The role of UL13 in host shutoff is unlikely to activate the UL41 gene product by phosphorylation

1.5.8. HSV gene expression during productive infection

During productive infection by HSV, genes are expressed sequentially and coordinately in a cascade fashion (Honess and Roizman, 1974, 1975). HSV genes can be classified broadly as α, β and γ (Honess and Roizman, 1974, 1975) or immediate-early (IE), early (E) and late (L) (Clements *et al.*, 1977). This classification is based on the kinetics of synthesis of individual viral transcripts and proteins and requirement for either *de novo* protein or DNA synthesis.

IE gene expression is detectable at 1h post-infection with peak synthesis occurring at 3-4h post-infection and declining rapidly thereafter, although low levels of synthesis are detectable at later times (Honess and Roizman, 1974). There are five IE genes (Table 1.2) some of whose products are the major regulatory proteins, required to induce synthesis of early and late transcripts (Honess and Roizman, 1974, 1975; Deluca *et al.*, 1985; O'Hare and Hayward, 1985; Sacks *et al.*, 1985). Transcription of IE genes is activated by a late structural protein, VP16 (Vmw65) (Batterson and Roizman, 1983).

Table 1.2. The IE genes of HSV-1 and their products

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

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

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

E genes are expressed prior to the onset of HSV DNA replication and require IE gene expression. Transcription of E genes and synthesis of proteins begin by 3h post-infection, peak at about 5-7 and declines thereafter (Honess and Roizman, 1974). These genes can be divided into subclasses (E1 and E2) on the basis of their times of expression and abundance. The appearance of E gene products signals the onset of viral DNA synthesis and most viral genes involved in viral nucleic acid metabolism appear to be in the E group.

L genes are maximally expressed after the onset of viral DNA replication and can be divided into 2 subclasses [L1 (EL or leaky late) and L2] on the basis of an obligatory requirement of DNA replication for expression (Costa *et al.*, 1981). Most L genes are structural genes and assembly proteins.

Physical mapping studies have shown that two of the five immediate early genes are diploid being located within the repeat sequences flanking the U_L and U_S regions of the genome, while E and L genes are located in U_L and U_S (Murchie and McGeoch, 1982; McGeoch *et al.*, 1985).

In contrast to the sequence of events that occurs during productive infection, HSV gene expression in latently-infected cells is limited mainly to the latency-associated transcripts (LATs), a family of transcripts ranging in size from 2.0 to >8Kb (Spivack and Fraser, 1987; Zwaagstra *et al.*, 1990) [see sections 1.6.1 and 1.6.3].

1.5.9. Regulation of HSV gene expression

Expression of HSV genes is controlled by both viral and cellular *trans*-acting factors which require *cis*-acting sites. A number of regulatory sequence elements are found in IE gene promoters. E promoters consist of a near upstream region composed of binding sites for cellular transcription factors linked to a TATA/cap site (McKnight and Kingsbury, 1982; Everett, 1984, McKnight and Tjian, 1986). True HSV-1 late promoters appear to have a simpler structure with a TATA element being the only consensus element identified in the upstream regulatory region (Wagner, 1985). Deletion studies have indicated that little sequence information upstream from this element is required

for efficient expression (Homa *et al.*, 1986, 1988).

One of the unique regulatory sequence elements found in IE gene promoters is TAATGARAT, R=Purine (Mackem and Roizman, 1982; Whitton and Clements, 1984; Gaffney *et al.*, 1985) which is required for the induction of IE genes by α-TIF (Vmw65). Induction by Vmw65 occurs through binding of the cellular transcription factor Oct-1 (Stern *et al.*, 1989) to the *cis*-acting site TAATGARAT and interaction of Vmw65 with Oct-1 to form a complex (Gerstar and Roeder, 1988; Kristie and Roizman, 1987; O'Hare and Goding, 1988; Preston *et al.*, 1988). An additional cellular factor termed complex forming factor (CFF) binds directly to Vmw65 in the absence of Oct-1 or DNA (Kristie *et al.*, 1989; Xiao and Capone, 1990; Katan *et al.*, 1990), therefore it is likely that Vmw65-CFF interaction is the first step in complex formation which subsequently recognises the DNA-bound Oct-1. Recently, Raiph *et al* (1994) demonstrated that a cellular factor in Vero and NB41A3 cells expressed after release from growth arrest can activate IE gene expression.

Sequence comparison of the TAATGARAT motif of IE promoters showed that some have a 5' extension with an ATGC sequence (ATGCTAATGARAT) (APRhys *et al.*, 1989). The extended (represented by RL2) and the standard (represented by RS1) motifs were tested by Douville *et al* (1995) for binding Oct-1/Vmw65 and for transcriptional activity. The extended RL2 sequence binds to Oct-1/Vmw65 with higher affinity and drives transcription more efficiently than RS1 which is poorly active on its own. Neighbouring DNA sequence motifs CGGAAR which bind the cellular GABPα/β factor act in synergy with the RS1 sequence and compensate for its weak activity.

A second sequence element, RTCGTCNNYNYSG, Where R=purine, Y=pyrimidine, S= C or G and Y is any base, is a high-affinity binding site for Vmw175 (Kristie and Roizman, 1986; DiDonato *et al.*, 1991) and is thought to be the major negative regulatory element for promoters (Roberts *et al.*, 1988; DiDonato *et al.*, 1991). A third element is the binding site for the cellular transcription factor Spl (Jones and Tjian, 1985; Jones *et al.*, 1985).

Three of the HSV IE nuclear proteins (Vmw110, Vmw175 and Vmw63) perform

regulatory functions cooperatively, but the mechanism underlying this is not understood. Vmw175 is the major transcriptional regulatory protein required to activate transcription of E and L genes for progression of the lytic cycle and to repress transcription from its own and other IE genes (Deluca *et al.*, 1985; Kristie and Roizman, 1986; DiDonato *et al.*, 1991). Vmw110 is able to transactivate all classes of HSV genes that exhibit a basal level of transcription. The mechanism by which Vmw110 activates viral gene expression is not known. Vmw110 and Vmw175 activate E and L gene expression synergistically (Everett , 1984; O'Hare and Hayward, 1985; Quinlan and Knipe, 1985). Yao and Schaffer (1994) reported that Vmw110 and Vmw175 interact physically. Vmw63 by itself appears to have little effect on the expression of HSV genes. However, it can act as a *trans*-repressor or a *trans*-activator in transfection assays in combination with Vmw110 and Vmw175 (Everett, 1986; McMahan and Schaffer, 1990; Sekulovich *et al.*, 1988; Su and Knipe, 1989). A recent study (Purves *et al.*, 1993) demonstrated that Vmw68 is also involved in the regulation of HSV gene expression by enhancing the level of Vmw110 expression.

1.6. HSV latent infection

One of the features of infection with most human herpesviruses is the ability to establish and maintain latency with subsequent reactivation. Latency has been defined as the persistence of the virus in a host in a non-infectious form. At the cellular level, latency has been defined as the presence of the viral genome in the absence of the production of infectious progeny (Fraser *et al.*, 1992). Latency is established following a primary infection. Skin, cornea and mucous membranes are the portals of entry in which HSV multiplies forming mucocutaneous lesions. After replication at the site of infection, virions are transported by axons to the cell body of sensory neurons within the dorsal root ganglia (Cook and Stevens, 1973). Latency is established in a range of sensory ganglia (Hill, 1985) e.g. trigeminal (Bastian *et al.*, 1972; Baringer and Swoveland, 1973; Baringer, 1974; Efstathiou *et al.*, 1986) and cervical ganglia (Balan *et al.*, 1994) in HSV-1 infection and sacral, lumbar and thoracic ganglia (Galloway *et al.*, 1979) in

HSV-2 infection. However both viruses can establish latent infection in any dorsal root ganglia (Stevens and Cook, 1971). Periodically, in response to a variety of stimuli, such as stress, fever, menstruation, therapeutic irradiation and UV light, latent virus can be reactivated and subsequently pass down the axon of the sensory neuron to replicate at or near the initial site of infection (Whitley, 1990). Depending on the host immune response, the resulting disease in most cases is cutaneous lesions. Occasionally acute fatal encephalitis occurs (Hill, 1985). The frequency of HSV reactivation depends upon the virus type and the site of infection (Lafferty *et al.*, 1987). Genital HSV-2 infections recur 8-20 times more frequently than HSV-1 genital infections with more than 80% of patients with primary HSV-2 genital infections having a recurrence within 12 months compared with 55% with HSV-1 genital infections (Lafferty *et al.*, 1987; Whitley, 1990). Conversely, oral HSV-1 infections recur much more frequently than oral HSV-2 infections (Spruance *et al.*, 1977; Lafferty *et al.*, 1987)

Latency is a complicated biological phenomenon which involves viral, cellular and host factors. It can be divided into three phases: establishment, maintenance and reactivation (Roizman and Sears, 1987).

1.6.1. Establishment and maintenance of latent infection

The initial work on establishment of latency came from a hypothesis that neurons harbouring latent virus lack host factors for the transcription of viral genes. An alternate hypothesis is that in the environment of the sensory neuron, a specific viral gene arrests the viral gene cascade which consequently leads to the establishment of latency (Roizman and Sears, 1987). The search for these putative viral genes has taken two directions. The first involves analysis of ganglia harbouring latent virus for the presence of viral gene products [see section 1.6.3]. The second involves analysis of HSV mutants for their ability to establish latency. Using deletion mutants, it has been shown that viral DNA replication and genes associated with initiation of lytic infection are not required for establishment of latency. These include thymidine kinase (Tenser *et al.*, 1979, 1981, 1989 ; Coen *et al.*, 1989; Katz *et al.*, 1990; Kosz-Vnenchak *et al.*, 1990), Vmw110

(Clements and Stow, 1989), Vmw68 (Sears *et al.*, 1985), Vmw65 (Steiner *et al.*, 1990; Valyi-Nagy *et al.*, 1991), Vmw175 (Katz *et al.*, 1990; Dobson *et al.*, 1990), viral DNA polymerase (Wilcox and Johnson, 1988) and ribonucleotide reductase (Katz *et al.*, 1990). Similarly, latent infection with wild-type HSV can be established in ganglionic neurons in which no prior viral IE gene expression or DNA replication has taken place (Speck and Simmons, 1991; Margolis *et al.*, 1992). This result indicated that the latent and productive pathways may diverge early in the acute ganglionic infection and raises the important question of which viral or cellular factor(s) is responsible for selection of the latent as opposed to the lytic pathway?. Data has emerged which suggests that the establishment and maintenance of neuronal latency is the result of failure of viral IE expression (Stevens *et al.*, 1987; Croen *et al.*, 1987; Margolis *et al.*, 1992). Failure of IE gene expression could be due to loss of Vmw65 when the virus is carried from the peripheral site to the nucleus of the sensory neuron (Roizman and Sears, 1987). This was supported by the observation that HSV-1 mutants which lack the *trans*-inducing function of Vmw65 establish latent infections capable of reactivation (Steiner *et al.*, 1990; Valyi-Nagy *et al.*, 1991). A contradictory finding that latency can be established in transgenic mice in which Vmw65 has been expressed in all cell types including sensory neurons was shown by Sears *et al* (1991). The block of IE expression may be due to the lack of Oct-1 which has been shown to be present at a very low levels in most neurons (He *et al.*, 1989). An alternative possibility is the presence of a neuronal specific octamer-binding protein which represses HSV-1 IE gene expression by preventing *trans*-activation by the Oct-1/Vmw65 complex. To study the nature of the octamer binding, a series of immortalised rat ganglionic neurons were prepared by fusing primary sensory neurons of neonatal rat DRG with the C1300 mouse neuroblastoma cell line. The resulting cell lines (ND) proliferate indefinitely and possess the characteristics of primary sensory neurons which are not found in the parental neuroblastoma (Suburo *et al.*, 1992). Transfection of these cells with a construct in which expression of the CAT gene is driven by the HSV IE promoter showed a very low level of IE promoter activity compared to that observed in permissive fibroblasts. It was suggested that neuronal cells

contain an inhibitory octamer binding protein which following infection, binds to the TAATGARAT motif of IE promoters to prevent binding of the *trans*-activating complex of Oct-1/Vmw65. This was tested by cotransfection of IE-CAT with increasing amounts of a plasmid containing a cloned TAATGARAT motif. A steady increase in the IE-CAT activity in neuronal cells was observed indicating removal of the inhibitor by excess TAATGARAT and activation of the IE promoter by the Oct-1/Vmw65 complex (Kemp *et al.*, 1990; Wheatley *et al.*, 1991). The neuronal octamer-binding repressor was identified by DNA mobility shift assays and found to be Oct-2 which showed a similar mobility to B cell Oct-2 and reacted with a polyclonal antibody to Oct-2 (Lillycrop *et al.*, 1991). The Oct-2 mRNA in ND cells was detected using PCR and the cDNA product showed that the ND cell Oct-2 protein was identical to the B cell protein in the central POU domain which mediates DNA binding (Lillycrop *et al.*, 1991; Latchman *et al.*, 1992). Deletion of a forty amino acid region in the N-terminus of Oct-2 abolishes the inhibitory effect on the IE promoters (Lillycrop *et al.*, 1994a). Two isoforms, Oct 2.4 and Oct 2.5, are overexpressed in neuronal cells (Wirth *et al.*, 1991) and are unable to form a complex with Vmw65 due to differences between the POU domains of Oct-1 and Oct-2 (Stern *et al.*, 1989; Gerster and Roeder, 1988). Unlike Oct-1, Oct 2.4 and Oct 2.5 lack a gene activation domain at the C terminus of the molecule (Wirth *et al.*, 1991) and therefore binding of Oct-2 to the TAATGARAT motif does not stimulate transcription but prevents binding of the activating complex of Oct-1/Vmw65 (Lillycrop *et al.*, 1991). In co-transfection assays using IE3-CAT, both Oct-2.4 and Oct-2.5 were able to inhibit the IE promoter while Oct-2.1, the predominant B cell isoform which possesses the C-terminal activation domain, activated the IE promoter (Lillycrop and Latchman, 1992; Lillycrop *et al.*, 1994a). The ability of Oct-2.4 and Oct-2.5 to inhibit the viral lytic cycle was further tested by Lillycrop *et al* (1994a) in permissive BHK cells which had been engineered to stably express either Oct-2.4 or Oct-2.5. Following infection with HSV-1(F), a clear reduction in the transcription level of all five viral IE gene was observed. The subsequent stages of the HSV lytic cycle were also affected as indicated by reduction in the expression level of E and L genes represented by TK, gB and gC. A

decline in viral glycoprotein production was paralleled by a decline in viral yield. In contrast, a small increase in IE gene expression and viral yield was observed by expressing Oct-2.1. Lillycrop *et al* (1994a) concluded that expression of these isoforms is likely to play a role in establishment of latency by making neuronal cells non-permissive for the viral lytic cycle. The role of Oct-2 in repressing IE gene expression in normal unimmortalised sensory neurons *in vivo* is not known yet, but Oct-2 mRNA has been detected in sensory neurons of rat DRG by *in situ* hybridisation, PCR and DNA mobility shift assays (Lillycrop *et al.*, 1991; Wood *et al.*, 1992).

Expression of a low level of HSV genes, in particular Vmw175, during latency has been demonstrated recently by Kramer and Coen (1995) using a sensitive quantitative RNA PCR. Vmw175 specific RNA was found in variable amounts in about 20% of ganglia with 1-7 RNA molecules per viral genome. These amounts were similar at 30 and 60 days postinfection. TK-specific RNA was found at a maximum level of 3.2×10^6 molecules per ganglia (500 molecules per viral genome). It was concluded that a low level of Vmw175 expression in the absence of detectable reactivation led to TK transcription. This finding confirms the previous detection by immunofluoresence and by *in situ* hybridisation of a low level of Vmw175 (Green *et al.*, 1981) and Vmw110 (Croen *et al.*, 1987).

1.6.2. The latent viral genome

Studies on latently infected ganglia of experimental animals demonstrated that the HSV genome is maintained in a nonlinear endless form (Rock and Fraser, 1983; Efstathiou *et al.*, 1986) due to joining of the termini (Rock and Fraser, 1985). Based on density gradient centrifugation experiments, Mellerick and Fraser (1987) provided evidence that the latent genome can be separated from host chromosomal DNA and thus persists in a non-integrated state. Additional experiments indicated that the latent HSV genome has a nucleosomal structure similar to that of cellular chromatin (Deshmane and Fraser, 1989) and is not extensively methylated (Dressler *et al.*, 1987). The estimated copy number of

viral DNA in a latently infected neuron in a sensory ganglion ranges from 10 to >100. This multiple copy number may arise by several possibilities: i) infection with an average m.o.i. of $10\text{-}10^2$ virions/cell, which would not subsequently increase in number, ii) a low level of genome replication prior to or during the latent phase (Roizman and Sears, 1987). As gene expression during latency is repressed, this replication could not be accomplished by viral enzymes which recognise the origins of viral DNA synthesis (Ori_L, Ori_S) used during the lytic infection. It was predicted that the viral genome contains at least one cellular origin for viral DNA synthesis, which is recognised by cellular enzymes. Sears and Roizman (1990) reported that the 1.6Kb *Bam*HI-*o* fragment mapping in U_L contains a host origin (Ori_H) of DNA synthesis. This fragment is within the ORF encoding the large subunit of ribonucleotide reductase. Ori_H was predicted by Sears and Roizman (1990) to play a role in reactivation.

1.6.3. Transcription during latency

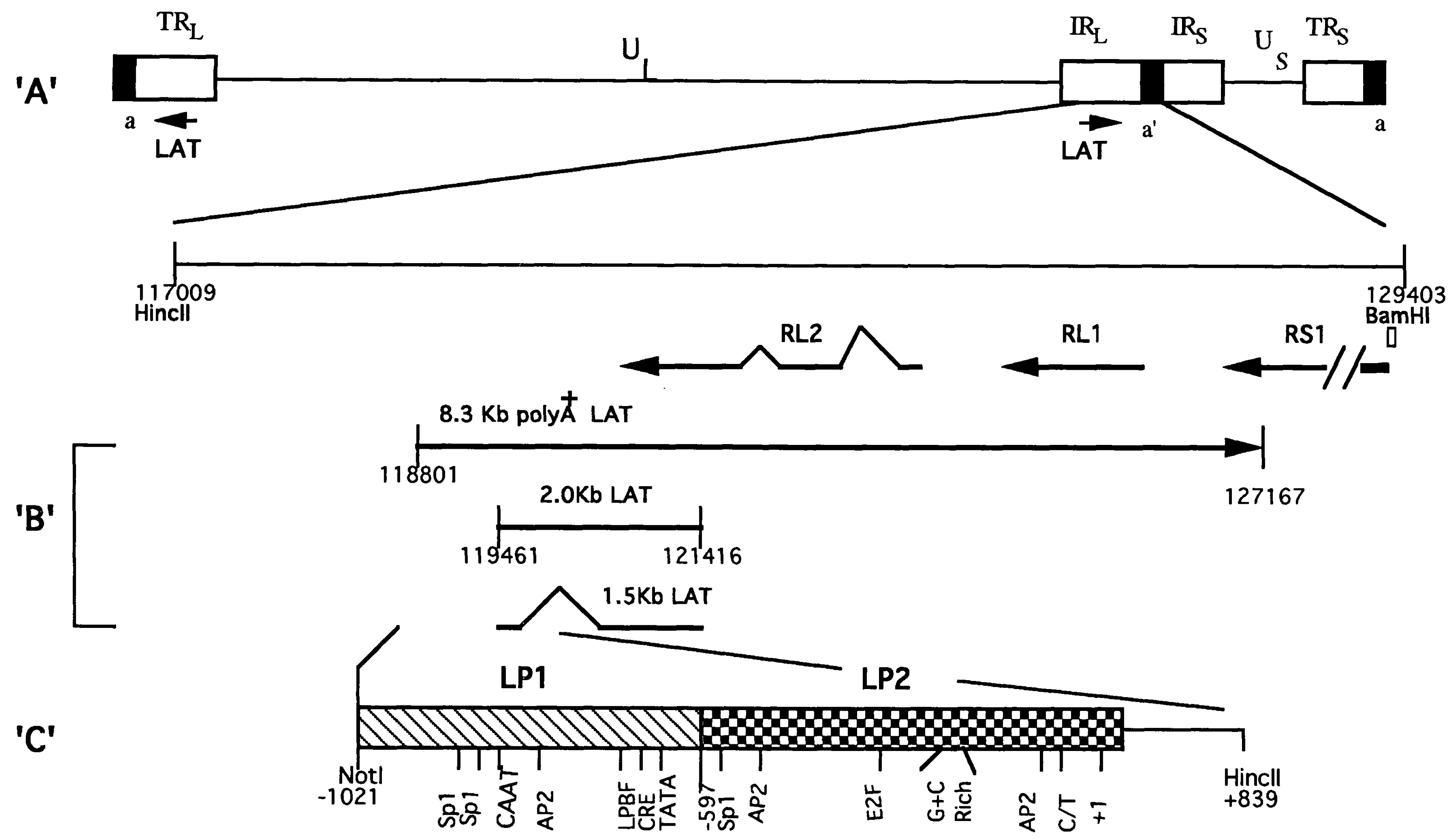
In both HSV-1 and HSV-2, a single region in R_L known as the latency associated transcript or LAT has been shown to encode RNA (Stevens *et al.*, 1987; Croen *et al.*, 1987; Deatly *et al.*, 1987; Rock *et al.*, 1987, Mitchell *et al.*, 1990a,b). This region has been sequenced for HSV-1 strains KOS(M), 17^+ and F (Wagner *et al.*, 1988; Perry and McGeoch *et al.*, 1988; Wechsler *et al.*, 1989) and for HSV-2 strain HG52 (McGeoch *et al.*, 1991). The location of the LAT promoter has been confirmed by transient CAT assays (Zwaagstra *et al.*, 1989, 1990) and analysis of mutant viruses with deletions encompassing the promoter region (Mitchell *et al.*, 1990c; Dobson *et al.*, 1989).

Nothern blots of RNA isolated from latently infected ganglia demonstrated the presence of uncapped 3' co-terminal poly(A^-) RNA species of sizes 1.45, 1.5 and 2.0Kb mapping in an antisense direction to part of the RL2 gene (Spivack and Fraser, 1987; Devi-Rao *et al.*, 1991). Subsequently, it has been reported that these transcripts may arise as stable introns from a larger (8.3Kb) capped polyadenylated transcript (Fig. 1.4) which extends antisense to both the RL1 and RL2 genes across the L-S junction (Spivack and Fraser, 1987; Zwaagstra *et al.*, 1990; Spivack *et al.*, 1991; Farrell *et al.*, 1991). The 2.0Kb

Figure 1.4. The LAT region of HSV-1

The HSV genome is shown (A). The LATs are transcribed from the repeat regions. The LAT region transcripts (B) overlapped by transcripts expressed during the lytic infection. The latency active promoter(s), LP1 and LP2 with *cis* elements (C) (Adapted from Goins *et al.*, 1994).

In this figure the numbering system used for LAT promoters has been changed. The 5' end of the 2Kb LAT is referred to as +1. This means for example that the DNA sequence recognised by LPBF referred to in the text at position -65–-72 is numbered -662–-669.


'A'
TRL
a
LAT
U
L
IRL
IRS
US
TRS
a'
a
117009
HincII
129403
BamHI
RL2
RL1
RS1
8.3 Kb polyA+ LAT
118801
127167
'B'
2.0Kb LAT
119461
121416
1.5Kb LAT
'C'
LP1
LP2
NotI
-1021
Sp1
Sp1
CAAT
AP2
LPBF
CRE
TATA
-597
Sp1
AP2
E2F
G+C
Rich
AP2
C/T
+1
HincII
+839

transcript is abundant and can be detected readily in neurons harbouring latent virus (Spivack and Fraser, 1987; Stevens *et al.*, 1987). The unspliced 8.3Kb RNA is much less abundant and the presumed 6.5Kb spliced species which gives rise to the 2.0- and 1.5-Kb LATs is not present in sufficient amounts to be detected (Mitchell *et al*, 1990b; Wagner *et al.*, 1988; Zwaagstra *et al.*, 1990). The relationship between these transcripts has not been completely elucidated. Recently, Lagunoff and Roizman (1994) demonstrated that the domain of the genome covered by the 8.3Kb transcript encodes in the same orientation a protein ORF P.

The role that LATs play in the latent state has been examined with LAT$^-$ mutants. Mutant viruses with deletions in the promoter or the transcribed region of LAT are able to establish and maintain latency and reactivate (Javier *et al.*, 1988b, Ho and Mocarski, 1989; Block *et al.*, 1990; Mitchell *et al.*, 1990c). Several laboratories have reported that LAT deletion mutants demonstrate a reduced capacity to reactivate and the kinetics of reactivation of these mutants is slower than wild-type (Steiner *et al.*, 1989; Leib *et al.*, 1989b; Hill *et al.*, 1990; Trousdale *et al.*, 1991; Devi-Rao *et al.*, 1994), suggesting that LAT expression is non-essential for establishment and maintenance of latency but plays a role in reactivation (Javier *et al.*, 1988b, Ho and Mocarski, 1989; Block *et al.*, 1990; Mitchell *et al.*, 1990c; Perng *et al.*, 1994). The observation that the 2.0Kb LAT is antisense to the 3' domain of the RL2 gene led to speculation that LAT transcripts enable the establishment of latency by suppressing the expression of RL2 and hence shutoff of the viral lytic cycle. Evidence for this has been shown by Farell *et al* (1991) in transient transfection assays. There is no evidence in animal models to support this finding.

To date, the precise function of LATs is unclear

.. Doerig *et al* (1991), using an antiserum prepared against a bacterially-expressed fusion protein containing part of a ORF within the 2.0Kb LAT, reported an HSV-1 latency associated protein in primary neurons which had been latently infected *in vitro*. This putative 80KDa latency-associated protein has not been identified in latently infected tissues.

1.6.4. Latency promoter

Two LAT promoters (Fig. 1.4) have been identified in HSV-1. The first is a TATA box-containing promoter (referred to as LP1), located approximately 700bp upstream of the 5' end of the major 2.0Kb LAT (Zwaagstra *et al.*, 1991; Batchelor and O'Hare, 1990). The second is a TATA-less promoter termed LP2, located downstream of LP1 and 5' proximal to the major 2.0Kb LAT (Goins *et al.*, 1994). In addition to the TATA box, LP1 contains Sp1-binding sites and a number of potential control elements which could have a role in the expression of LAT during latency. These include a binding site for a nuclear factor called the Latency Promoter Binding Factor (LPBF). The DNA sequence recognised by LPBF is located near the TATA box of the promoter, between -72 and -65 with the sequence CCACGTGG. Removal of this site results in up to a 32-fold loss of promoter activity in transient transfections (Zwaagstra *et al.*, 1991) of both neuronal and non-neuronal cells. LPBF has not yet been characterised and the significance of its binding to the LAT promoter region is unknown. Between the LPBF and the TATA box there is a cAMP response element (CRE) which stimulates LAT expression in the presence of cAMP in cell culture (Lieb *et al.*, 1991). There are potential binding sites for Vmw175 and Vmw65(VP16) in the LAT promoter region (Zwaagstra *et al.*, 1989; Batchelor and O'Hare, 1990). Vmw175 has been reported to repress LAT expression in transient assays (Batchelor and O'Hare, 1990; Rivera-Gonzalez *et al.*, 1994). A similar site for Vmw175, found at the cap site of RS1 is involved in its autoregulation (Roberts *et al.*, 1988). However, Vmw65 has little or no effect upon the level of reporter gene activity directed by the LAT promoter (Batchelor and O'Hare, 1990).

It is not clear what makes the LAT promoter functional during latency. Lokensgard *et al.* (1994) tried to address this issue by constructing several recombinant viruses, some of which contain the upstream HSV-1 LAT(LP1) promoter region combined to the Moloney murine leukemia virus (MMLV) long terminal repeat (LTR), driving expression of the *lac Z* reporter gene at the gC locus. The LTR of MMLV was able to maintain transcription of β-galactosidase during latency in murine DRGs compared to a decrease to undetectable levels in expression from the virus with the LAT promoter

alone. It was concluded that the LTR provides a DNA element designated long-term expression (LTE) element which functions to prevent promoter inactivation during latency. It is possible that the nucleosomal structure of the viral genome represses LAT transcription and the function of LTE may be to keep this region open specifically to allow LAT region transcription to continue. An analogous HSV-LTE region has not mapped, but is predicted to exist downstream of the LAT cap site.

1.6.5. Reactivation from latency

Reactivation of HSV results in the appearance of infectious virus at the site of initial primary infection where lytic phase replication can proceed until the host immune response clears it. It has been shown that very few latently infected neurons can be reactivated and express viral antigens after *in vivo* induction by neurectomy or by hyperthermia (McLennan and Darby, 1980; Sawtell and Thompson, 1992a,b). The molecular basis of reactivation is poorly understood. IE expression has been implicated in the switch from latency to the lytic phase. Leib *et al* (1989a) reported that RL2 deletion mutants of HSV-1(KOS) were able to establish latency in the mouse eye and footpad models but were impaired in reactivation from ganglion explants. In contrast, Clements and Stow (1989) demonstrated that a RL2 deletion mutant of HSV-1(17^+) reactivated efficiently from murine ganglia, although the kinetics of reactivation were slower. The authors suggested that this difference might be due to strain variation or to the extent of the deletions in the mutants. The possibility that the KOS mutants might contain a secondary mutation was not excluded. In an *in vitro* latency system of HSV-2, it has been shown that Vmw110 is required to reactivate latent HSV-2 (Harris *et al.*, 1989; Zhu *et al.*, 1990).

The process of reactivation has been investigated using RNA polymerase chain reaction (RNA-PCR) (Minagawa *et al.*, 1994). It was observed that Vmw110-specific RNA was first detected 24h after explantation while LATs remained detectable 120h after explantation. In contrast, RNAs encoding Vmw175, Vmw63, TK and Vmw65 were detectable within 48h, only following *in vivo* immunosuppressive and antimetabolic

treatment with cyclophosphamide and dexamethasone. It was suggested that reactivation of HSV-1 from trigeminal ganglia occurs in two steps: (i) initiation of Vmw110 gene transcription and (ii) accumulation of other viral transcripts which could be regulated by the host immune system.

The role of nerve growth factor (NGF) in maintenance of latency was demonstrated by Wilcox and Johnson (1987, 1988). The relationship between the levels of NGF and Oct-1 and reactivation was demonstrated by Wood *et al* (1992). In the absence of NGF from sensory neuron cultures, the level of Oct-2 mRNA decreased without affecting the level of Oct-1 which can bind to the TAATGARAT motif to activate gene expression and in turn reactivation (Wood *et al.*, 1992). HSV reactivation can take place by other mechanisms which do not involve the Oct-1/Vmw65 complex. Smith *et al* (1992) reported that treatment of sensory neurons with cAMP can reactivate HSV. An increase in the intracellular cAMP level stimulates transcription of IE genes via a cAMP response element in the IE gene promoter (Wheatley *et al.*, 1992). Moreover, the IE promoters are stimulated by another POU family transcription factor, Brn-3a which is expressed by specific sensory neurons *in vivo*. Brn-3a levels increase following treatment of neuronal cells with cAMP. The ratio of Brn-3a to Oct-2 in neuronal cells may determine whether lysis or latency results from an infection with HSV. A rise in the Brn-3a to Oct-2 ratio may stimulate IE promoters and consequently viral reactivation in latently infected cells which do not express Vmw65 and can not activate IE promoters via an Oct-1/Vmw65 complex (Lillycrop *et al.*, 1994b).

1.7. Pathogenicity of HSV

The pathogenicity of human herpesviruses, HSV-1 and HSV-2, is dependent upon the ability of the virus to replicate and establish latency in the host. Virulence provides a measure of the pathogenicity of a virus. Neurovirulence is the term normally used in connection with central nervous system (CNS) disease induced by a virus. Neurovirulence of HSV is the consequence of (i) peripheral replication (ii) invasion of the CNS (invasiveness) and (iii) growth in the CNS (neurogrowth) (Roizman and Sears,

1990).

A number of animal models have been used to study the neurovirulence of HSV. These include mice (Underwood and Weed, 1974), rabbits (Schlitt *et al.*, 1986) and guinea pigs (Scriba and Tatzber, 1981). The degree of virulence is dependent on host and viral factors. The host factors include age (Zawatzky *et al.*, 1982; Ben-Hur *et al.*, 1983), route of inoculation (Dix *et al.*, 1983), state of skin and mucous membranes (Sprecher and Becker, 1986, 1987), animal strain (Caspary *et al.*, 1980), interferon (Lopez, 1985), humoral immunity (McKendall *et al.*, 1979) and cytotoxic immunity (Nash *et al.*, 1985). Viral factors include the virus type and strain (Halliburton *et al.*, 1987). In some strains, individual plaque stocks of a HSV strain exhibit different degrees of virulence within the same host species (Taha *et al*, 1988). In addition, serial passage of the virus *in vivo* (Kaerner *et al.*, 1983), *in vitro* (Goodman and Stevens, 1986) and body temperature of the host (Thompson and Stevens, 1983a) also affect the outcome of infection.

As the virulence characteristics of each individual HSV strain must be genetically determined, many laboratories have been involved in identifying the genes and gene products that control pathogenicity. A number of genes can be assigned particular biological functions, each of which contributes to pathogenicity. A mutation in a gene that reduces the capacity of the virus to grow peripherally, to invade the CNS or to grow in the CNS could be designated a virulence gene (Roizman and Sears, 1990). These genes will be discussed in the following sections according to progression of the CNS disease in acute HSV infection, although some are involved in more than one virulence function e.g. UL23, UL39-UL40 and RL1 ($\gamma_1$34.5).

1.7.1. HSV genes involved in peripheral replication

Gene function *in vivo* has been defined in experimental animals following inoculation at different sites e.g. foot pad, ear, eye and peritoneum.

1.7.1.1. US7 and US8

The US7 and US8 genes encode the glycoproteins gI and gE, respectively (McGeoch *et*

al., 1985). Balan *et al* (1994) constructed mutants of HSV-1 strain SC16 lacking either gI or gE by inserting a *lacZ* gene. The virulence of the mutants was evaluated using the mouse ear model. At various times post-inoculation, virus titres were determined at the inoculation site, the innervating sensory ganglia (cII, cIII, cIV), spinal cord and brain stem. Mutants lacking gI or gE failed to replicate efficiently at the inoculation site and were barely detectable in sensory ganglia or in the CNS. In epithelial cells *in vitro*, it was found that the mutants were able to adsorb to and enter cells at a normal rate but formed minute plaques due to failure of cell-to-cell spread. It was concluded that the gE-gI complex plays a part in transmission of the virus from infected to uninfected cells by cell contact.

1.7.1.2. UL10

This gene was identified in the HSV-1(17$^+$) genome by McGeoch *et al* (1988a) and encodes a glycosylated membrane protein with 8 transmembrane domains and 2 glycosylation sites (MacLean, C *et al.*, 1991; Baines and Roizman, 1993). The product of the UL10 gene is present in virions, light particles and plasma membranes of infected cells (Baines and Roizman, 1993; MacLean *et al.*, 1993) and has been designated gM (Baines and Roizman, 1993). It is not essential for growth in tissue culture (MacLean, C *et al.*, 1991, 1993; Baines and Roizman 1991). Its role *in vivo* was investigated by MacLean *et al* (1993) using a UL10-deletion mutant of 17$^+$ in the mouse foot pad model. Compared to wild-type and revertant viruses, the mutant was found to be impaired in growth at the periphery, in spread to the nervous system and in reactivation from latency. It was also impaired in growth in the central nervous system and was 35 fold less virulent than the revertant virus.

1.7.1.3. UL56

Intraperitoneal (IP) injection is a common route of inoculation of HSV-1 into laboratory animals. Little is known about genes involved in IP virulence, but recently the UL56 gene has been implicated (Rosen-Wolff and Darai, 1991). The HSV-1 strain HFEM

with a 4.1 Kb deletion between 0.762 and 0.789 m.u. (Koch *et al.*, 1987) was shown to be avirulent in tree shrews (Rosen-Wolff and Darai, 1985) and mice (Becker *et al.*, 1986) following IP inoculation. Virulence was restored to HFEM when rescued with *Bam*HI-*b* (0.738-0.809 m.u.) or a *Mlu*I fragment from 0.7615-0.789 m.u. or *Hpa*I-*p* (0.762-0.787 m.u.) from the virulent strain F (Rosen *et al.*, 1985; Rosen *et al.*, 1986; Becker *et al.*, 1986). Comparative analysis of HSV-1 strain F and HFEM revealed the absence of a 1.5Kb RNA in cells infected with HFEM. This transcript was found to be that of the UL56 gene (Rosen-Wolff and Darai, 1991).

1.7.2. Genes involved in neuroinvasiveness of HSV

The neural spread of HSV from the site of primary infection to the central nervous system (CNS) requires viral functions which are discussed below:

1.7.2.1. UL23

UL23 encodes thymidine kinase (TK) which is a key enzyme in the *de novo* synthesis of precursors for DNA synthesis (Kit and Dubbs, 1965; Klemperer *et al.*, 1967). It has been shown that TK-deficient (TK^-) mutants of HSV-1 and HSV-2 are less virulent than TK^+ viruses following either peripheral (mouse ear) or intracerebral inoculation (Field and Wildy, 1978). Tenser *et al* (1979, 1981) reported that TK^- mutants of HSV-1 (KOS) replicate efficiently in the cornea but are defective in replication in the trigeminal ganglion. Consistent with this was the report by Stanberry *et al* (1985) that the TK producing HSV-2 strains 333 and MS replicated to high titre in the vagina and spinal cord of guinea pigs while TK^- viruses resulted in similar vaginal titres but low spinal cord titres. Latent and recurrent infections were observed in animals inoculated with either TK^+ or TK^- viruses. This demonstrated that TK is not essential for establishment of latent infections but may be necessary for efficient virus reactivation or virus replication following reactivation (Tenser *et al.*, 1989, Coen *et al.*, 1989; Efstathiou *et al.*, 1989).

The role of TK in viral pathogenesis is of special clinical interest because TK^- mutants

are resistant to the widely used anti-HSV drug, acyclovir (Field *et al.*, 1980). Such TK$^-$, acyclovir (ACV) resistant mutants can arise during treatment of HSV infection with ACV (Kit *et al.*, 1987). The resistance stems from the requirement for viral TK to activate ACV efficiently (Elion *et al.*, 1977).

1.7.2.2. UL30

UL30 encodes DNA polymerase, a key enzyme in replication of herpesvirus DNA *in vivo*. HSV strains with a defect in UL30 show reduced virulence as demonstrated from impaired replication in the central nervous system. Mutations in this gene also confer resistance to one or more antiviral compound. Larder *et al* (1986) reported that a defect in DNA polymerase accounted for the reduction in virulence of the variant RSC-26 of HSV-1 strain SC16 in the mouse ear model. This variant contains a single nucleotide change at position 1790 which confers resistance to ACV and phosphonoacetic acid (PAA). Recombinants generated from rescuing RSC-26 with a wild-type DNA fragment containing the DNA polymerase gene, restored the wild-type phenotypes of sensitivity to antiviral drugs, DNA polymerase activity and virulence. The pathogenicity of other polymerase mutants has been determined by Field and Coen (1986).

A connection was established between the neuroinvasiveness of a number of HSV strains and the DNA sequence between 0.31-0.44 m.u. of the HSV genome (Oakes *et al* 1986). HSV-2 strain 186 was found to be non-neuroinvasive following ocular inoculation although it has normal TK activity and is virulent following intracerebral inoculation. The HSV-1/HSV-2 intertypic recombinant HSV-R (B1E), in which HSV-2 DNA sequences between 0.31-0.44m.u. were exchanged with HSV-1 (17$^+$) DNA sequences, acquired the ability to spread from the cornea to the trigeminal ganglia and brain. This region of the genome is located within the sequences previously shown by Thompson *et al* (1986) to be responsible for invasion of the CNS following footpad inoculation (m.u. 0.25-0.53). This function was later attributed to the viral DNA polymerase gene (Day *et al.*, 1988).

1.7.2.3. UL50

This gene codes for the enzyme deoxyuridine triphosphate nucleotidohydrolase (dUTPase) which catalyses the hydrolysis of dUTP to dUMP and pyrophosphate (Wohlrab and Francke, 1980; Caradonna *et al.*, 1981). The gene encoding dUTPase has been mapped to UL50 (McGeoch *et al.*, 1988a). In tissue culture, dUTPase is not essential for HSV replication (Fisher and Preston, 1986; Williams, 1988; Barker and Roizman, 1990). Infection with wild-type HSV-1 results in down regulation of the cellular dUTPase while infection with dUTPase deficient mutants does not (Williams, 1988). In the mouse model, Pyles *et al* (1992) demonstrated that dUTPase is an important virulence and neuroinvasiveness determinant. Two dUTPase negative mutants (dUT^-) of HSV-1 were characterised *in vitro* and *in vivo*. The 1218 mutant contains an insertion of four codons in the dUTPase reading frame (Fisher and Preston, 1986) and the 17B1 mutant has a deletion of 928bp and encodes only the first six amino acids of the protein. Like the wild type HSV-1(17^+), both mutants replicate efficiently in primary mouse embryo cells. The mutants were 10-fold less virulent than 17^+ following intracerebral inoculation and 1000-fold less virulent following footpad inoculation. The replication kinetics of mutants compared to 17^+ were assayed following footpad inoculation to determine the anatomical location of the invasiveness restriction. It was found that both mutants replicated with wild type kinetics in non-neural tissues (footpad) and entered and replicated efficiently in the peripheral nervous system (sciatic nerve and dorsal root ganglia). Replication was reduced in the spinal cord indicating that the mutants were defective in entry into or replication in the central nervous system. However, the mutants were capable of establishing latent infection but demonstrated a reduced reactivation frequency. The reactivation frequency of the 17B1 mutant was half that of the wild-type by seven days post DRG explantation. The mutants were severely impaired in *in vivo* reactivation following hyperthermia induction (heat stress) (Sawtell and Thompson, 1992a). 19% and 21% of DRG infected respectively with 1218 and 17B1 reactivated compared to 82% of the 17^+-infected DRG. Restoration of the dUTPase gene resulted in wild-type levels of virulence, neuroinvasiveness and

reactivation frequency from latent infection.

1.7.2.4. US6

US6 encodes the glycoprotein D which is required for virus penetration into cells (Fuller and Spear, 1987; Highlander *et al.*, 1987) and has also been implicated in neuroinvasiveness. Kumel *et al* (1982) reported that the HSV-1 strain ANG is non-neuroinvasive following intraperitoneal, intravaginal or footpad inoculation. The generation of the neuroinvasive variant, ANG path following serial passage of the ANG strain in mouse brain was reported by Kaerner *et al* (1983). ANG path was able to spread from the site of inoculation to the central nervous system following intraperitoneal (Kaemer *et al.*, 1983) or footpad (Izumi and Stevens, 1990) inoculation. The increase in neuroinvasiveness of ANG path was attributed to a single amino acid substitution, from alanine to glycine, at position 84 of gD (Izumi and Stevens, 1990).

1.7.2.5. UL27

This gene codes for the glycoprotein B (gB) which is involved in viral fusion and entry into cells (Cai *et al.*, 1988). Little is known about the role of gB in the virulence of HSV. It was shown that the HSV-1 strain KOS is non-neuroinvasive being blocked at the level of the spinal ganglia following footpad inoculation. The neuroinvasiveness of KOS was enhanced when its genome was recombined with the *Hind*III-*a* fragment mapping between 0.25-0.53 m.u. (Thompson *et al.*, 1986). The UL27 gene is located in *Hind*III-*a* and it was shown to contain a single base change that appeared to affect the neuroinvasiveness phenotype of KOS. Another region of the genome which was responsible for the non-neuroinvasiveness of this strain was localised to the 6.3Kb *Bam*HI-*a*/*Eco*RI-*d* fragment (0.15-0.19 m.u.) containing the entire UL10, UL11, UL12, UL13 and part of the UL9 and UL15 open reading frames. It is not yet known which of these ORFs is responsible for the non-neuroinasiveness of KOS (Yuhasz and Stevens, 1993).

1.7.2.6. UL5

In previous studies, Javier *et al* (1988a) demonstrated that the HSV-1(17$^+$)/HSV-2(HG52) intertypic recombinant (R13-1) was attenuated by 10^5 fold in virulence following intracerebral inoculation of mice. Bloom and Stevens (1994), found that following footpad inoculation, no virus was detected in the brain and a reduced amount of R13-1 was found in the sciatic nerve, dorsal root ganglia and spinal cord. Examination of the spinal ganglia by immunofluorescence revealed that R13-1 is restricted in replication in neurons but not in glial cells. A few neurons of R13-1-infected ganglia expressed viral antigens but most antigens were found in supporting cells. It was found that the block in the infectious cycle occurred prior to the initiation of late events. R13-1 produced normal levels of early RNA, but production of DNA and late RNA was less than that of the wild-type. In R13-1, the UL5 gene of HSV-1 is replaced by that of HSV-2. Marker rescue of R13-1 with the HSV-1 UL5 gene restored the virulence of R13-1 to that of wild-type. The UL5 product is a component of the helicase-primase complex, essential for DNA replication. It interacts with two other virally encoded components, UL8 and UL52 to form this complex (Crute *et al.*, 1989). In R13-1, the origin-binding complex is composed of UL5 and UL8 products from HSV-2 and the UL52 product from HSV-1. It was postulated that replacement of UL5 with its HSV-2 counterpart results in the formation of an altered helicase-primase complex, although a mutation in HSV-2 UL5 was not ruled out.

1.7.3. HSV genes involved in intracerebral virulence (Neurogrowth)

1.7.3.1. UL53

This gene is known to be the locus of a mutation that causes a syncytial plaque phenotype (Debroy *et al.*, 1985; Pogue-Geile and Spear, 1987). UL53 encodes a glycosylated protein, gK (Hutchinson *et al.*, 1992b) which contains four transmembrane regions and two N-glycosylation sites (Debroy *et al.*, 1985; McGeoch *et al.*, 1988a). The product of UL53 was found to be responsible for intracerebral pathogenicity (IC) using

two intratypic recombinants, R-15 and R-19 (Moyal *et al.,* 1992). The molecular basis of pathogenicity of R-15 and R-19 was analysed by Ben-Hur *et al* (1987). Both R15 and R19 were generated by cotransfection of HFEM DNA with *Bam*HI-*b* (0.738-0.809 m.u.) from strain F, to replace the deletion in the HFEM genome (0.762-0.789 m.u.). On intracerebral inoculation of mice, R-19 was as virulent as the parental HFEM while R-15 was avirulent (Rosen *et al.*, 1985). The recombinant R-15 regained virulence when rescued with the *Bam*HI fragment *l* (0.70-0.738 m.u.) from R-19. The virulence function was localised to a 2.0Kb *Nru*I-*Bam*HI fragment which contains the UL53 gene. The virulence phenotype of the recombinants was linked with the morphology of the plaques. R-19 produces large syncytial plaques in monkey kidney BSC-1 cells while R-15 produces tiny syncytial plaques. Nucleotide sequence analysis was determined for the UL53 gene in R-15 and R-19 and three mutations were found in the amino acid sequence of UL53 of R-15 which were considered responsible for its avirulence phenotype and small plaque morphology (Moyal *et al.*, 1992).

1.7.3.2. UL39-UL40

These genes code for the ribonucleotide reductase (RR) which is another key enzyme in the *de novo* synthesis of precursors for DNA synthesis. RR reduces the four ribonucleotide diphosphates to 2'-deoxyribonucleotide diphosphates (Thelander and Reichard, 1979). The enzyme consists of two non identical subunits (Cohen *et al.*, 1985); the large subunit termed RR1 (*M*r 140000; also known as ICP6 for HSV-1 and ICP10 for HSV-2) and the small subunit termed RR2 (*M*r, 38000) which are encoded by the UL39 and UL40 genes, respectively (McGeoch *et al.*, 1988a). Despite the essential role of RR in DNA synthesis, in certain tissue culture systems RR is not essential for growth of HSV in dividing cells (Goldstein and Weller, 1988a). Studies with a deletion mutant (ICP6Δ) lacking 90% of the coding region of UL39 demonstrated that RR1 is essential for growth in dividing cells at high temperature (39.5°C) and in non-dividing cells under serum starved conditions. It was concluded that actively dividing cells contain a factor(s) which can complement the function of RR1 and that this factor(s) is

limited or absent in both growth restricted cells and cells at 39.5°C (Goldestein and Weller, 1988a,b). The enzyme appear to be essential for productive acute and reactivable latent infections in mice. In the eye model, Jacobson *et al* (1989) have shown that the RR deletion mutant ICP6Δ is severely impaired in growth in the eye and trigeminal ganglia and failed to establish reactivable latent infections. The mutant also grew poorly in human primary corneal fibroblasts, suggesting that RR may be required for virulence in human infections (Brandt *et al.*, 1991). This finding was supported by Yamada *et al* (1991) when testing the pathogenicity of RR-null mutants which failed to grow in the cornea and in the nervous tissue of 4-week old mice. Mutants were detected in brain tissues following superinfection with a TK-deficient mutant of HSV-1. In hairless guinea pigs, the ICP6Δ mutant produces cutaneous lesions as severe as those of the wild-type HSV-1 (Turk *et al.*, 1989). Cameron *et al* (1988) found that temperature sensitive mutants, *ts*1207 and *ts* 1222 with lesions in either subunit were about 10^6 fold less virulent in mice than the parental HSV(17^+) following intracerebral inoculation.

1.7.3.3. RL1 (γ_1 34.5)

Several laboratories have associated loss of capacity of HSV-1 or HSV-2 strains to cause death following intracerebral inoculation of mice, with genetic defects located at or near the termini of the long component of the genome. The existence of a virulence locus in the long repeat region of HSV was first demonstrated by Thompson and Stevens (1983b) using the 17^+/HG52 intertypic recombinant, RE6. Following intracerebral inoculation of mice, RE6 was avirulent with a LD_{50} of 3.2×10^7 pfu/mouse compared to 10 pfu/mouse for strains 17^+ and HG52. Neurovirulence was restored to RE6 by HSV-1 DNA sequences between 0.71 and 0.83 map units (Thompson *et al.*, 1983). Subsequently further mapping localised this to a *SstI/Xho*II (0.82 and 0.832 m.u.) of the *Hind* III-*c* subfragment (Thompson *et al.*, 1989). The same region was also implicated in virulence of HSV by Javier *et al* (1987) using another intertypic recombinant, RS6.

In our laboratory, a genomic region associated with HSV virulence has been determined using a deletion variant JH2604 of HSV-2 strain HG52 (Taha *et al.*, 1989a,b). This

variant has a deletion of 1488bp within each copy of *Bam*HI-*v* (0-0.02 m.u. and 0.81-0.83 m.u.) of R_L including one copy of the 17bp direct repeat element of the '*a*' sequence and terminating 522bp upstream of the 5′ end of the RL2 gene. JH2604 was shown to be avirulent in Balb/c mice with a $LD_{50} > 10^7$p.f.u./mouse compared to $<10^2$p.f.u./mouse for the wild-type HG52. When the deleted sequence was introduced into the parental virus genome, avirulent recombinants with an $LD_{50} > 10^7$ p.f.u./mouse were produced. Construction of revertants confirmed the localisation of a virulence locus. The avirulent phenotype of JH2604 was due to inability to replicate within neuronal cells of the brain as demonstrated by immunohistochemical staining using polyclonal anti-HSV serum. HSV antigens were widely distributed in neuronal and supporting cells inoculated with wild-type HG52. In JH2604 infected brains, virus antigens were located only at the site of inoculation (Taha *et al.*, 1990). The failure of JH2604 to multiply and spread in the CNS could have been due to poor packaging of the virus as a result of absence of the normal '*a*' sequence function. To exclude this possibility, another variant, 2616 which retained the DR1 element of the '*a*' sequence but had a deletion of 786bp extending from 782bp upstream of the 5′ end of RL2 to 463bp downstream from the '*a*' sequence was isolated. 2616 was also avirulent following intracerebral inoculation of mice. This result demonstrated that the deletion of the DR1 element of the '*a*' sequence did not contribute to avirulence (Harland and Brown, 1991). A sequence in an equivalent genomic location in HSV-1 strain 17^+ was shown to play a role in neurovirulence (MacLean, A *et al.*, 1991).

A diploid gene designated γ_1 34.5 (RL1) has been localised to R_L of the HSV-1 genome, between RL2 and the '*a*' sequence. The promoter and transcriptional initiation site of RL1 are located in the terminus of the '*a*' sequence (Chou and Roizman, 1986). In HSV-1 strain F, RL1 was predicted to encode the protein ICP34.5, a 43.5K polypeptide which contains the triplet Pro-Ala-Thr (PAT) repeated 10 times $[(PAT)_{10}]$ (Chou and Roizman, 1986). In a subsequent study, Chou and Roizman (1990) revised the sequence of γ_1 34.5, by making 25 alterations to the original sequence resulting in the gene containing only 263 codons instead of the previously published 358 codons

(Ackermann *et al.*, 1986). The revised sequence of HSV-1 strain F was almost identical to the published HSV-1 strain 17+ sequence (Perry and McGeoch, 1988), with the only major difference being a 2bp insert in the strain 17+ sequence. The two extra nucleotides rendered 60% of the 17+ sequence, including the PAT repeat, out of frame (Dolan *et al.*, 1992). Revision of the 17+ sequence identified an equivalent ORF, designated RL1, to that in strain F (Dolan *et al.*, 1992). RL1 in 17+ encodes a polypeptide of 248 amino acids which contains 5 copies (Fig. 1.5) of the PAT sequence (McKay *et al.*, 1993). In HSV-1 strain F the protein migrates at 39K and in 17+ at 37K (McKay *et al.*, 1993). The number of copies of the PAT sequence was found to be variable among other HSV-1 strains (Chou and Roizman, 1990) and is absent in RL1 of HSV-2 strain HG52 (McGeoch *et al.*, 1991).

The ICP34.5 protein was detected in lysates of Hep-2 cells infected with HSV-1(F) using an antiserum raised against a peptide consisting of 10 copies of the PAT repeat (Ackermann *et al.*, 1986). A similar antiserum recognised ICP34.5 from HSV-1 strain F infected BHK cell extracts but was approximately 30-fold less efficient at detecting the strain 17+ protein. This difference in detection level was attributed to either a lower level of ICP34.5 expression in HSV-1(17+)-infected cells or to a lower antibody affinity, as ICP34.5 of HSV-1(17+) contains only five copies of the PAT sequence (McKay *et al.*, 1993). To facilitate detection of ICP34.5 in strain 17+-infected cells, the RL1 gene was expressed in a bacterial expression system and the protein used to raise a polyclonal anti-ICP34.5 serum (McKie *et al.*, 1994). Using this antiserum, ICP34.5 HSV-1(17+) was detected at levels comparable to that of HSV-1(F) indicating that the previous differences were due to weak antibody affinity (McKay *et al.*, 1993). Ackermann *et al* (1986) and McKay *et al* (1993) using cell fractionation studies reported the accumulation of ICP34.5 largely in the cytoplasm of infected Hep-2 and BHK21/C13 cells, respectively. Using the polyclonal serum in fluorescence studies, the protein was localised to dense punctuate patches with minimal perinuclear staining in the cytoplasm of BHK21/C13 cells infected with HSV-1(17+) (McKie *et al.*, 1994).

Insertion of a translational stop codon at the beginning of the RL1 coding sequence

abolished the virulence phenotype of HSV-1 strains F and 17+. The mutant viruses grew well in culture but failed to grow and spread in the CNS of mice after intracerebral inoculation. Reinsertion of the wild-type genes into these mutants restored the virulence phenotype (Chou *et al.*, 1990; McKie *et al.*, 1994). Recently, Bolovan *et al* (1994) suggested a generalised function for ICP34.5. The requirement of ICP34.5 for productive infection of the cornea was demonstrated using the mutant 17termA of HSV-1(17+). This mutant contains an insertion of 20bp in both copies of RL1 at the *Bst*EII site which results in termination of ICP34.5 after the N-terminal 30 amino acids. In the eyes of four week-old Swiss Webster mice, the mutant did not replicate efficiently and could not be recovered from the eyes and the trigeminal ganglia 3 days post-inoculation, although 17+ was recovered until day 7 post-inoculation. Similar findings with the RL1 deletion mutant, 1716 and a stop codon insertion mutant, 1771 were demonstrated by Spivack *et al* (1995) in a mouse eye model. In contrast, the mutant 17termA was as efficient as 17+ in replication in the foot pad (Bolovan *et al.*, 1994). The role of ICP34.5 in neuroinvasiveness was also demonstrated by Bolovan *et al* (1994). The mutant 17termA was avirulent in Swiss Webster mice following footpad or ocular inoculation and the mice did not display any signs of central nervous system disorder. The reduction in the neuroinvasiveness of the mutant was due to severe restriction in replication in the dorsal root and trigeminal ganglia. A similar finding was reported for both 1716 and 1771 following footpad inoculation of mice (Robertson *et al.*, 1992; Spivack *et al.*, 1995).

Nucleotide sequence analysis of the corrresponding region of the HSV-2 strain HG52 genome identified an equivalent RL1 ORF. The HSV-2 HG52 RL1 coding sequence starts at an ATG which aligns with the initiating ATG of HSV-1 strain F (Chou and Roizman., 1990) and strain 17+ (McGeoch *et al.*, 1991). In HG52, the coding sequence is interrupted by a set of repeated sequences consisting of six complete copies and one partial copy of a 19bp element which contains a TAG stop codon blocking all forward reading frames (Fig. 1.5). McGeoch *et al* (1991) considered that this repeat family which is flanked by excellent candidate splice donor and acceptor sites must be an

intron, removal of which would bring the coding sequence back into frame. The spliced type 2 gene is predicted to encode a protein of 261 amino acids with 83% of the carboxy terminus being homologous (Fig. 1.5) to that of HSV-1 (McGeoch *et al.*, 1991) and to approximately 55% of the mouse protein MyD116 (McGeoch and Barnett, 1991). A corresponding domain is found in the LMW23-NL gene of the African Swine fever virus (Sussman *et al.*, 1992) and in the hamster GADD34 gene (Chou and Roizman, 1994) induced by growth arrest signals and DNA damaging agents (Fornace *et al.*, 1989).

1.8. Aims of the project.

The work presented in this thesis had the following objectives:

1. To confirm that the predicted ORF of RL1 in HSV-2 strain HG52 was correct by constructing a RL1 null mutant virus.

2. To confirm the presence of the intron in the predicted ORF of RL1 and investigate its role in gene expression by constructing an intronless mutant virus.

3. To determine whether the 189bp conserved region played a role in virulence of HG52 by making truncated versions of RL1.

4. To characterise the phenotype of mutant viruses *in vivo* and *in vitro* by comparison with the parental wild-type virus HG52.

CHAPTER 2

MATERIALS AND METHODS

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2.1. MATERIALS.

2.1.1. Cells

Baby hamster kidney 21 clone 13 (BHK21/C13) cells (MacPherson and Stoker, 1962) and 3T6 mouse embryo fibroblasts (Todaro and Green, 1963) were used throughout this study.

2.1.2. Viruses

The wild-type parental virus HSV-2 strain HG52 plaque 17 (Timbury, 1971; Taha *et al*., 1988) and the avirulent RL1 deletion mutant 2604 (Harland and Brown, 1985) were used. Viruses were propagated and titrated in BHK21/C13 cells.

2.1.3. Cell culture media

BHK21/C13 cells were grown in Eagle's medium (Gibco) supplemented with 10% (v/v) newborn calf serum (Gibco), 10% (v/v) tryptose phosphate broth, 200mM L-glutamine (Gibco), 10,000 μg/ml penicillin (Gibco) and 10,000 μg/ml streptomycin (Busby *et.al.*, 1964). This medium will be referred to subsequently as ETC10.

Variations on the basic growth medium were:

PIC	Phosphate-free Eagle's medium containing 1% newborn calf serum
EMC10%	Eagle's medium containing 1.5% carboxymethyl cellulose and 10% newborn calf serum.

3T6 mouse embryo fibroblasts were propagated in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% foetal calf serum (Gibco), 200mM glutamine (Gibco), 10,000 μg/ml penicillin (Gibco) and 10,000 μg/ml streptomycin (Brown *et al*., 1994a).

2.1.4. Experimental animals

Three week old female Balb/c mice (Bantin and Kingman; Harlan U.K. Ltd.) were used in this study.

2.1.5. Bacterial and phage strains

Escherichia coli strain NM522 [supE, thi, (lac-proAB), hsd5(r-, m-), (F' proAB, lac192 M15)] (Gough and Murray, 1983) and *E. coli* strain BW313 (recA1, spoT1, ung$^-$, dut$^-$, thi-, Hfr KL16(PO45) (Kunkel, 1985) were used for transformation and growth of recombinant plasmids and phagemids. The bacteriophages M13KO7 and R408 ($\geq 10^{11}$ p.f.u./ml), were used for preparation of uracil-rich single-stranded DNA templates for site-directed mutagenesis. Both phages and *E. coli* strain NM522 were obtained from Promega while *E. coli* strain BW313 was kindly provided by Dr. R. Thompson.

2.1.6. Bacterial growth media

The media used for bacterial growth were:

L-broth	10g/l NaCl, 10g/l bactotryptone (Difco), 5g/l yeast extract (Difco).
L-broth agar	L-broth containing 1% (w/v) bactoagar (Difco).
2xYT	5g/l NaCl, 16g/l bactotryptone (Difco), 5g/l yeast extract (Difco).
Top agar	0.6% (w/v) bactoagar (Difco) in water.

2.1.7. Oligonucleotides

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

2.1.8. Plasmids

The starting plasmid used for construction of RL1 containing recombinant phagemids was pAT153 BamHI *g* . This plasmid contains the wild-type HG52 BamHI *g* fragment and was kindly provided by Dr. A. Davison. Subfragments of BamHI *g* containing the RL1 ORF were cloned into the phagemid pGEM3zf(-) (Promega).

2.1.9. Enzymes

Restriction enzymes were obtained from New England Biolabs. Exceptions were:

Avian Myeloblastosis Virus reverse transcriptase Promega

Calf intestinal alkaline phosphatase (CIP)	Boehringer Mannheim Corporation
Lysozyme	Sigma Chemical Co.
Moloney Murine Leukemia virus reverse transcriptase	BRL-GIBCO
Retrotherm	Cambio
Ribonuclease A	Sigma Chemical Co.
T4 DNA ligase	Boehringer Mannheim Corporation
T4 polynucleotide kinase	Boehringer Mannheim Corporation
T7 DNA polymerase	Pharmacia

2.1.10. Radioisotopes

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

[^{32}P]-Orthophosphate	3000Ci/mmol
5' [$\alpha - {}^{32}$P] dNTPs	~3000Ci/mmol
[$\alpha - {}^{35}$S] dATP	400Ci/mmol

2.1.11. Chemicals

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

Acetic acid glacial	May and Baker Ltd., Dagenham, England
Ampicillin sodium B.P. (Penbritin)	Beecham Research
APS and TEMED	Bio-Rad Laboratories
Boric acid and glycerol	PROLABO
Caesium Chloride and acrylamide	Koch Light Ltd. (Suffolk, England)
2′-Deoxynucleotide-5′-triphosphate (dNTPs)	Pharmacia
Gene 32 protein (3.7mg/ml)	Pharmacia
Guanidine thiocyanate	Fluk Biochemika

Hexadeoxyribonucleotides [pd(N)6]	Pharmacia
Isopropyl -β-D-thiogalactoside	Bethesda Research Laboratories (BRL)
NuSieve agarose	FMC BioProducts

2.1.12. Commonly used buffers and solutions

Alkaline lysis solution I	10mM EDTA, 50mM glucose, 25mM Tris-HCl pH 8.0 and 5mg/ml lysozyme added prior to use.
Alkaline lysis solution II	200mM NaOH and 1% (w/v) SDS.
Alkaline lysis solution III	5M potassium acetate pH4.8.
Chloroform:isoamylalcohol	This is a 24:1 mixture of chloroform and isoamylalcohol
DEPC-treated water	0.5ml of DEPC in 500ml d.H_2O.
Formamide dye mix	95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol.
Gel soak I	3M NaCl, 1M NaOH.
Gel soak II	600mM NaCl, 1M Tris-HCl, adjusted to pH8.0 with HCl.
Hybridization buffer	7% SDS, 0.5M $NaP0_4$ (NaH_2PO_4; Na_2HPO_4), pH7.4.
Ligation buffer (10x)	200mM DTT, 100mM $MgCl_2$, 0.5M Tris-HCl, pH 7.8.
10xMOPS buffer	200mM MOPS, 50mM sodium acetate, 10mM EDTA.
Phenol chloroform (1:1)	This is a 1:1 mixture of phenol and chloroform.
PBS -A	170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH7.2.
PBS complete	PBS A plus 6.8mM $CaCl_2$, 4.9mM $MgCl_2$.
PBS /calf serum	PBS complete containing 5% newborn calf serum.
Random primer buffer (5x)	Three solutions A, B, C at a ratio of 10:25:15 respectively: solution A: 0.25M 2-mercaptoethanol, 0.5M dGTP or dCTP, 0.5M dATP, 0.5M dTTP, 1.25M Tris-HCl, pH7.6, 0.125M MgCl2.

	solution B: 2M HEPES, pH6.6.
	solution C: Hexadeoxyribonucleotides resuspended in TE at 90 OD units/ml.
RE stop	100mM EDTA, 10% (w/v) Ficoll 400, 0.25% (w/v) bromophenol blue, 5x TBE.
RNA-loading buffer	30% glycerol, 5% bromophenol blue
Saturated phenol	Phenol saturated by mixing 1:1 with phenol saturation buffer, (10mM Tris-HCl, pH7.5, 10mM EDTA, 100mM NaCl).
SSC (20x)	300mM trisodium citrate, 260mM NaCl.
T4 DNA polymerase buffer (10x)	1M Tris-HCl pH7.9, 1M $MgCl_2$, 1M DTT.
TBE (10x)	2mM EDTA, 89mM boric acid, 89mM Tris-HCl pH8.0.
TE	1mM EDTA, 10mM Tris-HCl pH8.0.
Tris-saline	140mM NaCl, 30mM KCl, 280mM Na_2HPO_4, 1mg/ml glucose, 0.0015% (w/v) phenol red, 25mM Tris-HCl (pH7.4), 100 units/ml penicillin, 0.1 mg/ml streptomycin.
Trypsin	0.25% (w/v) Difco trypsin dissolved in Tris-saline.
Trypsin-Versene	1 volume trypsin + 4 volumes versene.
Versene	0.6mM EDTA dissolved in PBS containing 0.002% (w/v) phenol red.
X-gal	25mg/ml in dimethylformamide.

2.1.13. Miscellaneous

Hybond N hybridization transfer membranes	Amersham
Giemsa stain	BDH
Polaroid- 667 film	Polaroid (U.K.)

Repelcote	BDH Chemicals
XS-1 film	Kodak

2.2. METHODS

2.2.1. Growth of cells

BHK21/C13 cells were grown in 80oz roller bottles containing 100ml ETC10 medium at 37°C for 3 days in an atmosphere of 95% air and 5% CO_2 (MacPherson and Stoker, 1962). Confluent cells were harvested by washing the monolayers twice with 25ml of trypsin-versene, and resuspending the detached cells in 20ml of ETC10. Cells were stored for a short-term (one week) at 4°C in the refrigerator . When required, they were passed and for some experiments were plated on 60mm or 30mm petri dishes or Linbro wells at a density of $4x10^6$, $2x10^6$ and $5x10^5$ cells per plate, respectively.

3T6 mouse embryo fibroblasts were grown and harvested under the same conditions using Dulbecco's Modified Eagle's medium. The yield of cells was determined as described by Freshney (1994) using the improved Neubauer counting chamber (Weber, England).

2.2.2. Long-term storage of cells

To provide a large supply of cells for experimental purposes, personal user stocks of cells were prepared and stored in a liquid nitrogen freezer. For this purpose, cells were grown in a 600ml culture flask under the conditions described in Section 2.2.1. Confluent cells were harvested, resuspended in 5ml medium containing 10% DMSO and aliquoted at $1x10^6$ cells/ml into glass black capped vials. The vials were wrapped with cotton wool and placed in a polystyrene foam box lined with several layers of napkins to provide sufficient insulation during freezing at -70°C at a rate of 1°C/min. Following overnight freezing at -70°C, vials of cells were transferred rapidly to a liquid nitrogen freezer (Freshney, 1994). When required, cells were recovered by rapid thawing in a 37°C water bath. Cells were transferred to a 50ml culture flask and warm medium was added. Following 8-24h incubation at 37°C, medium was removed from the flask and replaced with a fresh DMSO-free medium. Incubation at 37°C was continued for a further 2-3

days. Confluent monolayer of cells was trypsinized and cells were grown at 37°C in a 600ml culture flask. The yield from this flask was split between other flasks and a large supply of cells was grown.

2.2.3. Growth and harvest of HSV

Virus stocks were grown as described by Brown *et al* (1973). Confluent roller bottles were infected with 0.003 p.f.u./cell of HSV in 20ml of ETC10, assuming 1×10^8 cells per roller bottle. These were incubated at 31°C for 4 -5 days, until c.p.e. was complete, when the cells were shaken into the medium. The cells were pelleted in 250ml plastic Falcon tubes by spinning at 2K for 10 minutes at 4°C in a Fison's Coolspin. The supernatant and cell pellet were separated and two individual virus stocks prepared:

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

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

In many instances the cell-associated and the supernatant stocks were mixed before aliquoting and storing at -70°C.

2.2.4. Sterility checks on virus stocks

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

To check for fungal contamination of virus stocks, a small aliquot was streaked in duplicate onto BHI plates, which were sealed with parafilm and incubated at RT. Bacterial contamination was detected by plating onto BHI blood agar and incubating at 37°C. If no

colonies were visible after 7 days incubation, the stocks were considered sterile.

2.2.5. Titration of virus stocks

Virus stocks were serially diluted 10-fold in PBS/calf serum. 100µl aliquots were added to 75% confluent monolayers of BHK21/C13 cells on 60mm petri dishes from which the medium had been removed. The plates were incubated at 37°C for 45-60 min to allow absorption of the virus to the cells, before overlaying with 6ml EMC10%. Plates were incubated at 37°C or 38.5°C for 2 days, or at 31°C for 3 days. Monolayers were fixed and stained with Giemsa at RT for 1h. After washing, plaques were counted using a dissecting microscope and virus titres calculated as p.f.u./ml.

2.2.6 Temperature sensitivity

Confluent BHK/C13 cells were infected at a multiplicity of 5 p.f.u./cell. Following separate 24h incubation at 31°C, 37°C, or 38.5°C, the monolayers were harvested, sonicated and the progeny virus titrated on BHK/C13 cells at 37°C.

2.2.7. Preparation of HSV DNA

The DNA of HSV stocks was prepared according to the method of Wilkie (1973) and Brown *et al* (1984). To prepare a large scale HSV DNA stock, 10 roller bottles containing almost confluent monolayers of BHK21/C13 cells were infected at 31°C at a m.o.i. of 0.003 p.f.u./cell. The infection was allowed to proceed until c.p.e. was extensive (4-5 days). The cells were shaken into the medium and spun at 2K for 10 minutes in a Fison's Coolspin. The supernatant was kept on ice while the nuclei were extracted from the cell pellet by treatment with 0.5% (w/v) NP40 in RSB (10mM KCl, 1.5mM $MgCl_2$, 10mM Tris-HCl, pH 7.5) followed by centrifugation (2K for 10 minutes) to pellet the cell debris and nuclei. This was repeated before pooling the supernatants and spinning to pellet the virus at 12K for 2h in a Sorvall GSA rotor.

The virus pellet, containing cell released and cytoplasmic virus was resuspended in NTE buffer (10mM Tris-HCl, pH7.5, 10mM NaCl, 1mM EDTA) before adding EDTA and

SDS to a final concentration of 10mM and 2% (w/v) respectively to lyse the virus. Viral DNA was extracted 3-4 times with saturated phenol and once with chloroform: isoamylalcohol, prior to precipitating with 2 volumes of ethanol at RT for 5 minutes. DNA was pelleted at 2K for 10 minutes, washed with 70% ethanol, dried at 37°C and resuspended in a minimal volume of H_2O containing RNase A (50µg/ml).

2.2.8. *In vivo* ^{32}P labelling of viral DNA

This is a modification of the method described by Lonsdale (1979). Confluent monolayers of BHK21/C13 cells in Linbro wells were infected with either 100µl of a plate stock or $2x10^6$ p.f.u. of a virus stock. After absorption for 45 minutes at 37°C, the virus was removed and the cells washed with and maintained in PIC for 2h at 31°C. One µCi of orthophosphate (^{32}P) in 50µl PIC was added per well and incubation continued at 31°C for 2-5 days. At the end of the incubation period, 0.5ml of 5% (w/v) SDS was added to each well. Trays were incubated at 37°C for 5-10 minutes before scraping the cells off the wells and adding them to 1ml of phenol. The samples were inverted and incubated at RT for 10 minutes, with a further inversion after 5 minutes. They were centrifuged at 2K for 10 minutes at RT in a Fison's Coolspin before removing the top layer into 2 volumes of ethanol. After gently inverting the tubes, they were spun as before. The ethanol was poured off and the DNA dried for 10 minutes at 37°C with the tubes in an inverted position. Two hundred µl of a RNAse A (50µg/ml) solution were added to each sample and the DNA left to dissolve for 2h at 37°C. The DNA was then ready for digestion with the appropriate enzyme(s).

2.2.9. Transfection of DNA into cells using the calcium phosphate technique

This method is a modification of that described by Stow and Wilkie (1976). Five hundred µl HEBS, pH7.05, (130mM NaCl, 4.9mM KCl, 1.6mM Na_2HPO_4, 5.5mM D-glucose, 21mM HEPES) containing 10µg/ml calf thymus DNA [section 2.2.11], 0.2-2µg intact virus DNA, 1-10µg plasmid DNA and 130mM $CaCl_2$, were added to semi-confluent monolayers of BHK21/C13 cells on 60mm petri dishes, from which the medium had been

removed. Following incubation at 37°C for 45-60 minutes, cells were overlaid with 6ml ETC10.

Four to eight hours later the media was removed from the plates which were washed once with ETC10. 1.5ml 25% (v/v) DMSO in HEBS was added and the plates incubated at RT for 4 minutes. The DMSO was removed and the plates washed twice and overlaid with, 6ml ETC10. Incubation was continued at 37°C until c.p.e. was complete.

2.2.10. Isolation of single plaques from a transfection

Once c.p.e. from a transfection was complete, the cells were scraped into the growth media, transferred into a black-cap vial and sonicated until homogeneous. Serial 10-fold dilutions of each transfection were made in PBS/calf serum and the 10^{-3} to 10^{-7} dilutions from each plated onto 70% confluent BHK21/C13 cells. After absorption at 37°C for 1h, the plates were overlaid with 6ml EMC10% and incubation continued at 37°C for a further 48h. The plates were washed twice and overlaid with 1-2ml of PBS/calf serum. To prepare plate stocks, single plaques were isolated and grown in Linbro wells containing BHK21/C13 cells. Plates were incubated for 3-4 days at 37°C then stored at -70°C.

2.2.11. Preparation of calf thymus DNA

One gram of double-stranded calf thymus DNA (Sigma) was dissolved in 300ml of dH_2O containing 2% SDS at RT for 1-2 days. DNA was extracted twice with an equal volume of chloroform:isoamylalcohol (24:1), mixed for 10-15min and spun at 2000 rpm for 10min. The top aqueous layer containing the DNA was removed and 2 volumes of ethanol were added. The DNA precipitate was spooled out using a glass rod and lyophilised in the freeze drier. The dried DNA was dissolved in 300ml dH_2O (without SDS), digested with 50µg/ml RNase A for 30min at 37°C.

The extraction, precipitation and drying of DNA was repeated again as above. Finally, the DNA was redissolved gradually in 25ml of dH_2O and quantitated spectrophotometrically using the formula $1OD_{260} = 40\mu g/ml$. The concentration was adjusted by the addition of dH_2O to 10mg/ml. This was then aliquoted and stored at -20°C.

2.2.12. Ligation

Several strategies of ligation (Sambrook *et al* , 1989) were adopted to ligate fragments of HSV DNA to plasmid/phagemid vectors. Both vector and insert, were cut with the appropriate restriction enzymes. If the vector was cut with a blunt-cutting enzyme its digestion was usually carried out in the presence of 1 unit calf intestinal phosphatase (Boehringer Mannheim) to prevent recircularization during ligation. The vector was purified as described in section 2.2.18 and the fragment as described in section 2.2.20. Three types of fragment ends could be generated:

a) Blunt or compatible ends: In this case various quantities of fragment were ligated to 100ng of vector DNA at 14°C overnight. Ligation was carried out in 1x ligation buffer [see section 2.1.12] in the presence of 2 units of T4 DNA ligase (Boehringer Mannheim).

b) 5' overhangs: In the absence of a compatible site in the vector: if the fragment to be cloned had 5' overhangs these had to be filled in before cloning into the vector which had been cut with a blunt-cutting enzyme e.g. Sma I. Prior to running on a gel, 0.1 volume of 2.5mM dNTPs and 1unit of Klenow fragment of DNA polymerase I which carries the 5—>3′ exonuclease activity was added to the digestion mixture. Following incubation at 37°C for 30 minutes, the digest was run on a gel. The appropriate fragment was purified as described in section 2.2.20 and ligated to the vector as described in (a) above.

c) 3' overhangs: In the absence of a compatible site in the vector: if the fragment to be cloned into the vector had 3' overhangs, these had to be exonucleased. The digestion mixture was purified as described in section 2.2.18. and redissolved in an appropriate volume of dH_2O. To this was added 1 unit T4 DNA polymerase which has a strong 3′—>5′exonuclease activity and 0.1 volume of T4 polymerase buffer. Following incubation at 37°C for 1hr, purification and ligation of the fragment was carried out as in (i) above.

2.2.13. Glycerol / DMSO stocks of bacteria

Glycerol stocks of bacteria were prepared by centrifugation of an overnight culture (10ml) at 2000 rpm for 10 min. The pellets were resuspended in 5ml L-broth, mixed with 5ml of

99.5% glycerol and stored at -20°C. DMSO stocks were prepared using the same procedure except that the pellets were resuspended in L-broth containing 10% (v/v) DMSO and stored at -70°C.

2.2.14. Preparation of competent bacteria and transformation

Ten ml of 2xYT broth was inoculated with 15µl of a glycerol stock of bacteria and incubated with shaking at 37°C overnight to produce a saturated culture. One ml of this culture was used to inoculate 100ml of 2xYT broth which was shaken at 37°C for 2.5-3h to get the bacterial cells in mid-log phase. The bacterial cells were pelleted by spinning at 2K for 10 minutes in a Fison's Coolspin and resuspended in 1/10th volume of transformation and storage buffer (10mM $MgCl_2$, 10mM $MgSO_4$, 10%(w/v) PEG 3,500, 5% (v/v) dimethyl sulphoxide). After 10 minutes on ice, the bacteria were considered competent for transformation.

Typically, 1µl and 10µl of a ligation mix or plasmid preparation were incubated for 30 minutes on ice with 100µl of competent *E.coli*. One ml of L-broth was added to the transformation mix and incubation was continued for a further hour at 37°C. For selection of transformants, 100µl of the transformed bacteria were plated onto L-broth agar plates containing, if appropriate 10µg/ml ampicillin ± 25µg/ml X-gal. Plates were allowed to dry at room temperature, before incubating in an inverted position at 37°C overnight (Chung and Miller, 1988).

2.2.15. Small scale plasmid preparation

Single, transformed, bacterial colonies were picked and resuspended in 5ml of L-broth containing 100µg/ml ampicillin. Following growth at 37°C for 8-16h, 1.5ml aliquots of each culture were spun at low speed (6500g) in a microfuge, the supernatant discarded and the cells resuspended in 100µl of alkaline lysis solution I [see section 2.1.12]. Following 5 minutes incubation at RT, 200µl of alkaline lysis solution II [section 2.1.12] was added, and the cultures mixed vigorously. After a further 5 minutes incubation at RT, 150µl of alkaline lysis solution III [section 2.1.12] was added, mixed by vortexing and incubated at

RT for 5 minutes. A white precipitate consisting of the cell debris was pelleted at high speed (13000g) in a microfuge. Plasmid DNA was extracted from the supernatant using an equal volume of phenol:chloroform followed by ethanol precipitation at RT for 1 minute. The DNA was pelleted by spinning at 13,000g for 5 minutes in a microfuge, washed in 70% ethanol, dried in a Speedivac and resuspended in 100µl H_2O containing 50µg/ml RNase A (Sambrook, 1989).

2.2.16. Large scale plasmid preparation

The method used was essentially as described by Birnboim and Doly (1979), and modified by Maniatis *et al* (1982). A single transformed bacterial colony from a L-broth agar plate was inoculated into 5ml of L-broth containing the appropriate antibiotic and shaken at 37°C for 8-16h. The culture was transferred into 500ml L-broth containing the appropriate antibiotic in a 2 litre dimpled flask and shaken at 37°C overnight.

The bacteria were pelleted by centrifugation at 8K for 10 minutes in a Sorvall GSA rotor, the pellet resuspended in 7ml alkaline lysis solution I [see section 2.1.12] and incubated at RT for 10 minutes. Freshly made alkaline lysis solution II (14ml) was added and incubation continued for a further 10 minutes on ice. Ice-cold alkaline lysis solution III (10.5ml) was added, incubation continued on ice for 10 minutes and the bacterial debris pelleted by centrifugation at 12K for 10 minutes in a Sorvall SS34 rotor. DNA was extracted twice with an equal volume of phenol:chloroform and once with an equal volume of chloroform. The DNA was precipitated by the addition of 2 volumes of ethanol, centrifuged at 12K for 30 minutes in a Sorvall SS34 rotor at RT, washed in 70% ethanol, pelleted as before, dried in the incubator and dissolved in water containing 50µg/ml RNase A.

In some instances, to remove residual host DNA and RNA, the DNA was further purified by isopycnic banding on caesium chloride gradients. In this case, the DNA was only extracted once with phenol:chloroform. Caesium chloride was added until the buoyant density was 1.55g/ml and ethidium bromide was added to a final concentration of 0.5 mg/ml. The mixture was pipetted into Oakridge tubes, which were capped, sealed and

centrifuged at 45,000 r.p.m. overnight in a Sorvall Ti50 rotor. The DNA was visualised on a long wave U.V. light box and the lower band which contained the supercoiled plasmid DNA was removed using a needle and syringe. After extracting at least 3 times with isoamyl alcohol, the DNA was ethanol precipitated as described above. The DNA concentration was quantitated by running a small quantity on an agarose gel alongside standards of known concentrations (Sambrook *et al*., 1989).

2.2.17. Restriction enzyme digestion of DNA

The manufacturer's recommended buffers and conditions were generally used for each individual restriction enzyme. To achieve complete digestion, 1μg samples of HSV or plasmid DNA were digested with 2-5 units of restriction enzyme for 4h at the appropriate temperature. If the digested DNA was to be run on an agarose gel, 1/5- 1/6 volume of RE stop was added prior to loading, otherwise the digested DNA was recovered as described in section 2.2.18.

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

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

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

2.2.19. Agarose gel electrophoresis

Restriction endonuclease digested viral or plasmid DNA was analysed on 0.4-1.5% (w/v) agarose gels in 1xTBE buffer. When cool to about 45°C, the boiled agarose solutions were poured onto glass plates (16.5x26.5cm) whose edges had been sealed with tape and onto which 12-26 tooth combs had been placed. For non-radioactive samples, ethidium bromide was added to the agarose solution to a final concentration of 0.5μg/ml before pouring. The gels were allowed to set at RT for 1h and then transferred to horizontal tanks containing 1xTBE buffer. DNA samples were mixed with one-fifth volume of RE stop, loaded into the gel tracks and electrophoresed at 40-120V for 4-16h (Sambrook *et al* ,1989).

For quantitation of fragment and vector DNA prior to transfection or ligation, gels containing ethidium bromide were poured into mini-gel kits (Bio-Rad). Samples (generally 5-7μl) were run at 50V for 1-2h, visualized using a short wave (260) U.V. lamp and photographed on Polaroid film. A 1Kb DNA marker (BRL) of known concentration was run alongside the DNA samples to enable confirmation of the vector/fragment size and quantitation of each sample.

2.2.20. Recovery and purification of DNA from agarose gels

Generally the DNA to be recovered had been digested with restriction enzymes [section 2.2.17] before running on an agarose gel. In order to cause the minimum possible damage to the DNA, it was visualised using a long wave (300-360nm) U.V. lamp. A gel slice containing the desired DNA fragment was cut out with a sharp scalpel and the DNA recovered and purified using the Prep-A-Gene DNA Purification Kit (BIO-RAD) as directed. For this purpose, the gel slice was cut into small pieces and dissolved at 55°C in the presence of Prep-A-gene binding buffer. The required amount of Prep-A-gene matrix was added, the mixture vortexed briefly, incubated at RT for 10 min and spun at 2000rpm for 2 min. The pellet containing the bound DNA was washed twice with the binding buffer and three times with the wash buffer. After the last wash, the bound DNA was eluted at 37°C using the elution buffer. The yield was estimated as described in section

2.2.19.

In many instances, if the DNA fragment was small, it was recovered from the gel using the Spin-X™ centrifuge filter unit (Costar). The agarose retained on the filter was washed with 0.6M sodium acetate. The DNA was phenol:chloroform extraced, precipitated with ethanol plus 3M sodium acetate, washed with 70% ethanol, dried and dissolved with dH_2O.

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

Gel purified fragments of plasmid DNA were ^{32}P labelled using the method of nick-translation (Rigby *et al*, 1977). DNA (10-100ng) was boiled for 10 minutes and radiolabelled in a reaction mix containing: 1µl of 1% BSA, 20mµCi ^{32}P dCTP, 5µl of 5x random primer buffer and 2 units Klenow polymerase in a final volume of 25 µl. The mixture was incubated at 37°C for at least 30 minutes and boiled prior to use for Southern blotting.

2.2.22. Southern blotting

Southern blotting was carried out as described by Sambrook *et al* (1989). Purified virus, infected cell DNA or plasmid DNA was digested with the appropriate restriction enzyme(s) before running on an agarose gel. The gel was visualised under short-wave U.V. light to confirm DNA digestion before placing in 500ml Gel Soak I [see section 2.1.12] for 1h, rinsing with deionised water and transferring to Gel Soak II [section 2.1.12] for 1h. After rinsing as before, it was transferred to 500ml 20xSSC for a further 1h. The gel was now ready for blotting onto either one or two sheets simultaneously of Hybond™ N hybridization transfer membrane. For each transfer, one sheet of membrane and five sheets of Whatmann 3MM chromatography paper, all cut to the exact size of the gel were required.

A bundle of 'Hi-Dry' towels was placed on the bench followed by three sheets of dry then two sheets of 20xSSC soaked 3MM paper. The membrane was placed on top of the filter papers followed by the gel, thus ensuring that there were no air bubbles between the gel

and the membrane. A glass plate and heavy weight were placed on top and the blot left for at least 4h during which time the DNA was drawn out of the gel onto the adjacent side of the nitrocellulose. To transfer DNA to two membranes this procedure was repeated on top of the gel.

The DNA was cross-linked to the membrane using a U.V. Stratalinker (Stratagene, USA) and hybridized to the labelled probe [section 2.2.21] in a sealed bag containing 20ml hybridization buffer [see section 1.1.12]. Hybridization was generally carried out overnight at 65°C. The membrane was washed for 3x30 min with 1 litre 2xSSC, 0.1%(w/v)SDS before drying and setting up for autoradiography against Kodak XS-1 film.

2.2.23. Purification of a synthetic oligonucleotide

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

The synthetic oligonucleotides were deprotected at 65°C for 5-6h, frozen on dry ice and dried in the speedivac overnight. Each oligonucleotide was resuspended in 50µl H_2O. Glycerol (5µl) was added to 30µl of oligonucleotide solution and loaded immediately onto the gel. Formamide dye mix (2µl) was loaded in a separate well to act as a molecular weight marker and the gel was run at 10mA for 3-4h in 1xTBE. Electrophoresis was stopped when the dye had migrated approximately two thirds of the length of the gel. The gel was removed, wrapped in cling-film and viewed against a white chromatographic plate using a hand-held long-wave U.V lamp (Sambrook *et al.*, 1989). If the synthesis had been successful, then a predominant band, with possibly a few lower molecular weight bands, was observed. The top band was cut out with a scalpel, mashed with a glass rod, and incubated overnight at 42°C in 1ml elution buffer (0.5M ammonium acetate, 0.25mM EDTA, 20% (w/v) SDS). This was filtered through a SPIN-X™centrifuge filter unit to remove the acrylamide, phenol: chloroform extracted, ethanol precipitated, washed in 70% ethanol, dried and dissolved in dH_2O. The concentration of the oligonucleotide was

determined spectrophotometrically using the formula: 1OD = 20µg/ml.

2.2.24. Linker-insertion mutagenesis

Insertional mutagenesis was performed according to the method of Sambrook *et al* (1989). For this purpose, an oligonucleotide containing the desired mutation was synthesized [section 2.1.7], purified [section 2.2.23],phosphorylated [section 2.2.25(a)] and used to prepare the double stranded linker. This linker was prepared by heating the phosphorylated oligonucleotide to 70°C and allowing it to cool slowly to RT. The annealed DNA was ligated to blunt ended termini of a plasmid vector as described in section 2.2.12 (a).

2.2.25. Single-stranded site directed mutagenesis

This technique was carried out as described by Carter (1991), based on the method of Kunkel *et al* (1987). It includes the following:

a) Phosphorylation of the mutagenic oligonucleotide

The oligonucleotide (60 pmole) was purified [section 2.2.23] and phosphorylated by 10 units of T4 polynucleotide kinase in a mixture containing 500mM ATP, 100mM DTT and 10x Kinase buffer (500mM Tris.HCl pH 8, 100mM $MgCl_2$) at 37°C for 1h. The reaction was terminated by heating at 65°C for 15 min.

b) Preparation of uracil-rich single-stranded DNA template

Recombinant pGEM3zf(-) phagemid DNAs were transformed into competent cells of *E.coli* strain BW313 (ung^-, dut^-). A bacterial colony was grown overnight at 37°C in 5ml 2xYT medium containing 100mg/ml ampicillin. This culture was used to inoculate 250 ml 2xYT medium containing 0.25 mg/ml uridine and 100µg/ml ampicillin. Growth was allowed to proceed at 37°C with vigorous shaking to an OD_{600} of 0.3-0.35. The culture was then infected with 1ml of the helper phage M13KO7 or R408 ($\geq 10^{11}$ p.f.u./ml). Following incubation at 37°C for 30 min, kanamycin (75µg/ml; depending on the helper phage) was added to the culture and incubation continued overnight. The bacterial cells

were pelleted by centrifugation at 12000 rpm (Sorval GSA rotor) for 15 min at 4°C. The supernatant was poured into a fresh centrifuge tube and spun for 15min. The phage in the supernatant was precipitated by adding 0.25volume of a solution containing 20% PEG6000 and 3.5M ammonium acetate. At the end of 30min incubation on ice, the mixture was centrifuged for 30min at 12000 rpm at 4°C. The pellet was drained well, resuspended in 500μl of TE and transferred into a sterile microfuge tube. An equal volume of phenol:chloroform (1:1) was added, the tube was vortexed for one full minute and centrifuged at 13000g for 5min. The upper aqueous phase containing the single-stranded phagemid DNA (ssDNA) was transferred to a fresh tube leaving the large interphase behind. This extraction was repeated approximately 5-6 times until there was no visible material at the interface. The ssDNA was then extracted with an equal volume of chloroform, precipitated with 0.1 volume of 3M sodium acetate and 2 volumes of ethanol on dry ice for 30min and pelleted at 13000g for 15min. The pellet was washed with 95% ethanol, dried in a vacuum desiccator and dissolved in 20μl dH20. The yield was estimated by measuring the OD at 260 nm (1 OD260 = 40μg/ml). The quality of the ssDNA was evaluated by agarose gel electrophoresis. A major band of ssDNA was usually observed on a 1% agarose gel if the DNA was of good quality. In some cases, major helper phage DNA and a small amount of large chromosomal DNA was observed.

c) Annealing of the phosphorylated mutagenic oligonucleotide to the template

The ssDNA template was added to a 20, 30, 50-fold molar excess of the phosphorylated mutagenic oligonucleotide in the presence of 5xSEQ buffer (200mM Tris-HCl pH7.5, 80mM MgCl2). The sample tubes were incubated in a water bath at 65°C for 10min, then allowed to cool slowly to RT over a period of about 30min. The tubes were spun for a few seconds to recover condensation from the lids and placed on ice for 5min.

d) Synthesis of the complementary mutagenic DNA strand and transformation of the reaction products

The primer extension reaction was carried out by adding 10μl of the annealing reaction

mix to: 2µl 5xSEQ buffer, 1µl 5mM ATP, 1µl 100mM DTT, 1µl dNTPs, 1µg/µl acetylated bovine serum albumin (Sigma), 1µg/µl gene 32 protein, 3U T4 DNA ligase, 5U T4 polynucleotide kinase, 1U T7 DNA polymerase. Following incubation at 37°C for 1h, half of the reaction mix was transformed in to *E.coli* NM522 (dut^+, ung^+) and plated on to L-broth agar containing 100µg/ml ampicillin. Individual colonies were picked and the mutants were identified by restriction enzyme [section 2.2.17] and by DNA sequence analysis [section 2.2.29] of the plasmid DNA.

e) Controls

i) Negative control

The reaction mixture containing all ingredients except the primer was used to test for non-specific endogenous priming in mutagenesis experiments. This priming which is caused by contaminating nucleic acids in the template preparation may result in lowering the mutation efficiency.

ii) Positive control.

U-phagemid DNA (BIO-RAD) in conjunction with the reverting primer (BIO-RAD) were used in the mutagenesis experiments to test for the efficiency of the reagents, enzymes and competent cells. U-phagemid DNA is pTZ18U (2860 base) containing an amber mutation at codon 16 of the lacZ fragment. The UGG codon (tryptophan) was converted to the chain terminating codon UAG. Since the phagemid contains an amber mutation in the lacZ fragment, it is therefore unable to complement $lacZ^-$ host strains and yields white colonies on plates containing ampicillin and X-gal. The reverting primer is a phosphorylated 16-base oligonucleotide (5′pGGTTTTCC<u>C</u>AGTCACG3′) which converts the amber mutation UAG back to the wild type UGG codon for tryptophan. Thus blue colonies are produced on plates containing X-gal. The underlined base is the one which is converted in the amber phagemid by this primer.

f). Preparation of helper phage stocks

The procedures for growth of the 2 helper phages are slightly different due to their differences in genotype. M13KO7 has a Kanamycin resistance marker that is

spontaneously deleted with high frequency in the absence of antibiotic selection. Therefore, a single kanamycin resistant colony is used to propagate this virus while R408 can be propagated from a single plaque.

i) Growth of phage M13KO7

Phage stock (Promega) was serially diluted in L-broth. A total of five 100 fold serial dilutions were prepared in separate sterile vials. 100μl of each dilution was added to 200μl of log phase *E.coli* NM522 cells (prepared by adding 1ml of overnight cells to 10ml 2xYT and shaken for 60 min). Following 5 min incubation at RT, 50μl of the cell and phage mixture was plated on L-broth agar containing 70μg/ml Kanamycin. Plates were incubated overnight at 37°C. A single colony was picked, transferred to 50ml of 2xYT broth and shaken at 37°C for 8-12h with vigorous agitation. Bacterial cells were pelleted by centrifugation at 3K for 15min in sterile tubes. The supernatant was poured into a fresh tube and spun again for 15min. To kill any remaining bacterial cells, the supernatant was heated at 55°C for 30min before storage at 4°C.

ii) Growth of phage R408

The phage stock (Promega) was serially diluted 100 fold and used to infect NM522 cells as described above for M13K07. Following 5min incubation at RT, 4ml of top agar was added to each cell/phage mixture and poured on L-broth agar lacking Kanamycin. Plates were incubated overnight at 37°C. A single plaque was picked and used to inoculate 50ml of 2xYT broth. Isolation and storage of the phage was continued as described above (i).

g) Titrating phage M13KO7 and R408 stocks

The prepared phage stocks [section 2.2.25(f)] were serially diluted 100 fold in L-broth. 200μl of log phase NM522 bacteria were infected with 100μl of each dilution and incubated at RT for 5min. Molten top agar (5ml) was added to each cell/phage mixture, poured on L-broth agar and incubated overnight at 37°C. The plaques were counted and the number of plaque forming units/ml (pfu/ml) was calculated.

2.2.26. Construction of M13 recombinant plasmids

M13mp18 and mp19 double-stranded DNA was commercially obtained from Pharmacia. Typically 10μg DNA was digested with the appropriate restriction enzyme e.g. SmaI. Following extraction as described previously [section 2.2.18] the DNA was resuspended in 10μl H_2O. One μg aliquots of M13 and a wide range of fragment amounts (0.1-10μg) were generally used in each ligation mix. Standard cloning methods were employed (Sambrook *et al.*, 1989).

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

Competent cells of *E.coli* strain NM522 were prepared as described in section 2.2.14. Typically 1μl and 10μl of ligation mix were added separately to 200μl of competent cells and incubated on ice for 30 minutes. Five ml melted top agar at 42°C, containing 30μl of 25mg/ml IPTG in water and 30μl of 25mg/ml X-gal. was added and the mixture poured on to 90mm L-broth agar plates and incubated at 37°C overnight.

2.2.28. Preparation of M13 single stranded DNA

Single, colourless, transformed M13 colonies were used to inoculate 1.5ml 2xYT broth containing 1/100 dilution of an overnight saturated bacterial culture. Following 4.5-6h incubation with shaking at 37°C, the culture was spun at 13,000g in a microfuge to pellet the bacteria. The supernatant, which contained the M13 bacteriophage, was retained and respun to ensure no bacterial contamination. M13 was precipitated by the addition of 200μl of a solution containing 20% (w/v) PEG6000 and 3.5M ammonium acetate. Following incubation on ice for 10 minutes, bacteriophage were pelleted by centrifugation at 13,000g in a microfuge. The supernatant was discarded and the pellet respun before removing any excess liquid with an eppendorf tip. After resuspending the pellet in 200μl TE, the DNA was extracted once with an equal volume of phenol-chloroform, before precipitating with 2 volumes of ethanol and 0.1 volumes of 3M sodium acetate on dry-ice for 30 minutes. The DNA was pelleted by centrifugation at 13,000g for 5 minutes in a microfuge, washed with 70% ethanol, dried and resuspended in 15μl H_2O containing

50μg/ml RNase A (Sambrook *et al.*, 1989).

2.2.29. Sequence analysis of recombinant M13 clones

Sequence analysis was carried out using the Sequenase Version 2.0 kit (Amersham) in accordance with the manufacturer's instructions. Recombinant bacteriophage single-stranded DNA (5μl) was annealed to 3ng of 17-mer universal sequencing primer which hybridizes upstream of the multicloning site in a 5x annealing buffer (200mM Tris.HCl , pH7.5, 100mM $MgCl_2$ and 250mM NaCl) in a total volume of 10μl. Annealing was carried out by first heating to 55°C for 5 minutes in a 1.5ml eppendorf tube, then allowing to cool to room temperature over a period of 30 minutes. The templates were briefly chilled on ice, before being labelled and extended using a buffer which contained 1.5μM dCTP, 1.5μM dGTP, 1.5μM dTTP, 5μCi ^{35}S dATP, 6mM DTT and 3 units of Sequenase T7 DNA polymerase in a total volume of 16μl. Labelling was carried out at RT for 2-5 minutes. Each template was extended and terminated by addition of 3.5μl of labelled template to 2.5μl of each of the 4 ddNTP termination mixes in microtitre wells, which had been pre-warmed to 37°C. Each ddNTP termination mix contained one ddNTP at a concentration of 8μM and dNTPs at a concentration of 80 μM. The plate contents were mixed by centrifugation in a Beckman benchtop centrifuge and the reactions carried out at 37°C for 10-30 minutes. Four μl formamide dye mix was added to each sample. The samples were boiled for 2 minutes before loading onto a gel for electrophoresis.

2.2.30. Electrophoresis and autoradiography of sequencing gels

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

2.2. 31. Computing

DNA sequence analysis of various RL1 fragments of HSV-2 HG52 was carried out on the Digital MicroVAX 11 and Digital AXP 21001/A500mp computers in this Institute, using the University of Wisconsin Genetics Computer Group software package.

2.2.32. Isolation of total RNA

The single-step method of RNA isolation by acid guanidinium thiocyanate (Chomczynski *et al.*, 1987) was used to isolate the total RNA from BHK21/C13 cells infected with HG52. Cells were grown in 140mm-tissue culture dishes at a density of 5×10^7 cells/dish and were infected with the virus at a multiplicity of infection of 10 pfu/cell. Uninfected cells (mock) were used as a negative control. Following incubation at 37°C, the ETC10 medium was poured off, the cells were then washed with 10ml of ice-cold phosphate-buffered saline (PBS-A). The cells were scraped off, transferred to a sterile 15-ml polypropylene tube and spun at 2000 r.p.m for 5min at 4°C in a Fison's Coolspin. Cells were lysed in 2ml of guanidine thiocyanate (4M guanidine thiocyanate, 25mM sodium citrate (pH7.0), 0.5% Sarkosyl (Sigma), 0.1M mercaptoethanol). The lysate was drawn in to a hypodermic syringe fitted with a 21-guage needle and then expelled into a polypropylene tube. This step was repeated 3-4 times to shear the cellular DNA. The proteins were removed by extracting once with phenol:chloroform. The aqueous and organic phases were separated by centrifugation at 8K for 10min at 4°C in a Sorvall SS34. The aqueous phase was transferred to a fresh tube and an equal volume of ice-cold isopropanol was added. Total RNA was precipitated overnight at -20°C and then recovered by centrifugation at 8K for 10min in a Sorvall SS34. The pellet was washed with 70% ethanol and dissolved in 200µl of Tris-HCL (pH7.8), 1mM EDTA (pH8.0). $MgCl_2$ and dithiothreitol were added to a final concentration of 10mM and 0.1mM, respectively. The placental RNAase inhibitor (RNAsin; Promega) and RQ1 RNase-free DNase (Promega) were added, each to a final concentration of 1000U/ml. The mixture was incubated for 60min at 37°C, then EDTA and SDS were added to final concentrations of 10mM and 0.2%, respectively. The solution was extracted once with an equal volume

of phenol:chloroform and the aqueous phase was transferred to a fresh tube. To precipitate the RNA, 2.5 volumes of ice-cold ethanol and 0.1 volume of 3M sodium acetate were added. Following overnight incubation at -20°C, RNA was collected by centrifugation at 8K for 5min at 4°C. The RNA pellet was washed in 70% ethanol and resuspended in 100µl DEPC-treated dH_2O. The concentration of RNA was determined by measuring the OD_{260} of an aliquot of the final preparation. A solution of RNA whose $OD_{260} = 1$ contains approximately 40µg of RNA per milliliter. For storage of RNA, 2.5 volumes of ethanol were added to the RNA solution and stored at -70°C. When needed, it was recovered by adding 0.1 volume of 3M sodium acetate (pH5.2) before spinning at 13,000 r.p.m for 5min at 4°C in a microfuge.

2.2.33. Electrophoresis of RNA through gels containing formaldehyde

The quality of the isolated RNA was checked by denaturing agarose gel electrophoresis (Sambrook *et al.*, 1989). The gel tank was treated with 3% H_2O_2 overnight and filled with 1xMOPS buffer. The gel was prepared by dissolving one gram of agarose in the appropriate amount of sterile d.H_2O and cooled to 65°C. 10xMOPS buffer [section 2.1.12] and formaldehyde (Sigma) were added to give final concentrations of 1x and 2.2M, respectively. The gel was cast in a chemical hood and allowed to set for at least 30min at RT. RNA samples (20µg) were prepared by mixing with 5µl deionized formamide (Sigma), 1µl 10xMOPS buffer and 1.6µl formaldehyde. Samples were incubated at 65°C for 5min, chilled on ice and then 2µl of RNA-loading buffer [section 2.1.12] + 1µl of ethidium bromide (1mg/ml) were added. The gel was loaded with the samples and run in 1xMOPS buffer. When visualized under the UV, the 18S and 28S rRNAs were seen and the mRNA appeared as a smear.

2.2.34. Purification of cytoplasmic mRNA directly from tissue culture cells

The Dynabeads mRNA purification kit (Dynal) was used to isolate the polyadenylated mRNA [poly $(A)^{+}$RNA] from cells infected with HG52. BHK21/C13 cells were grown in 100mm tissue culture dishes at a density of $3x10^6$ cells/dish, then infected and collected

as described for total RNA isolation [section 2.2.32]. As directed by the manufacturer, the cell pellet was resuspended in 100μl of 1xlysis buffer (10mM Tris-HCl (pH7.5), 0.14M NaCl, 5mM KCl, 1% Triton X-100). Following incubation on ice for 1min, the nuclei were pelleted by centrifugation at 6500 r.p.m.for 30sec at 4°C in a microfuge. The cytoplasmic supernatant was transferred to a sterile eppendorf tube containing an appropriate amount of washed dynabeads oligo $(dT)_{25}$ in 100μl 2xbinding buffer (20mM Tris-HCl (pH7.5), 1.0M LiCl, 2mM EDTA, 1%SDS). The Dynabeads bind approximately 2μg poly $(A)^+$ RNA per mg of beads. The mRNA was allowed to bind for 5min at RT, then the eppendorf tube was placed in the Dynal magnetic particle concentrator (Dynal-MPC) for at least 30sec. The supernatant was removed by aspiration with a pipette while the tube remains in the Dynal-MPC and then the Dynabeads were washed 3 times with 200μl washing buffer. To elute the mRNA, 10μl of 1x elution buffer (2mM EDTA pH7.5), was added and beads were incubated at 65°C for 2min.

2.2.35. RNA-PCR

a) Selection of primers for RNA-PCR

Appropriate RNA-PCR sense and antisense oligonucleotide primers were designed according to:

a) Computer program (Oligo; MedProbe A.A).

This program was used to scan the nucleic acid sequence of the RL1 gene and to select the possible pairs of primers suitable to direct efficient cDNA synthesis and amplification.

b) Primers of Wallace temperature $\geq$ 80°C [4°Cx(G+C)+2°Cx(A+T)] were designed as recommended by Dutton *et al* (1993) for amplifying regions of very high G+C content.

b) cDNA synthesis

First strand cDNA was synthesized as described by Saiki *et al* (1988) using one of the following enzymes: (i) Avian Myeloblastosis virus reverse transcriptase (AMV-RT), (ii) Moloney Murine Leukaemia virus reverse transcriptase (MuLV-RT), (iii) Retrotherm reverse transcriptase. The reagent mixture consisted of 1-2μg total RNA [section 2.2.32]

or (0.6-3μg) mRNA [section 2.2.34], 20pmole of antisense oligonucleotide primer or random primer (Promega), 200μM dNTPs (Pharmacia), 1X RT buffer (purchased with the enzyme) in a final volume of 20μl. The mixture was heated at 70°C for 5min and then cooled immediately on ice for 5min. RNasin (20U; Promega) was added first and then 20U of either AMV- or MulV-RT. Depending on the enzyme, the reaction was carried out at either 37°C (MuLV-RT) or 42°C (AMV-RT) for 60min and then stopped by heating at 65°C for 10min. When Retrotherm RT (2U) was used, the reaction was carried out for 10min at >60°C depending on the primer-annealing temperature which was calculated as recommended by the manufacturer.

c) cDNA-PCR

Aliquots (5μl, 20μl) of cDNA from step (b) was subjected to PCR amplification under various cycling conditions. PCR was carried out as described by Saiki *et al* (1988) in a final volume of 50μl overlaid with 50μl mineral oil (Sigma). The PCR reaction consisted of 1xPCR buffer (purchased with the enzyme), 2.5mM $MgCl_2$ or 1mM $MgSO_4$ (depending on the enzyme), 200μM dNTPs, 50pmole each of the sense and antisense oligonucleotide primers, 2U of either Taq DNA polymerase (Promega) or Vent DNA polymerase (Biolab). In some experiments, a denaturant such as 5% or 10% DMSO was added to the reaction mixture. Appropriate positive and negative PCR controls were included in all experiments. Thirty to thirty five cycles of amplification were performed with a OmniGene (Hybaid) thermal cycler followed by a cycle of final extension. The amplification conditions used depend on the size of the primers (Wallace Temperature) and the type of the PCR DNA polymerase. The following are examples of amplification protocols:

i) Vent DNA polymerase: 1 cycle (98°C, 5min); 35 cycles [(98°C, 70sec), (55°C, 2min), (75°C, 3min)]; 1 cycle.(75°C, 10min).

ii) High G+C PCR Protocol: (98°C for 1min plus 70°C for 5min) for 30 cycles (Dutton *et al*,. 1993).

iii) Taq DNA Polymerase: 1 cycle (95°C, 5min), 30 cycles [(95°C, 70sec), (55°C, 90

sec), 72°C, 2min)]; 1 cycle [(95°C, 70sec), (55°C, 90sec), (72°C, 10min)].

Following amplification, samples were prepared for electrophoresis by chloroform extraction of the mineral oil. To visualize the amplified PCR product, one-tenth of the product was mixed with RE stop [section 2.2.19] and run on 3% NuSieve agarose (FMC BioProducts) gel. A 123 DNA marker (BRL-GIBCO) was run alongside the PCR samples to confirm the size of the desired amplified fragment.

2.2.36. Animal studies

a) Neurovirulence

Three week old female BALB/c mice were inoculated intracerebrally with individual virus stocks. Mice were anaesthetized with halothane and 25µl of the appropriate virus dilution in PBS/calf was inoculated into the central region of the left cerebral hemisphere. Four or five mice were inoculated with each virus dilution. Depending on the known, or postulated neurovirulence phenotype of the viruses being tested, doses were in the range 10^2 to 10^7 p.f.u./mouse. Stocks were always retitrated immediately post-injection to ensure that the correct dose had been administered. The mice were monitored for 21 days and scored for death between days 1 and 21. The neurovirulence of viruses was determined by calculating the LD_{50} values according to the formula of Reed and Muench (1938).

b) Virus growth properties *in vivo*

The ability of mutant and wild-type virus to replicate in mouse brains following intracerebral inoculation was assessed using a mouse model as described by Taha *et al.* (1989a). Twenty-five µl aliquots of wild-type virus (10^2 p.f.u./mouse) and mutant virus (10^5 p.f.u./mouse) were injected into the left cerebral hemisphere of 3-week old BALB/c mice. At 0h, 12h, 24h, 48h, 72h, 96h, 120h and 144h post-inoculation, 2 mice per virus group were killed, their brains removed, homogenized in 0.5ml PBS/calf and sonicated. The final volume was measured and recorded, before storing at -70°C. When all samples had been collected, they were thawed and progeny virus titrated on BHK21/C13 cells at

37°C. Final titres were calculated as mean p.f.u./mouse.

c) Virus growth properties *in vitro*

Virus growth experiments *in vitro* were carried out essentially as described by Brown and Harland (1987) and Brown *et al* (1994a). For multi-cycle growth experiments, confluent BHK21/C13 and 3T6 cell monolayers were infected in separate experiments at a multiplicity of 0.01 p.f.u./cell of stock viruses. Single-cycle growth experiments were carried out by infecting confluent BHK21/C13 monolayers at a multiplicity of 5 p.f.u./cell. At specific times post-infection, cells were harvested, sonicated and stored at -70°C. At the end of the experiments, viruses were titrated on monolayers of BHK21/C13 cells at 37°C.

d) Marker rescue

In vitro and the *in vivo* marker rescue methods was used to restore the neurovirulence phenotype of the mutant viruses. DNA (1μg) from a mutant virus was transfected onto BHK21/C13 cells with a 1-, 10- and 100- fold molar excess of a wild-type fragment containing the RL1 ORF [section 2.2.9]. After 2 days, the monolayer in which c.p.e. was most extensive was harvested and sonicated briefly.

For *in vitro* generation of rescuants, the progeny virus of a transfection mix was titrated on confluent 3T6 cells. Large plaques were isolated, purified three times and their DNA was analyzed by either *in vivo* ^{32}p DNA- labelling [section 2.2.8] or Southern blotting [section 2.2.22].

For *in vivo* marker rescue experiments, 10^{-1} and 10^{-2} dilutions of a transfection mix were prepared in PBS/calf serum and 25μl aliquots of each were injected intracerebrally into 3-week old BALB/c mice. Deaths from encephalitis were scored up to 21 days post inoculation. Mice which showed signs of encephalitis were put down according to the regulations for conducting animal experiments. Brains were removed, homogenised in PBS/calf serum, and the resultant viruses were titrated on 3T6 cells. Single large plaques were isolated, purified once and their genomes analysed to identify wild-type rescuants

before growing a virus stock.

CHAPTER 3

RESULTS

CHAPTER 3: RESULTS

3.1 Strategy

The strategy employed to achieve the objectives of this project was:

1. Construction of RL1 recombinant phagemids which have been used in mutagenesis experiments to generate RL1 mutant phagemids.
2. Isolation of RL1 mutant viruses by individual recombination of the mutated phagemids with the wild-type genome.
3. Evaluation of the virulence of the mutant viruses compared with the parental wild-type HG52.
4. Confirmation of the virulence phenotype of the mutant viruses by isolating wild-type rescuants.
5. *In vivo* and *in vitro* characterisation of the mutant viruses compared to the parental wild-type HG52. The characterization involved determination of :

a) the growth patterns in brains of 3-week old female Balb/c mice over a period of seven days.

b) the growth patterns in BHK21/C13 cells and in 3T6 mouse embryo fibroblast cells.

c) the sensitivity to various temperatures (31°C, 37°C and 38.5°C).

6. *In vitro* characterisation of the wild-type rescuants in 3T6 mouse embryo fibroblast cells.
7. cDNA-PCR of the mRNA flanking the proposed RL1 intron in an attempt to provide evidence that the mature mRNA of the gene is spliced.

The construction of each recombinant phagemid, mutant virus, wild-type rescuant and the *in vitro* and *in vivo* characterisation of mutants are described in the following sections of this chapter.

3.2. Construction of RL1 containing recombinant phagemids

All RL1 recombinant phagemids were constructed using pGEM3zf(-) which contains a bacteriophage f1 DNA replication origin to allow rescue of ssDNA for site-directed

mutagenesis and (if required) for sequencing. The starting plasmid was pAT153 containing the *Bam*HI*g* fragment (0.81-0.85 m.u.) (Davison, 1981) of HG52. This fragment (n.p. 10592-16449) contains the IR_L copy of RL1 (n.p. 13179-12243) (McGeoch *et al*, 1991). The recombinant plasmid pAT153-*Bam*HI*g* was digested in separate experiments by *Sph*I and *Xho*I restriction enzymes [section 2.2.17] and run on a 0.6% agarose gel. The RL1 containing *Sph*I and *Xho*I fragments were cut out and purified as described in section 2.2.20.

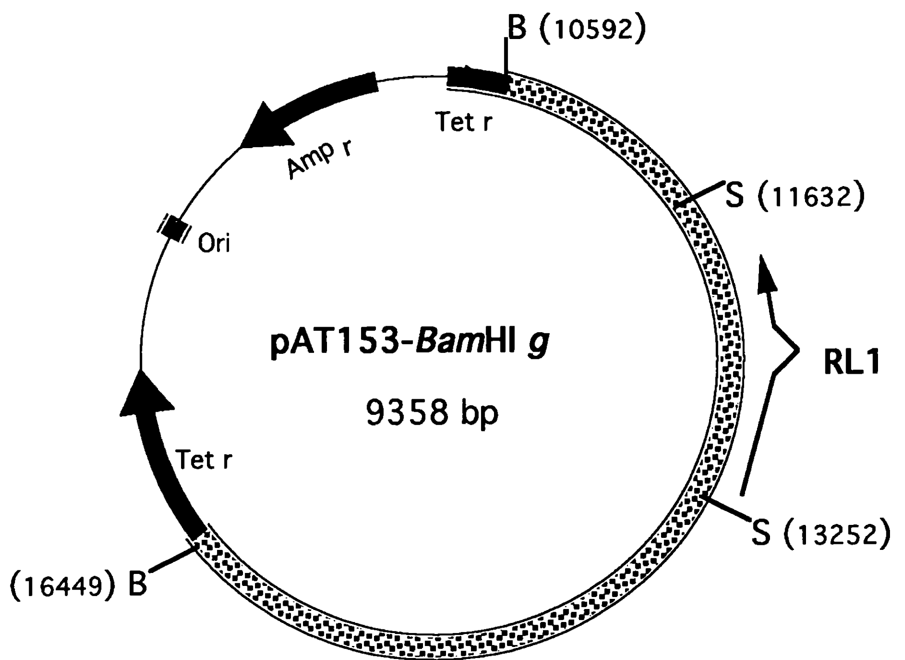
A 1.62Kb *Sph*I fragment of pAT153-*Bam*HI*g* was cloned into pGEM3zf(-) at the *Sph*I site of the multicloning region [section 2.2.12.(a)]. The resulting construct was designated pSB (Fig. 3.1) and was analysed with various restriction endonucleases (data not shown). pSB was used: (i) to delete the proposed intron [section 3.3.3.] and (ii) to insert separate stop codons in exon I, within the intron and within the conserved region of exon II [section 3.3.1.]. However, pSB was not suitable for insertion of a stop codon at the start of RL1 as there is insufficient sequence upstream of the RL1 initiation codon for homologous recombination to occur (Fig. 3.1). Therefore, a second phagemid, designated pXB, containing a 3.343Kb *Xho*I sub-fragment of pAT153-*Bam*HI*g* was constructed. The *Xho*I fragment was cloned into pGEM3zf(-) at the *Sma*I site (Fig. 3.2) after blunting with the Klenow fragment of DNA polymerase I [section 2.2.12(b)]. The recombinant pXB was used to introduce a stop codon 9bp downstream of the RL1 initiation codon [section 3.3.2] and to isolate wild-type rescuants in marker rescue experiments [sections 3.7 & 3.9].

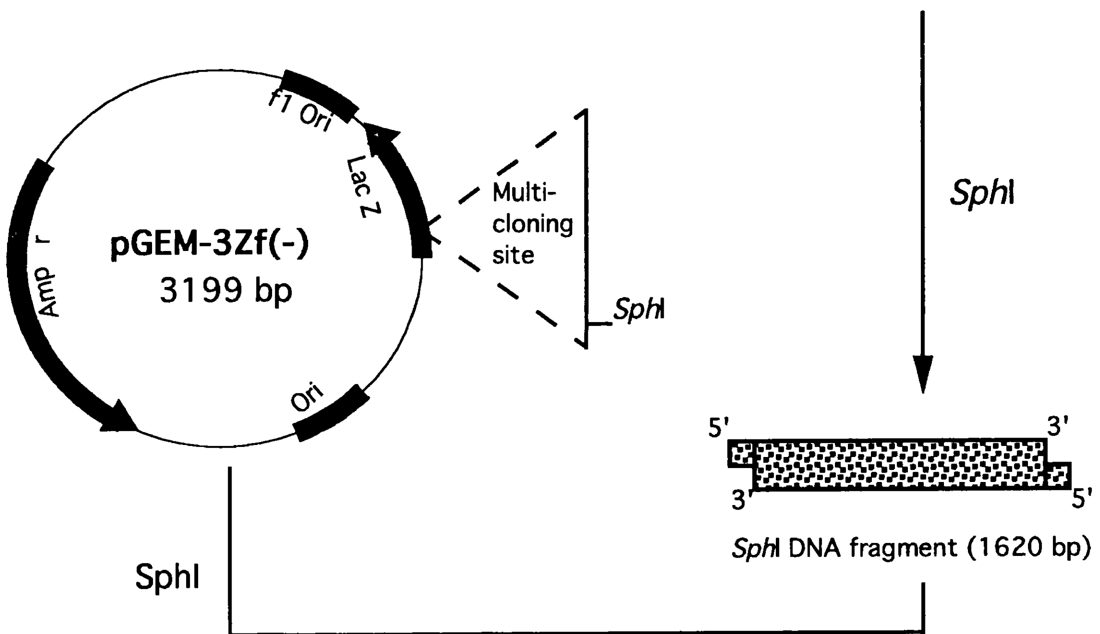
3.3. Construction of RL1 mutant phagemids

Six mutant phagemids were constructed using the recombinant phagemids pSB and pXB: one in which the putative RL1 intron has been deleted and five in which stop codons were inserted at different sites in the predicted ORF of RL1. Three were used to generate individual mutant viruses by recombination with the wild-type genome.The construction of the mutant phagemids is described in the following sections:

Figure 3.1. Construction of the RL1-recombinant pSB phagemid

A 1.620 Kb *Sph*I (S) fragment containing the HG52 RL1 gene was cut from the recombinant plasmid pAT153-*Bam*HI*g* and cloned into the *Sph*I restriction enzyme site in the multicloning site of pGEM-3zf(-) to generate the phagemid pSB. The position of the *Sph*I restriction enzyme sites, the location of the HG52 RL1 gene within the *Sph*I fragment and the positions of RL1 initiation and stop codons are marked. The nucleotide numbers described here represent the numbering system of the HG52 DNA sequence reported by McGeoch *et al* (1991).


B (10592)
Tet r
Amp r
S (11632)
Ori
pAT153-BamHI g
9358 bp
RL1
Tet r
S (13252)
(16449) B


f1 Ori
Lac Z
Multi-
cloning
site
SphI
pGEM-3Zf(-)
3199 bp
Amp r
Ori
SphI
5'
3'
3'
5'
SphI DNA fragment (1620 bp)
SphI

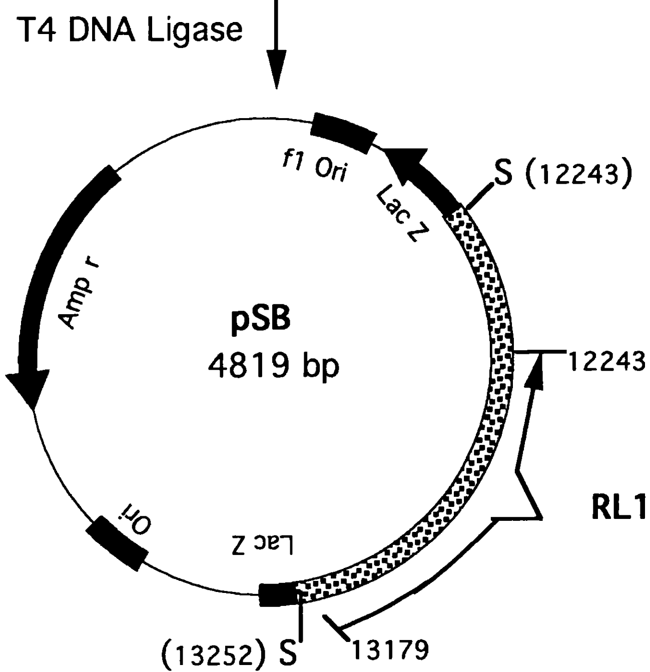
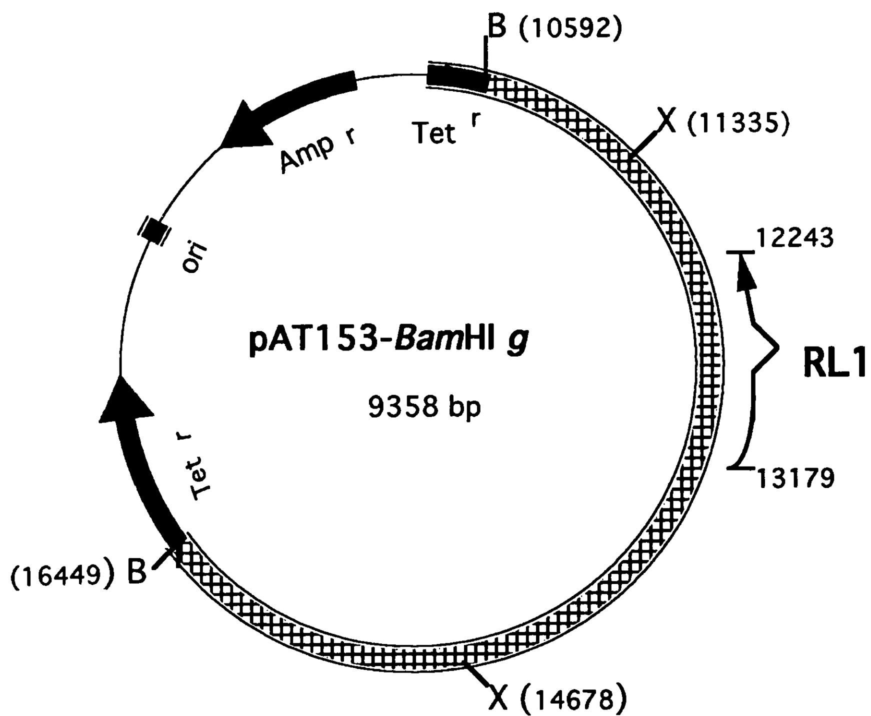
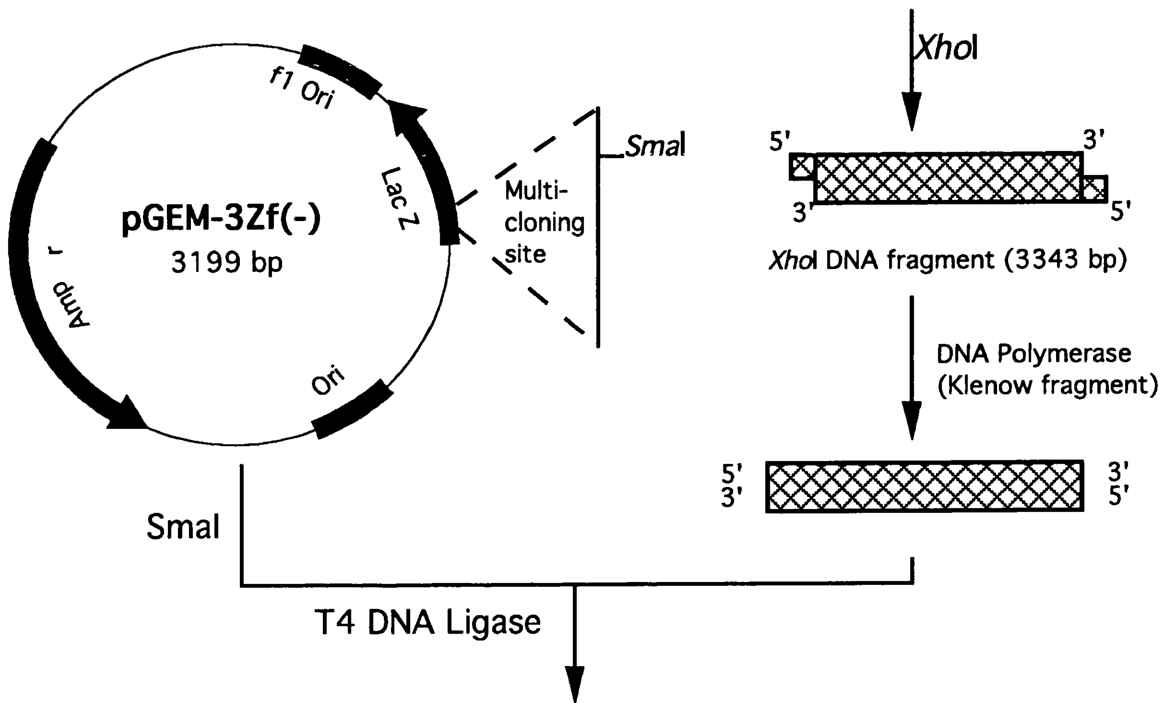
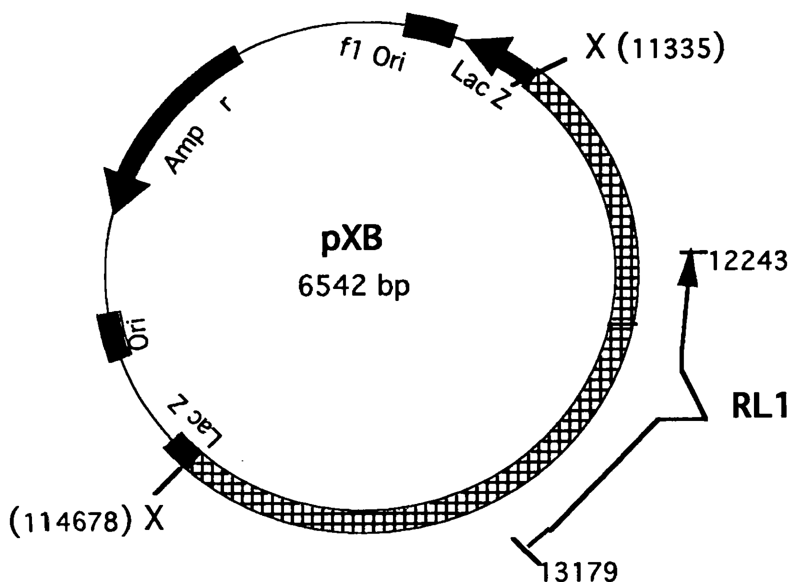

T4 DNA Ligase
f1 Ori
S (12243)
Lac Z
Amp r
pSB
4819 bp
12243
RL1
Ori
Lac Z
(13252) S
13179

Figure 3.2. Construction of the RL1-recombinant pXB phagemid

A 3.343 Kb *Xho*I (X) fragment containing the HG52 RL1 gene was cut from the plasmid pAT153-*Bam*HI*g*. The termini of this fragment was blunted with the Klenow fragment of DNA polymerase and cloned into the *Sma*I site, in the multicloning region of pGEM3zf(-) to generate the phagemid pXB. The position of the *Xho*I restriction enzyme sites and the location of the RL1 gene are illustrated. The nucleotide numbers described here represent the numbering system of the HG52 DNA sequence reported by McGeoch *et al* (1991).


B (10592)
X (11335)
Amp r
Tet r
ori
pAT153-BamHI g
9358 bp
12243
RL1
13179
Tet r
(16449) B
X (14678)


XhoI
f1 Ori
Lac Z
pGEM-3Zf(-)
3199 bp
Amp r
Ori
Multi-
cloning
site
SmaI
5'
3'
3'
5'
XhoI DNA fragment (3343 bp)
DNA Polymerase
(Klenow fragment)
5'
3'
3'
5'
SmaI
T4 DNA Ligase


f1 Ori
Lac Z
X (11335)
Amp r
pXB
6542 bp
12243
Ori
RL1
Lac Z
(114678) X
13179

3.3.1. Construction of individual RL1 mutant phagemids with stop codons in exon I, in the intron and within the conserved region of exon II

To determine the functional domains of RL1, it was decided to generate HSV-2 HG52 variants that produce truncated versions of ICP34.5. Translational stop codons were introduced at unique restriction enzyme sites in the RL1 gene of pSB. In exon I at the *Fsp*I site, in exonII within the conserved region at the *Pfl*MI and *Rsr*II sites. The positions of these sites are illustrated in Figure 3.3. As an alternative to intron deletion, it was decided to introduce a stop codon into the intron at the *Drd*I site (Fig. 3.3). If the predicted ORF is correct and this is a true intron, it will be spliced out and the inserted stop codon should not affect the gene function.

Initially, linker-insertion mutagenesis [section 2.2.24] was carried out using the 24-mer linker (5′-TCTAG*ACTAGCTAGCTAGTCTAGA-3′). This linker contains the stop codon TAG in all six frames in addition to two *Xba*I restriction sites which facilitate detection of mutant phagemids and viruses. To insert the ds-linker into RL1, the recombinant phagemid pSB was linearised in separate experiments with *Fsp*I, *Drd*I, *Pfl*MI and *Rsr*II restriction enzymes in the presence of CIP. *Fsp*I is a blunt-cutting enzyme which generates linear pSB with blunt-ended termini. The resulting 5′ overhangs of pSB linearised with *Rsr*II were filled in with the Klenow fragment of DNA polymerase I [section 2.2.12(b)]. The 3′ overhangs of pSB linearised with *PflM* I and *Drd*I were excised with T4 DNA polymerase [section 2.2.12(c)].

Insertional mutagenesis was unsuccessful except at the *Rsr* II site (Fig. 3.3). The mutant pSB containing the above linker at the *Rsr* II site (the stop codon in the predicted RL1 frame is marked *) was analysed by *Xba*I and other restriction endonucleases (data not shown). Before recombination back into wild type HG52 DNA, the mutation was confirmed by DNA sequence analysis. For this purpose, the blunt-ended *Rsa*I-*Sma*I fragment (344bp) (Fig. 3.4) of the mutated pSB was cloned into the *Sma*I site of M13mp18 [section 2.2.26] and sequenced using the universal M13 primer [section 2.2.29]. As shown in Figure 3.5, the sequence confirms insertion of the linker within the conserved region and the stop codon is in the predicted RL1 frame. This mutated pSB was used to generate the mutant virus 2620

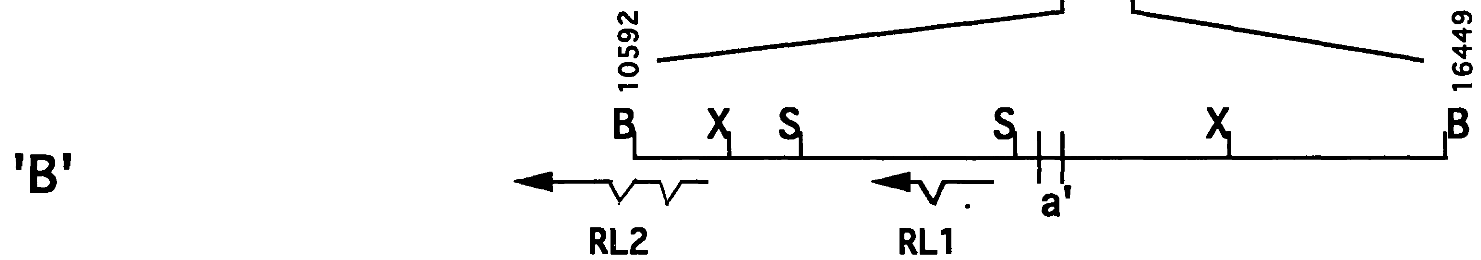
Figure 3.3. Location of translational stop codons in the ORF of the RL1 gene

A. A schematic diagram of the HSV-2 genome with the inverted regions depicted as open boxes.

B. An enlargement of the *Bam*HI*g* fragment at the L-S junction. The positions of the *XhoI* (X) and the *Sph*I (S) restriction enzyme sites are marked.

C. An expansion of the RL1 gene. The intron is indicated by dashed lines, exons by solid lines and the conserved region by a solid box. The positions of the inserted stop codons in the pSB and pXB phagemids are illustrated. The nucleotide numbers described here represent the numbering system of the HG52 DNA sequence reported by McGeoch *et al* (1991).

'A'
a
TR
L
U
L
IR
L
a'
IR
s
U
s
TR
s
a


BamHI
g
'B'
10592
16449
B
X
S
S
X
B
a'
RL2
RL1

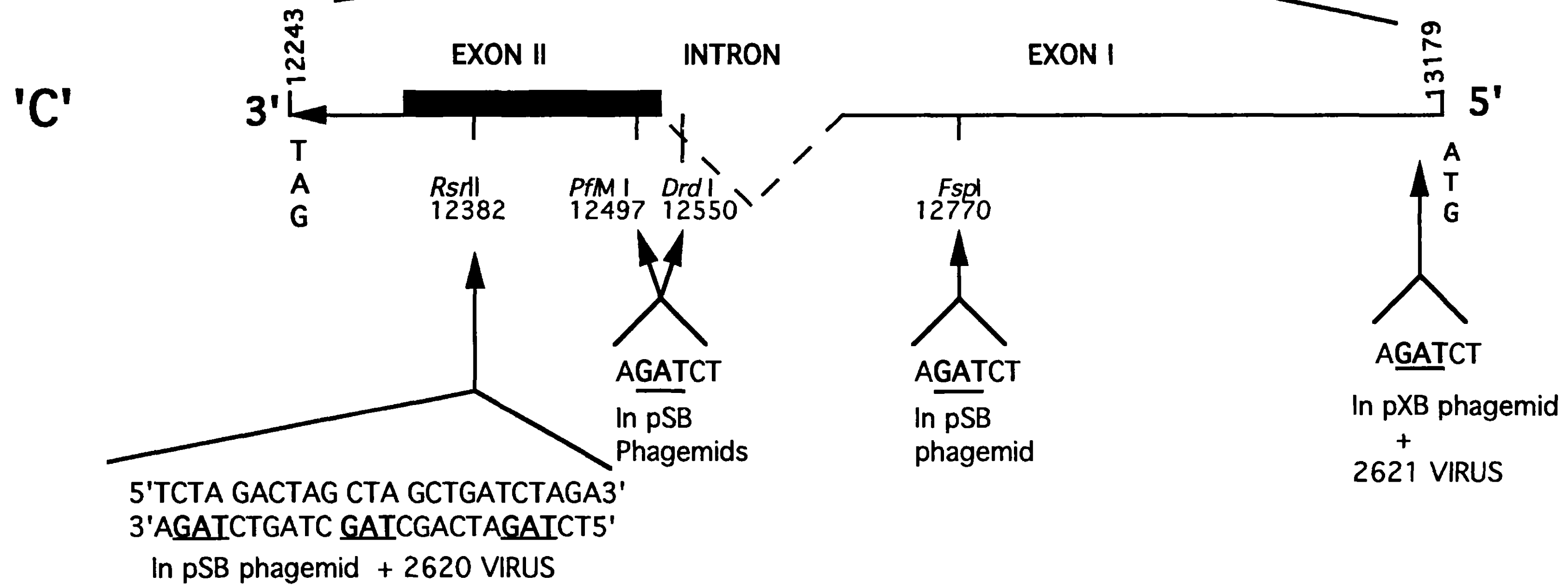
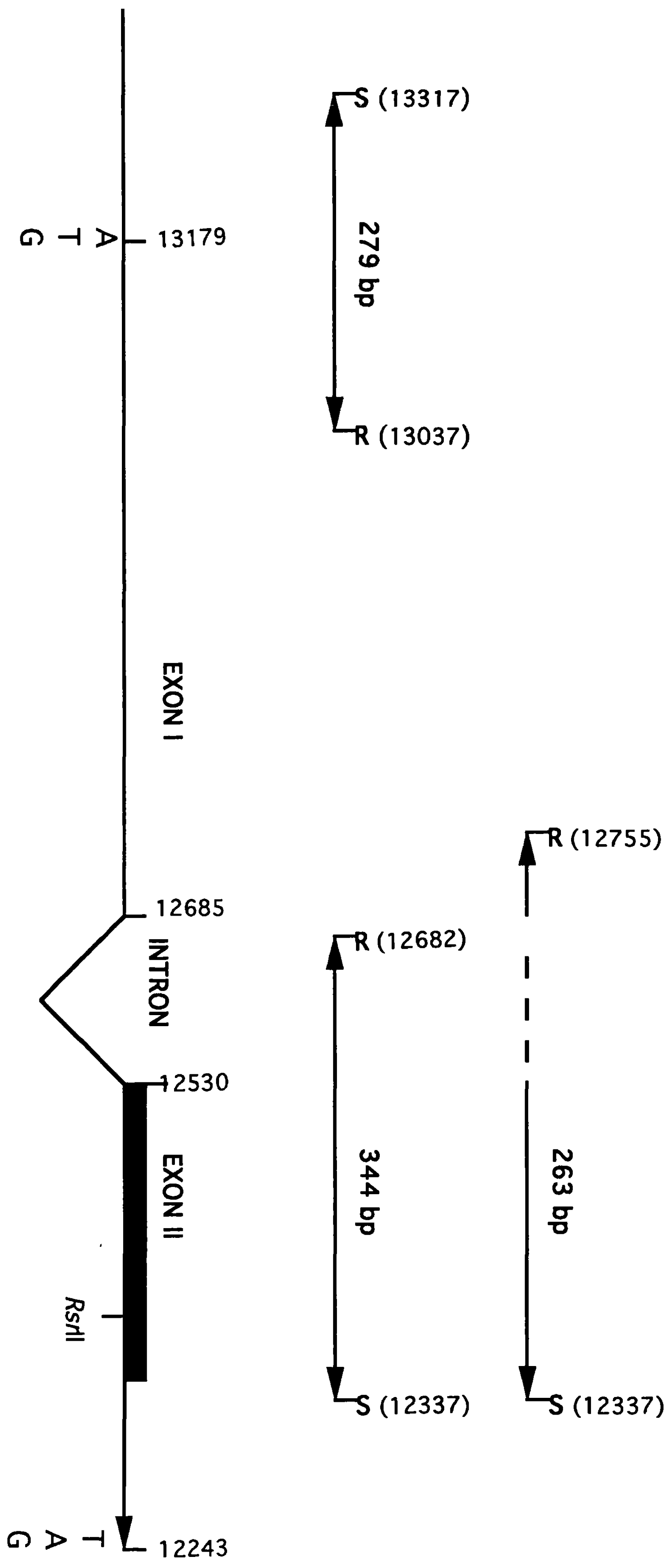

'C'
12243
13179
EXON II
INTRON
EXON I
3'
5'
T
A
G
RsrII
12382
PfM I
12497
Drd I
12550
FspI
12770
A
T
G
AGATCT
In pSB
Phagemids
AGATCT
In pSB
phagemid
AGATCT
In pXB phagemid
+
2621 VIRUS
5'TCTA GACTAG CTA GCTGATCTAGA3'
3'AGATCTGATC GATCGACTAGATCT5'
In pSB phagemid + 2620 VIRUS

Figure 3.4.The location and the size of RL1 DNA fragments used in DNA sequencing and Southern blotting

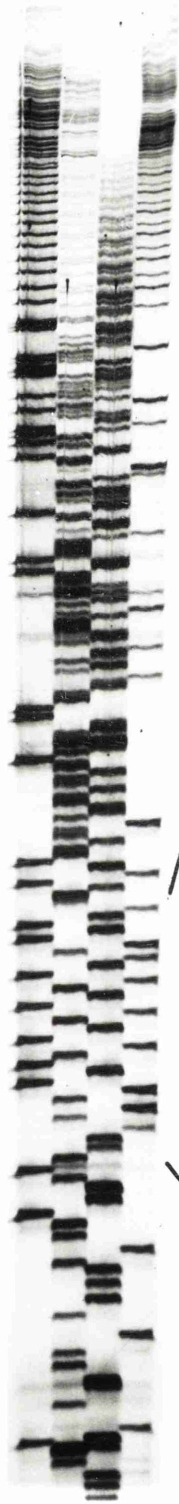
This figure illustrates the location and the size of RL1 DNA fragments generated from digestion with *Sma*I (S) and *Rsa*I (R) restriction enzymes. These fragments were used in DNA sequencing to confirm the appropriate mutations in pSB and pXB phagemids. Also the 345bp DNA fragment was ^{32}P-labelled and used as a probe in Southern blotting. The deleted intron in the 263bp fragment is indicated by a dashed line. This size refers to the fragment seen in the intronless pSB.



S (13317)
279 bp
R (13037)
A
T
G
13179
EXON I
12685
INTRON
12530
EXON II
RsrII
12243
T
A
G
R (12682)
344 bp
S (12337)
R (12755)
263 bp
S (12337)

Figure 3.5. DNA sequence of the mutated pSB phagemid in the conserved region of the RL1 gene

A 345 bp *RsaI/SmaI* subfragment of the mutated pSB phagemid was cloned into M13mp18 and sequenced. The DNA sequence of the non-coding strand of the RL1 gene in the conserved region is illustrated. The stop codon, TAG (ATC) in 6 reading frames within the inserted 24-mer linker is indicated by an open box. The dot (●) represents insertion of 3 bases into the RL1 sequence downstream of the stop codons, due to blunting of the pSB termini.



A C G T
T
G 12383
A
G
A
T
C
T
A
G
T
C
G
A
T
C
G
A
T
C
A
G
A
T
C
T
C
T
G
G 12382
C

[section 3.4.1] which was used to study the role of the conserved region in the virulence of HG52.

Since this approach was unsuccessful for insertion of the 24-mer linker at the other sites of RL1, single-stranded site-directed mutagenesis [section 2.2.25] was adopted using the oligonucleotides shown in Table 3.1. The oligonucleotides were individually used with the ssDNA of pSB in separate attempts to isolate the mutant phagemids. This technique was successful in inserting the stop codons in a single frame at the *Fsp*I, *Drd* I and *Pfl*MI sites (Fig. 3.3). Following demonstration of the avirulent phenotype of the mutant virus 2620 [section 3.5], the mutated pSB phagemids with stop codons at these sites were not used to generate HSV-2 HG52 variants.

3.3.2. Construction of a mutant phagemid containing a stop codon at the start of RL1

To provide evidence that HG52 encodes ICP34.5, the RL1 gene in pXB was mutated by introducing in a single frame, the translational stop codon TAG, 9bp downstream of the initiation codon (Fig. 3.3). The 44-mer oligonucleotide (5′-CAG CCG CCC GCC ATG TCC CGC CTC TAG AGC CGG GGT CCC CGC CG-3′) which was used for single-stranded site-directed mutagenesis is equivalent to part of the RL1 sequence of HG52 plus a 6bp insert representing an *Xba*I site (TCTAGA) for generation of mutant phagemid and virus. Positive clones were identified by restriction enzyme analysis (data not shown).

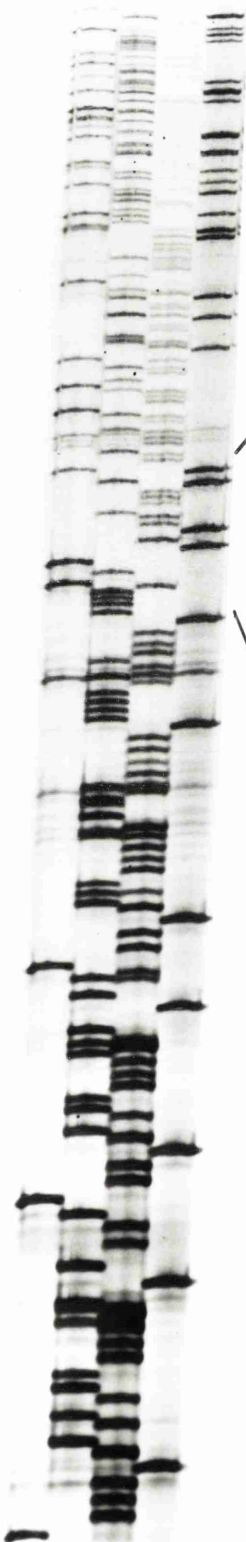
The mutation in pXB was confirmed by DNA sequencing of the region around the RL1 initiation codon. The blunt-ended *Rsa*I-*Sma*I fragment (279bp) (Fig. 3.4) of the mutated pXB was cloned into the *Sma*I site of M13mp18 [section 2.2.26] and sequenced [section 2.2.29]. The sequence of the coding strand of RL1 at the start of the gene is shown in Figure 3.6. It confirms the correct position of the stop codon TAG with respect to the RL1 initiation codon ATG. In addition, it shows deletion of two bases downstream of the stop codon, which has arisen during site-directed mutagenesis and which puts RL1 out of frame. This deletion is probably due to a defect in the synthesised oligonucleotide.

Table 3.1. The DNA sequence and the size of the synthetic single-stranded oligonucleotides used in site-directed mutagenesis to insert the stop codon TAG at the indicated restriction enzyme sites in the ORF of the RL1 gene.

Restriction enzyme site	Oligonucleotide position	Oligonucleotide size (mer)	Oligonucleotide sequence
Fsp I	12793-12748	52	5'-GCCCCACCTGGCGCTACGGCTGCTC**TAG** AGCACCACGACGGAGTACCTGGCG-3'
Drd I	12573-12522	58	5'-ACCCCCTGACGCCCCTCCGACCCCCTC **TAG**AGTGTCTCCCCGCCCGCAGGTGTG CTTC-3'
PfM I	12516-12471	52	5'-GCGCGTGCAGGTGCGCCATCTGGTC**TAG** ATGGCCTGGGAGACGGCCGCGCGC-3'

Figure 3.6. DNA sequence of the mutated pXB phagemid in the region of the RL1 ATG initiation codon

A 279bp *RsaI/SmaI* subfragment of the mutated pXB phagemid with a stop codon at the start of the RL1 gene was cloned into the *Sma* I site of M13mp18. It was sequenced using a M13 universal primer to confirm insertion of the stop codon, TAG, 9 bp downstream of the ATG initiation codon. Products were fractionated by electrophoresis in a 6% polyacrylamide gel containing 9M-urea. Both ATG and TAG are indicated by open boxes. The (●) represents deletion of two bases of the RL1 sequence which has occurred during site-directed mutagenesis. Otherwise, the sequence was identical to the wild-type sequence published by McGeoch *et al* (1991).


A G C T
A 13179
T
G
T
C
C
C
G
C
C 13170
T
C
T
A
G
A
G
C
C 13167
G
G
G
G
T

3.3.3. Construction of a RL1 intronless phagemid

An intronless phagemid was constructed to investigate the role of the putative intron in expression of RL1. The intron was deleted precisely to preserve the translational reading frame across the predicted splice junctions. The ss-DNA of the pSB phagemid was used in site-directed mutagenesis [section 2.2.25] in the presence of a 52-mer oligonucleotide (5′-CGC CCG CGG ACG CGC CGC GCG GGA AGG TGT GCT TCT CGC CGC GCG TGC AGG T-3′). This oligonucleotide corresponds to the DNA sequence across the intron junctions (Fig. 3.7). The deletion within the pSB phagemid was confirmed by restriction enzyme analysis (data not shown) and DNA sequencing [section 2.2.29] of the *Rsa*I-*Sma*I fragment (263bp) (Fig. 3.4) spanning the deletion. As shown in Figure 3.8, the DNA sequencing confirmed the precise deletion of the intron at the splicing sites predicted by McGeoch *et al* (1991).

3.4. Isolation of HSV-2 HG52 mutant viruses

To generate mutant viruses, the mutated phagemids were linearised with *Hind*III which cuts once in the multicloning site of pGEM3zf(-). Following phenol-chloroform extraction [section 2.2.18], the linearised phagemids were cotransfected in separate experiments with intact HSV-2 strain HG52 DNA as described in section 2.2.9. When extensive cytopathic effect was observed, the transfection plates were harvested and the progeny virus titrated on BHK21/C13 cells. Single virus plaques [section 2.2.10] were picked and mutant viruses were identified either by ^{32}P-DNA labelling [section 2.2.8] or Southern blotting [section 2.2.22]. Mutants with the desired genotype were plaque purified three times before growing a stock. Isolation of the mutant viruses is described in the following sections:

3.4.1. Isolation of the mutant virus 2620 with a stop codon within the conserved region of exon II

Comparative sequence analysis of the long repeat regions of HSV-1 /HSV-2 showed that HG52 possesses a counterpart of the HSV-1 RL1 gene (McGeoch *et al.*, 1991). DNA sequence analysis of the RL1 ORF revealed that the C-terminus of the predicted ICP34.5 of

Figure 3.7. A schematic diagram of the RL1 gene of HSV-2 strain HG52 and the oligonucleotide used to delete the predicted intron

This diagram illustrates the sequence and the location of the two components of the oligonucleotide used to delete the intron. The intron of RL1 is indicated by dashed lines and exons by solid lines. The nucleotide numbers represents the numbering system of the HG52 DNA sequence reported by McGeoch *et al.* (1991).

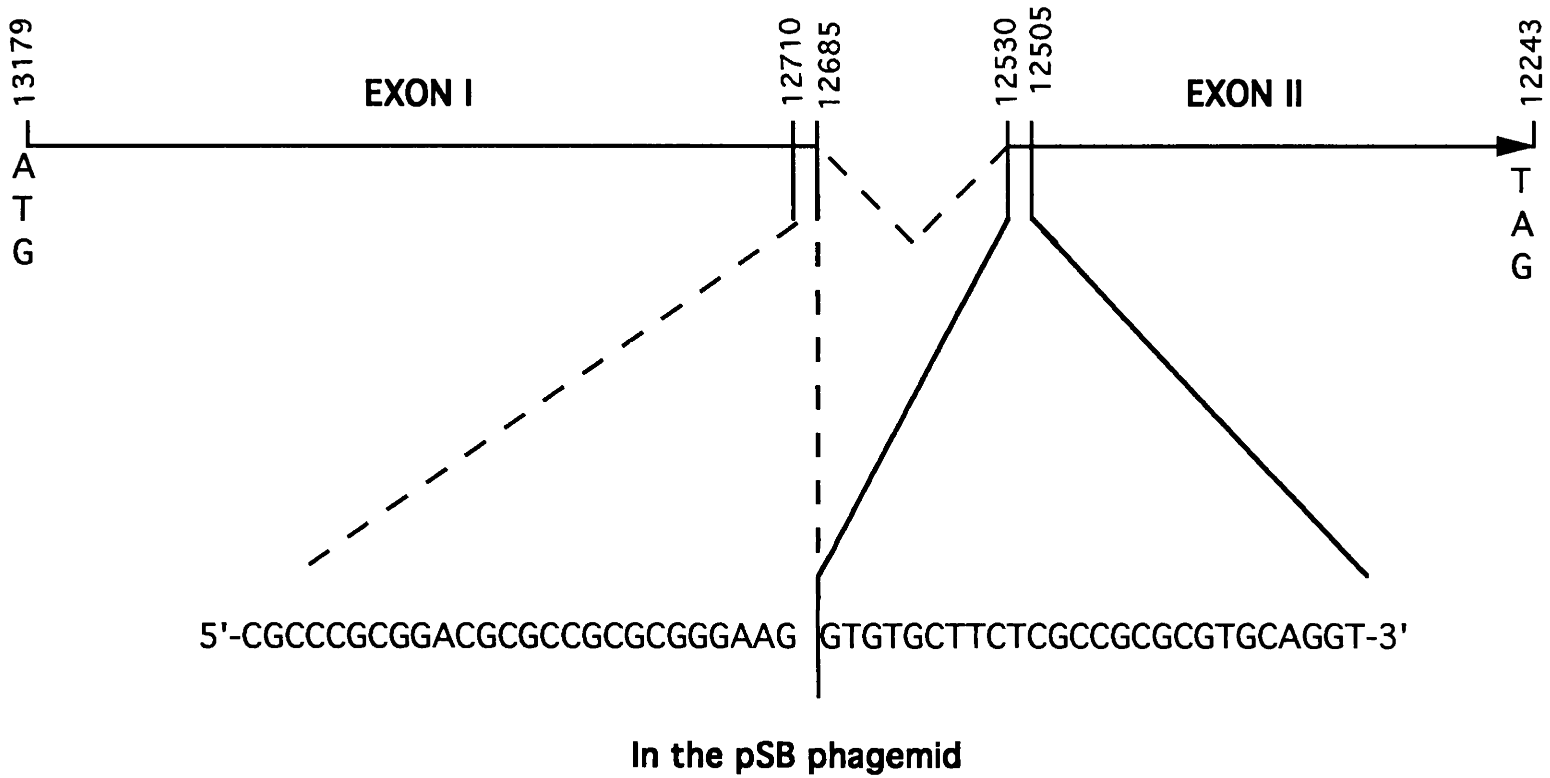
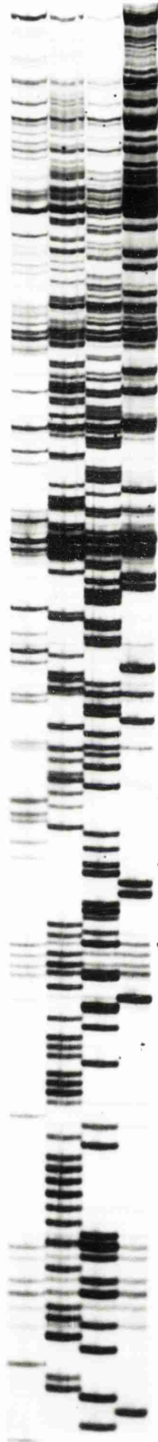

13179
EXON I
12710
12685
12530
12505
EXON II
12243
A
T
G
T
A
G
5'-CGCCCGCGGACGCGCCGCGCGGGAAG
GTGTGCTTCTCGCCGCGCGTGCAGGT-3'
In the pSB phagemid

Figure 3.8. DNA sequence of the intronless pSB phagemid

A 263 bp *RsaI/SmaI* subfragment of the intronless pSB phagemid was cloned into M13mp18 and sequenced. The DNA sequence confirmed deletion of the intron at the indicated splicing sites predicted by McGeoch *et al* (1991).



T C G A
C
T
T
C
G
T
G
T
12530 G EXON II
12685 G EXON I
A
A
G
G
G
C
G
C

* One hundred and twenty plaques were picked and the DNA was digested with *XbaI*.

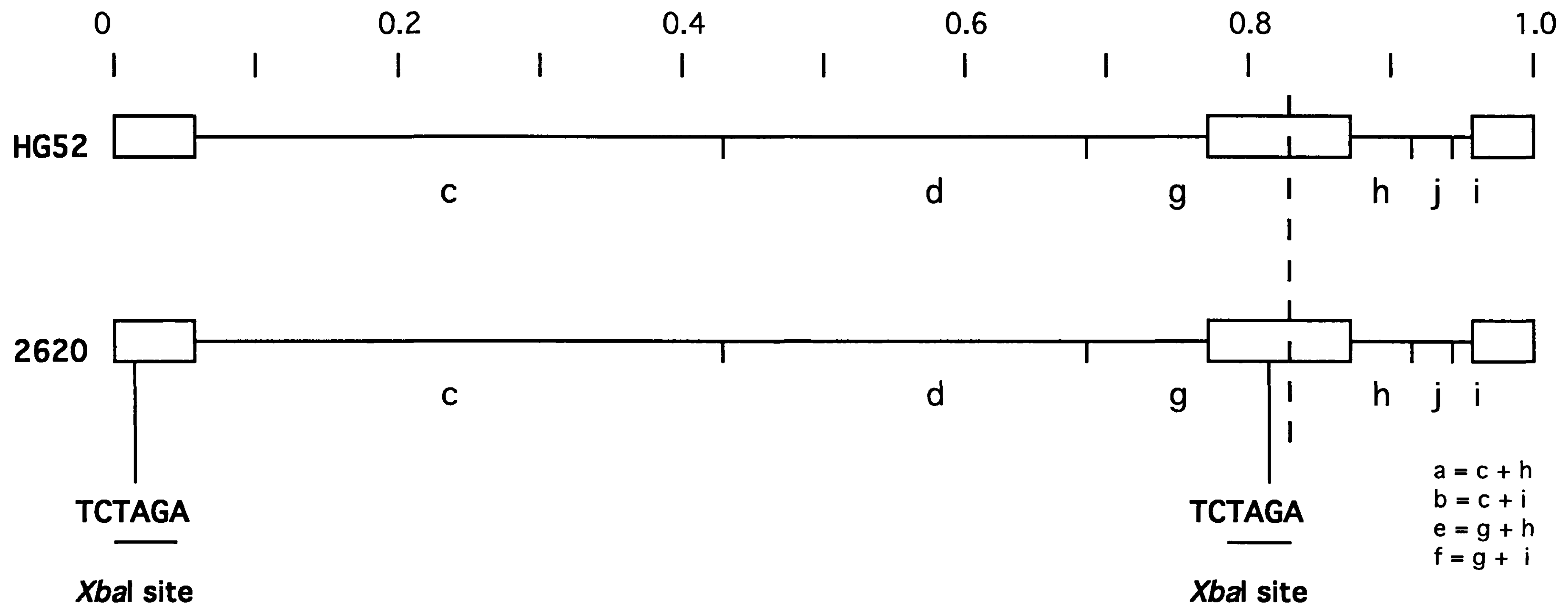
HG52 is 83% homologous to that of HSV-1 and approximately 50% to the C-terminus of the mouse protein MyD116 (McGeoch and Barnett, 1991). On this basis, it was important to study the role of the conserved region in the virulence of HSV-2 strain HG52. Therefore, the mutant virus 2620 was constructed. This mutant was selected by *Xba*I digestion of ^{32}P-labelled DNA of 192 plaques isolated from cotransfection of HG52 DNA and the pSB phagemid containing a stop codon in all six open reading frames at the *Rsr*II site within the conserved region (Fig. 3.3) [section 3.3.1]. Each inserted oligonucleotide (in TR_L and IR_L) contains two *Xba*I sites. On digestion of the recombinant virus, each pair will give the profile of one extra *Xba*I site. One site is 1122bp from the L terminus and the second, 1122bp from the L-S junction (Fig. 3.9). Therefore, on digestion of 2620 DNA with *Xba*I (Fig. 3.10), digestion also occurs at the novel *Xba*I sites. This results in digestion of the normally 0.5M terminal *c* and *g* fragments of the wild-type, to 1M bands, *c'* and *g'* that are 1122bp smaller, plus two new 1 M bands of 1122bp that cannot be seen on the gel. Correspondingly, the 0.25M L-S junction fragments *a, b, e* and *f* of the wild type disappear from their normal positions to be replaced by two 0.5M novel bands running above the *i* and *h* bands. These were designated *i'* and *h'* and are 1122bp bigger than the *i* and *h* fragments. The relevant *Xba*I restriction maps for HG52 and 2620 are shown in Figure 3.9.

3.4.2 Isolation of the RL1 null mutant virus 2621

Previous studies demonstrated that the HSV-1 RL1 gene is responsible for the virulence phenotype. Hence, insertion of translational stop codons at the beginning of the coding sequence abolished the virulence of HSV-1 strains F and 17$^+$ (Chou *et al.*, 1990; McKie *et al.*, 1994). To validate the HG52 RL1 sequence analysis, it was decided to generate the RL1 null mutant virus 2621 using the pXB phagemid containing a stop codon in a single frame 9bp downstream of the RL1 initiation codon (Fig. 3.3) [section 3.3.2]. On digestion of 2621 DNA with *Xba*I, the profile was essentially the same as 2620. In this case, novel bands *i'* and *h'* are 195bp bigger than the corresponding *i* and *h*. The *g'* and *c'* bands are 195bp smaller than *g* and *c* of the wild type (Fig. 3.11).

Figure 3.9. Position of the *Xba*I restriction enzyme sites in HSV-2 strain HG52 and the mutant virus 2620 genomes in the prototype orientation

The wild-type restriction enzyme sites are marked (Cortini and Wilkie, 1978) and the two stop codons (TAG) which have been introduced into the 2620 genome are underlined. The dashed vertical line indicates the position of the L-S junction.



0
0.2
0.4
0.6
0.8
1.0
HG52
c
d
g
h
j
i
2620
c
d
g
h
j
i
TCTAGA
XbaI site
TCTAGA
XbaI site
a = c + h
b = c + i
e = g + h
f = g + i

Figure 3.10. *XbaI* restriction enzyme profiles of HSV-2 strain HG52, the mutant virus 2620 and the rescuant R2620

Autoradiographs of *Xba*I digests of viral DNA ^{32}P-labelled *in vivo*. 2620 contains two detectable extra novel *Xba*I sites 1122bp from the L-terminus and from the L-S junction. Missing bands are indicated by an arrow head (◀). Novel bands are indicated by a dot (●) and are designated by the letter of the band from which they were derived plus a prime symbol ('). The DNA profile of R2620 is identical to that of the wild-type. Lane 1, 2620; Lane 2, HG52; Lane 3, R2620.

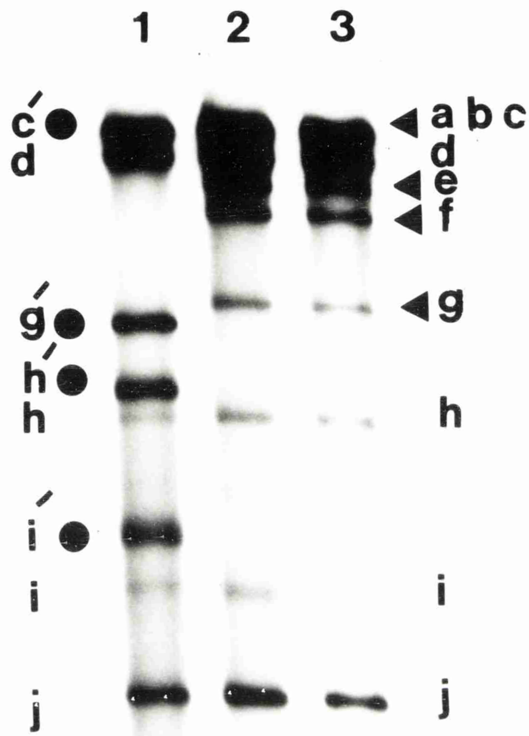
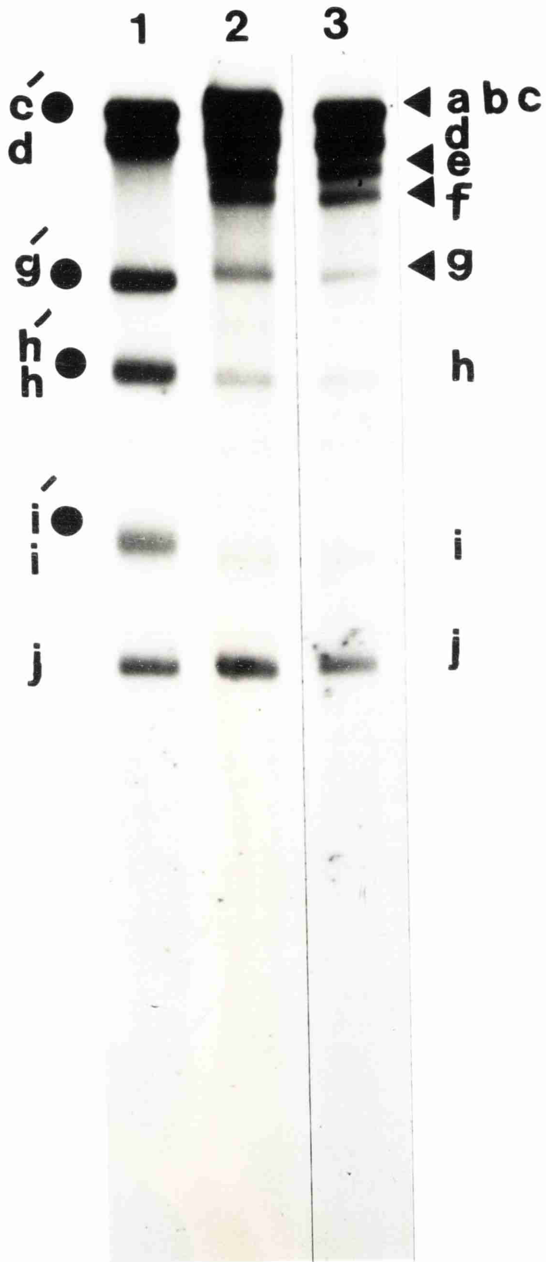

1
2
3
c' ●
d
g' ●
h' ●
h
i' ●
i
j
◀ a b c
d
◀ e
◀ f
◀ g
h
i
j

Figure 3.11. ***Xba*I restriction enzyme profiles of the DNA of HSV-2 strain HG52, the mutant virus 2621 and the rescuant R2621**

Autoradiograph of *Xba*I digests of viral DNA ^{32}P-labelled *in vivo*. 2621 contains two novel *Xba*I sites 195bp from the L-terminus and from the L-S junction. Missing bands are indicated by an arrow head (◀). Novel bands are indicated by a dot (●) and are designated by the letter of the band from which they were derived plus a prime symbol ('). The DNA profile of R2621 is identical to that of HG52. Lane 1, 2621; Lane 2, HG52; Lane 3, R2621.


1
2
3
c′
d
g′
h′
h
i′
i
j
a b c
d
e
f
g
h
i
j

3.4.3. Isolation of the potential intronless RL1 mutant virus 2622

It was predicted from the sequence analysis that the ORF of HG52 RL1 gene is interrupted by an intron which does not exist in the RL1 gene of the sequenced HSV-1 strains (Chou and Roizman, 1990; McGeoch *et al.*, 1991). It was therefore important to provide evidence that the mRNA of RL1 is spliced. As described in section 3.17 we tried to use PCR to amplify the reverse-transcribed cDNA product of the HG52 mRNA.

In parallel it was decided to delete the predicted intron at the proposed splicing sites to study its role in the expression of RL1. The mutant 2622 was generated by cotransfection of BHK21/C13 cells with intact HSV-2 HG52 DNA and the DNA of the intronless pSB phagemid (Fig. 3.7) [section 3.3.3.]. Single 393 plaques were isolated and their ^{32}P-labelled DNA subjected to *Bam*HI digestion (data not shown). Because the deletion was small (154bp), it was difficult to observe any alteration in the size of the *Bam*HI*g* fragment, therefore analysis was carried out by double restriction enzyme digestion and Southern blotting [section 2.2.22] using the ^{32}P-labelled *Rsa*I-*Sma*I fragment (344bp) (Fig. 3.4) as a probe [section 2.2.21]. As shown in Figure 3.12, digestion of 2622 DNA with *Sma*I and *Sac*I apparently generated an 802bp fragment spanning the deletion compared to the 957bp wild-type fragment.

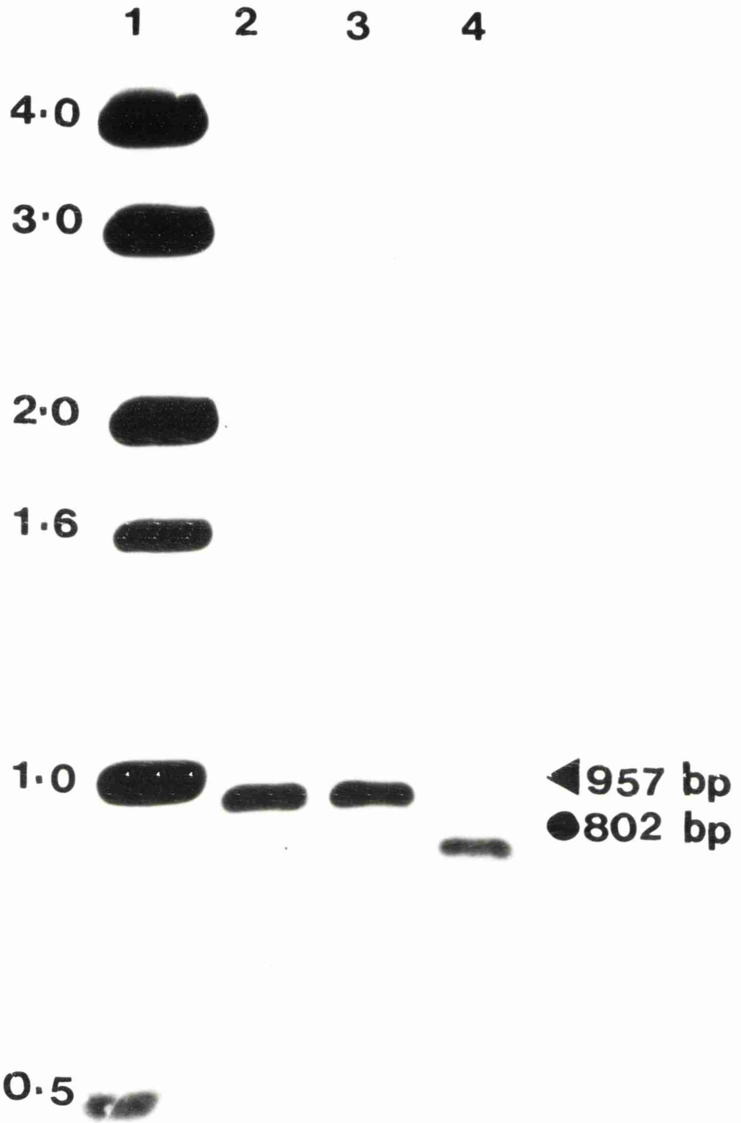
3.5. The virulence of RL1 mutant viruses

The virulence of the mutants was determined by calculating the LD_{50} values in Balb/c mice following intracerebral inoculation. The LD_{50} values were compared to those of the parental wild-type HG52 and the RL1 deletion mutant 2604. The genotype of 2604 was described in detail by Harland and Brown (1985) and Taha *et al* (1989b). Essentially it has a 1488bp deletion in both copies of *Bam*HI*v* which is located between 0-0.02 and 0.81-0.83 m.u. of TR_L and IR_L, respectively. As it is avirulent on intracerebral inoculation of mice (Taha *et al.*, 1989a), it was used as a negative control.

Separate groups of 3-week old Balb/c mice were intracerebrally inoculated [section 2.2.36(a)] with HG52 and RL1 mutant viruses. As the LD_{50}s of HG52 and 2604 were known from previous studies (Taha *et al.*, 1989a,b), mice were inoculated with these

Figure 3.12. Southern blot of a *SacI/SmaI* restriction enzyme digest of the DNA of HSV-2 strain HG52, the mutant virus 2622 and the rescuant R2622

The genomes of HG52, 2622 and R2622 were digested with *Sma*I and *Sac*I restriction enzymes. The intron sequence was detected using the 344bp *SmaI/RsaI* fragment of pSB (Fig 3.4). HG52 and R2622 produced a 957bp fragment while 2622 apparently produced a 802bp fragment. Fragments were separated on a 1% agarose gel. Lane 1, size marker; Lane 2, HG52; Lane 3, R2622, Lane 4, 2622.


1
2
3
4
4·0
3·0
2·0
1·6
1·0
0·5
957 bp
802 bp

viruses at doses of 10^2-10^3 and 10^5-10^7 p.f.u./mouse, respectively. Since the virulence of the constructed mutant viruses could not be anticipated, mice were injected with a range of doses, 2620 10^2-10^6 p.f.u./mouse while 2621 and 2622 10^2-10^7 p.f.u./mouse. Mice were monitored for 21 days and their clinical state recorded. Those which showed neurological dysfunction and signs of encephalitis were put down. The symptoms included: ruffled fur, hunched back, reluctance to move, tendency to be isolated, lack of interest in food and rarely limb paralysis. The results are shown in Table 3.2. It can be seen that HG52 and 2604 had LD_{50}s of $<10^2$ p.f.u./mouse and 3.16×10^6 p.f.u./mouse, respectively. These values are comparable to those determined previously (Taha *et al.*, 1989a,b). The ICP34.5 null mutant 2621 was avirulent with a LD_{50} value $>10^7$ p.f.u./mouse, thus this mutant was at least 10^5-fold less virulent than HG52. This result indicates that loss of HG52 virulence is due to lack of ICP34.5 expression and confirms the existence of the HG52 RL1 ORF predicted by McGeoch *et al* (1991).

The intronless variant 2622 had an intermediate LD_{50} value of 10^5p.f.u./mouse indicating that either (i) the predicted intron in HG52 is functional and required for full expression of RL1 (ii) the proposed splicing sites of the intron are not correct (iii) the deleted DNA sequence is not an intron but a functional domain of RL1, suggesting a sequencing error in the predicted ORF of HG52 RL1 (iv) there is an undetected secondary mutation in the gene.

Insertion of a stop codon at the *Rsr*II site within the conserved region abolished the virulence phenotype of HG52. The mutant 2620 was avirulent with a LD_{50} of 2.37×10^6 p.f.u./mouse. This result indicates that the conserved region and/or the C-terminal domain of ICP34.5 are essential for gene function. This is supported by the recent finding of Chou and Roizman (1994) who demonstrated that the conserved carboxyl terminal domain of the HSV-1 RL1 gene is necessary to preclude the total shutoff of protein synthesis in the neuroblastoma cell line SK-N-SH.

Due to the avirulent phenotype of 2620, construction of mutant viruses with stop codons upstream of the *Rsr*II site was not pursued. This included those with stop codons inserted at the *Pfl*M I, *Drd* I and *Fsp*I restriction sites described in section 3.3.1. Instead, it was decided to try to construct a mutant virus with a stop codon precisely at the terminus of the

Table 3.2. The LD50 values of HSV-2 strain HG52, 2604, 2621, 2622 and the rescuants following intracerebral inoculation of 3-week old Balb/c mice.

Virus \ Dose	10^2	10^3	10^4	10^5	10^6	10^7	LD50 p.f.u./mouse	LD50 mutant/ LD50 rescuant
HG52	4/4*	4/4	ND	ND	ND	ND	$<10^2$	____
2604	ND	ND	ND	0/4	0/4	4/4	3.16×10^6	____
2620	0/5	0/5	0/5	0/5	1/5	ND	2.37×10^6	____
2621	0/4	0/4	0/4	0/4	0/4	0/4	$>10^7$	____
2622	0/4	0/4	0/4	2/4	4/4	4/4	10^5	____
R2620	0/4	1/4	1/4	ND	ND	ND	2.15×10^4	1.1×10^2
R2621	0/3	0/3	2/3	ND	ND	ND	5.62×10^3	$> 1.78 \times 10^3$
R2622	0/4	1/4	3/4	ND	ND	ND	3.16×10^3	31.6

ND = not done

* = number of deaths/number of animals inoculated

conserved region. This virus would have confirmed the role of the conserved region in function of HG52 ICP34.5. However, despite trying several conditions of single-stranded site-directed mutagenesis, all were unsuccessful and the mutant phagemid was not obtained.

3.6. *In vivo* growth of HG52 and RL1 mutants in mouse brain

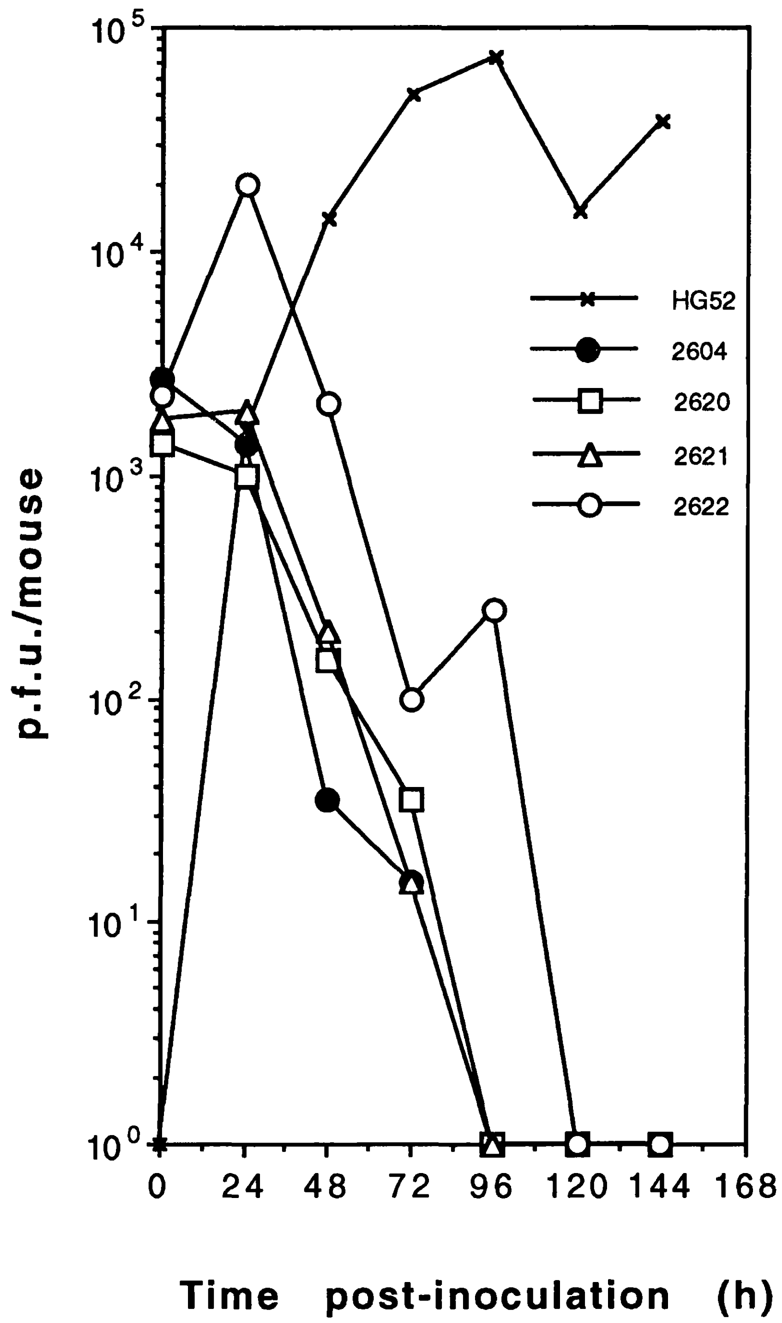
It has been shown previously that the HG52 variant 2604 is avirulent following intracerebral inoculation of mice, due to its inability to replicate in brain tissue (Taha *et al.*, 1989a). Similar results were observed with the HSV-1 strain 17^{+} variants 1716 and 1771 (MacLean, A *et al.*, 1991; McKie *et al.*, 1994). Therefore, it was likely that the differences in the LD_{50} values (Table 3.2) between the RL1 mutants (2620, 2621,2622) and the parental wild type HG52 pl.17 were also due to differential abilities to replicate in mouse brain. Separate groups of 3-week old female Balb/c mice were intracerebrally inoculated with HG52 at an input dose of 10^2 p.f.u./mouse and with 10^5 p.f.u./mouse of each of the RL1 mutants 2604, 2620, 2621 and 2622. At various times post-inoculation, two mice per virus were put down, their brains removed, homogenised, sonicated in PBS/calf serum and stored at -70°C. The virus progeny in each brain was titrated on BHK/C13 cells at 37°C and the mean ated for each virus as p.f.u./mouse (Table 3.2a).The *in vivo* growth kinetics of HG52 and RL1 mutant viruses are illustrated in Figure 3.13. As demonstrated previously for HG52 and 2604 (Taha *et al.*, 1989a), the wild type grows exponentially reaching a titre of $8x10^5$ p.f.u./mouse by 120h post-inoculation. In contrast, 2604 showed no evidence of replication, the titre diminished progressively with time and the virus was cleared by 96h post-inoculation. Similarly, the mutants 2620 and 2621 showed no growth. The growth of the mutant 2622 was intermediate between HG52 and 2604 and there was no detectable virus by 120h post-inoculation. The differential abilities of HG52 and the RL1 mutants to replicate in mouse brains is consistent with their LD50 values (Table 3.2).

3.7. *In vitro* marker rescue of the mutant viruses 2620, 2621 and 2622

To confirm that the virulence phenotypes of the mutant viruses were due solely to the RL1 mutations, wild-type rescuants R2620, R2621 and R2622 were constructed. The differential

Figure 3.13. ***In vivo* growth kinetics of HG52 and RL1 mutant viruses**

The *in vivo* growth kinetics of HG52, 2604, 2620, 2621 and 2622 were determined by injection of viruses into the left cerebral hemispheres of 3 week old Balb/c mice. The input dose was 10^2 p.f.u./mouse for HG52 and 10^5 p.f.u./mouse for mutant viruses. At the indicated times post inoculation (X-axis), two mice for each time point were sacrificed, their brains homogenised, sonicated and progeny virus titrated on BHK21/C13 cells at 37°C. The titres are given as p.f.u./mouse (Y-axis).



10^5
10^4
10^3
10^2
10^1
10^0
p.f.u./mouse
HG52
2604
2620
2621
2622
0
24
48
72
96
120
144
168
Time post-inoculation (h)

Table 3.2 a. Titer of virus in individual mouse brain following intracranial inoculation.

Virus	Mouse 1 (p.f.u./mouse)	Mouse 2 (p.f.u./mouse)	Mean (p.f.u./mouse)	Time post inoculation (h)
HG52	<10	<10	<10	0
2604	2.2×10^{3}	3.3×10^{3}	2.7×10^{3}	
2620	1.2×10^{3}	1.6×10^{3}	1.4×10^{3}	
2621	1.9×10^{3}	1.7×10^{3}	1.8×10^{3}	
2622	2.7×10^{3}	1.9×10^{3}	2.3×10^{3}	
HG52	1.4×10^{3}	1.8×10^{3}	1.6×10^{3}	24
2604	--	1.4×10^{3}	1.4×10^{3}	
2620	8×10^{2}	1.2×10^{3}	1.0×10^{3}	
2621	1.4×10^{3}	2.7×10^{3}	2.05×10^{3}	
2622	1.9×10^{4}	3.8×10^{4}	2.85×10^{4}	
HG52	1.7×10^{4}	1.2×10^{4}	1.4×10^{4}	48
2604	2.0×10^{1}	5.0×10^{1}	3.5×10^{1}	
2620	2.0×10^{2}	1.0×10^{2}	1.5×10^{2}	
2621	2.0×10^{2}	3.0×10^{2}	2.5×10^{2}	
2622	1.9×10^{3}	2.2×10^{3}	2.05×10^{3}	
HG52	4.2×10^{4}	5.0×10^{4}	4.6×10^{4}	72
2604	2.0×10^{1}	1.0×10^{1}	1.5×10^{1}	
2620	7.0×10^{1}	<10	3.5×10^{1}	
2621	2.0×10^{1}	1.0×10^{1}	1.5×10^{1}	
2622	1.0×10^{3}	2.0×10^{3}	1.5×10^{3}	
HG52	--	7.5×10^{4}	7.5×10^{4}	96
2604	<10	<10	<10	
2620	<10	<10	<10	
2621	<10	<10	<10	
2622	4×10^{2}	1.0×10^{2}	2.5×10^{2}	
HG52	--	1.5×10^{4}	1.5×10^{4}	120
2604	<10	<10	<10	
2620	<10	<10	<10	
2621	<1.0	<10	<10	
2622	<1.0	<10	<10	
HG52	3.8×10^{4}	3.9×10^{4}	3.85×10^{4}	144
2604	<1.0	<10	<10	
2620	<10	<10	<10	
2621	<10	<10	<10	
2622	<10	<10	<10	

growth of the wild type and RL1 negative mutants in 3T6 (Brown *et al.*, 1994a) was used to isolate rescuants. Mutants in RL1 which fail to express ICP34.5 form minute abortive plaques in confluent 3T6 cells while wild-type virus forms normal sized plaques.

Rescuants were generated in separate experiments [section 2.2.36(d)] following transfection of BHK/C13 cells with intact mutant DNA and the pXB phagemid linearized with *Hind*III. The RL1 recombinant pXB phagemid [section 3.2] was used in transfection because the *Xho*I fragment is small (3.3Kb) thus minimising the chances of correcting a secondary mutation that might exist outwith the RL1 gene. Progeny virus was titrated on confluent 3T6 mouse embryo fibroblast cells. Differentiation was based on size and morphology of plaques and individual large plaques were isolated and screened. Viruses with a wild type genotype were identified using the same technique previously described for isolation of the mutant viruses [section 3.4]. The *Xba*I profiles of the DNA of the rescuants R2620 and R2621 are shown in Figures 3.10 and Figure 3.11, respectively. In both rescuants, the *XbaI a, b, e, f* fragments reappeared and the *g* and *c* fragments returned to their wild-type position. The DNA profile of R2622 digested with *Sma*I and *Sac*I restriction enzymes and hybridised to the ^{32}P-labelled probe (344bp) (Fig. 3.4) is indistinguishable from that of HG52 (Fig. 3.12). All rescuants were plaque purified three times before growing stock virus.

3.8. The virulence of the rescuants generated by *in vitro* marker rescue

The virulence of the three times plaque purified rescuants generated by *in vitro* marker rescue was evaluated by calculating the LD_{50} values following intracerebral inoculation of Balb/c mice [section 2.2.36(a)]. Groups of 3-week old mice were inoculated separately with the rescuants at doses of 10^2-10^4 p.f.u./mouse. The LD_{50} values of the rescuants compared to the mutants, parental wild-type HG52 and 2604 are shown in Table 3.2. It can be seen that the rescuants demonstrated an intermediate level of virulence with their LD_{50} values ranging from $3.16x10^3$ to $5.62x10^3$ p.f.u./mouse. R2620 demonstrated about a 110 fold increase in virulence over 2620. R2621 demonstrated more than a $1.78x10^3$ fold increase over the virulence of 2621 and R2622 about a 31 fold increase over the virulence of 2622.

The failure of rescuants to regain the virulence of the wild-type HG52 suggested the

following possibilities: (i) a mutation in the pXB *Xho*I fragment used in the marker rescue experiments or (ii) a secondary mutation in either the RL1 gene or elsewhere in the genome of the mutant viruses and/or the genome of the rescuants due to either heterogeneity in the HG52 stock or in passage of the viruses during the 3 rounds of purification. These possibilities were analysed further.

3.9. *In vivo* marker rescue of the mutant viruses

As the rescuants generated by *in vitro* marker rescue did not return to the wild-type phenotype, it was decided to reisolate them by *in vivo* marker rescue following intracerebral inoculation of groups of mice with: (i) the same transfection mix as used for the *in vitro* marker rescue experiments [section 3.7] (ii) a transfection mix of mutant DNA plus the pAT *Bam*HI*g* plasmid (Fig. 3.1] and (iii) a transfection mix of mutant DNA plus the *Bam*HI*g* fragment, purified from digested HG52 DNA. The fragment *Bam*HI*g* was used because it has been shown previously to restore virulence to 2604 (Taha *et al.*, 1989b). The *in vivo* system should select rescuants generated from recombination of the wild-type DNA fragment with a mutant genome having no additional defects. Using a different wild-type DNA fragment should determine if there was a secondary mutation in the *Xho*I fragment of pXB. Separate transfections were carried out as described in section 2.2.9 using a 10-fold molar excess of either pXB or pAT-*Bam*HI*g* plasmids and 1:1 ratio of the HSV-*Bam*HI*g* fragment. Before animal inoculation, the presence of rescuant viruses with the wild-type profile in the transfection mixes was first tested by titration on 3T6 mouse embryo fibroblast cells. The DNA of large plaques was confirmed by either ^{32}P-DNA labelling or Southern blotting. As we expected a low frequency of revertants in the HSV-*Bam*HI*g* transfection mixes, 3-week old Balb/c mice were injected intracerebrally with 25µl of a neat and 10^{-1} dilution of each transfection mix (Table 3.3). As a higher frequency of revertants was expected in other transfection mixes with the pXB and pAT-*Bam*HI*g* plasmids, mice in this case were inoculated with 10^{-1} and 10^{-2} dilutions (Table 3.3). Mice were monitored for 10 days and their clinical state recorded. All mice inoculated with neat HSV-*Bam*HI*g* transfection mixes showed early signs of neurological dysfunction and were put down 24-

Table 3.3. *In vivo* marker rescue.

Plasmid/ DNA rescuant fragment \ Dose	N	10^{-1}	10^{-2}	Putative rescuant
pXB	ND	0/2*	0/2	R2620
pAT-*Bam*HI*g*	ND	0/2	0/2	
HSV-*Bam*HI*g*	2/2	0/2	ND	
pXB	ND	2/2	1/2	R2621
pAT-*Bam*HI*g*	ND	2/2	0/2	
HSV-*Bam*HI*g*	2/2	0/2	ND	
pXB	ND	2/2	0/2	R2622
pAT-*Bam*HI*g*	ND	2/2	0/2	
HSV-BamHI*g*	2/2	2/2	ND	

N = neat
ND = not done
* = number of deaths/ number of animals inoculated

30h post inoculation. It was considered that the early illness of these mice was due to a high dose of input virus from the transfection mixes.

In the case of R2620, apart from mice put down 30h post inoculation, none showed signs of illness indicating that R2620 with a wild-type phenotype had not been generated. Therefore, R2620 detected in the transfection mixes is likely to contain a defect which modulates virulence. To confirm this, it was decided to isolate a rescuant from the brain of a mouse, put down 30h post inoculation of the HSV-*Bam*HI*g* transfection mix (neat).

In the case of R2621, mice especially those inoculated with 10^{-1} of either pXB or the pAT-*Bam*HI*g* transfection mixes (Table 3.3) began to show signs of neurological dysfunction 3-7 days post inoculation indicating probable generation of wild type revertants. At this point the mice were put down, their brains removed and stored at -70°C. Similar findings were observed for R2622 (Table 3.3) in mice inoculated with 10^{-1} of either pXB or *Bam*HI*g*.

The wild-type rescuants R2621 and R2622 were isolated from the brains of mice which were put down 3-7 days post inoculation. This time point was based on the growth patterns of HG52 and the RL1 mutant viruses in mouse brain [section 3.6]. It was obvious that the avirulent variants of HG52 can not be detected by day 4 post inoculation and the intermediate variant, day 5 post inoculation (Fig. 3.13). Therefore, neurological symptoms observed before this time (Table 3.3) might be due to either the growth of rescuants or the high dose of input virus.

Two putative R2621 rescuants were isolated from brains of mice which were put down day 6 post inoculation. One from a brain inoculated with the pAT-*Bam*HI*g* transfection mix and the other from a brain inoculated with the pXB transfection mix (Table 3.4). Similarly, three putative R2622 rescuants were isolated, one from brain of a mouse which was put down 4 days post inoculation of the HSV-*Bam*HI*g* transfection mix and two from the brain of a mouse which was put down 5 days post inoculation of the pXB transfection mix (Table 3.4).

Brains were homogenised in 500μl PBS/calf serum, progeny virus titrated on 3T6 cells and single large plaques were isolated and tested as previously described for isolation of mutants [section 3.4]. One isolate with a wild-type restriction enzyme profile was selected from each

Table 3. 4. LD_{50} values after intracerebral inoculation of HG52 rescuants isolated by *in vivo* marker rescue using different DNA fragments.

Dose / Virus	Plasmid/DNA fragment	10^2	10^3	10^4	10^5	10^6	LD_{50} p.f.u./mouse	LD_{50} mutant/ LD_{50} rescuant
HG52	———	1/4*	4/4	ND	ND	ND	$2.15X10^2$	———
2620	———	ND	ND	ND	0/4	0/4	$>10^6$	———
2621	———	ND	ND	ND	0/4	0/4	$>10^6$	———
2622	———	ND	ND	ND	0/4	1/4	$2.15X10^6$	———
R2620	HSV-*Bam*HIg	0/4	0/4	0/4	1/3	ND	$1.77X10^5$	> 5.64
R2621/A	pAT-*Bam*HIg	4/4	4/4	3/3	4/4	ND	$<10^2$	$> 10^4$
R2621/B	pXB	2/4	3/4	4/4	4/4	ND	10^2	$> 10^4$
R2622/A	HSV-*Bam*HIg	0/4	2/4	4/4	4/4	ND	10^3	$2.15x10^3$
R2622/B	pXB	0/4	1/3	2/4	4/4	ND	10^4	$2.15x10^2$
R2622/C	pXB	0/4	4/4	4/4	4/4	ND	$3.16X10^2$	$6.8x10^3$

ND = not done

* = number of death/number of animals inoculated

brain (data not shown) and grown into a virus stock. Following one round of purification, the LD_{50} values [section 3.10] of these rescuants were determined. The rounds of purification were minimized to avoid a reduction in the virulence of the rescuants. The three rounds of purification of mutant viruses and rescuants generated *in vitro*, involved seven passages in BHK21/C13 cells.

3.10. Virulence of the rescuants R2620, R2621 and R2622 generated by *in vivo* marker rescue

The virulence of the rescuants R2620, R2621 and R2622 generated by *in vivo* marker rescue was evaluated to determine if their wild-type phenotype had been restored. The LD_{50} values were determined for groups of four Balb/c mice as described in section 2.2.36(a). Mice were intracerebrally inoculated with HG52 at doses of 10^2-10^3 p.f.u./mouse and with mutants (2620, 2621 and 2622) at doses of 10^5-10^6p.f.u./mouse. The doses were based on the known virulence phenotype of HG52 and the mutant viruses (Table 3.2). The rescuants were injected at doses of 10^2-10^5p.f.u./mouse, as it was anticipated that the rescuants would be as virulent as HG52 or 10-200 fold less virulent. The LD_{50} values of the rescuants compared to HG52 and the mutant viruses are shown in Table 3.4. For this experiment, the commercial supplier of mice had changed which could account for the slight increase in the LD_{50}s of HG52 and the mutants. HG52 was highly virulent giving a LD_{50} of 2.15×10^2 p.f.u/mouse. No significant difference was seen between the LD_{50}s of 2620, 2621 and 2622 and those observed previously (Table 3.2). As expected, the R2620 isolated using the *Bam*HI*g* fragment did not return to the wild-type and had a LD_{50} value of 1.77×10^5 p.f.u./mouse. This was only slightly more than a 5 fold increase in virulence compared to 2620. The two isolates of R2621 regained the wild-type level of virulence and had a LD_{50} values of $\leq 10^2$ p.f.u./mouse. This was a $>10^4$ increase in virulence compared to 2621. In the case of R2622, one isolate (R2622/C) demonstrated a full virulence phenotype and had a LD_{50} of 3.16×10^2 p.f.u./mouse and others behaved intermediately and had LD_{50}s of 10^3 and 10^4 p.f.u./mouse (Table 3.4).

The results from the *in vivo* marker rescue experiments (i) confirmed the avirulent phenotype

of the mutant 2621 and the intermediate phenotype of the mutant 2622 (ii) indicated a secondary mutation in the genome of the mutant 2620 which requires to be reconstructed (iii) ruled out the possibility of a mutation in the pXB *Xho*I fragment .

3.11. Virulence heterogeneity of individual plaque isolates within the HG52 plaque 17 stock

Heterogeneity in virulence among individual plaque isolates of the elite stock of HSV-2 strain HG52 has been demonstrated previously (Taha *et al.*, 1988). At that time, one plaque stock, number 17 was shown to be of high virulence and has subsequently been used as our parental virus for all virulence experiments. In this study, all rescuants generated *in vitro* (Table 3.2) and R2620, R2622/A and R2622/B generated *in vivo* (Table 3.4) did not regain the wild-type level of virulence. This could be attributed to heterogeneity of the parental wild-type HG52 pl.17 and possibly heterogeneity of the mutants. Therefore it was decided to assess heterogeneity within the pl.17 stock. The plaque 17 stock was diluted, plated out on BHK21/C13 cells and 6 well separated individual plaques were picked and subjected to three rounds of purification before growing stock virus and injecting into mice. Separate groups of 3-week old female Balb/c mice were intracerebrally inoculated with the 6 stock viruses at doses of 10^2-10^4 p.f.u./mouse. The LD_{50} values of the stocks compared to the parental pl.17 are illustrated in Table 3.5. The stocks were assigned to one of 3 classes depending on the LD_{50} values: (i) Class I of high virulence ($LD_{50} \leq 10^2$ p.f.u./mouse) contains plaque stocks 2, 5 and 6 (ii) Class II of intermediate virulence ($LD_{50} = 10^4$p.f.u./mouse) contains plaque stock 1. (ii) Class III of low virulence ($LD_{50} > 10^4$p.f.u./mouse) contains plaque stocks 3 and 4. It can be seen that again heterogeneity is demonstrable with LD_{50} values ranging from $\leq 10^2$ to $\geq 10^4$p.f.u./mouse.

It has been shown previously that the elite stock of HG52 contains variants with detectable genomic deletions at a frequency of 24% (Harland and Brown, 1985). To investigate the possibility that there were gross sequence alteration, the genomes of the 6 individual plaques of HG52 pl.17 were analysed by *Bam*HI restriction enzyme digestion. The analysis revealed no obvious deletion in the DNA of any plaque regardless of its virulence phenotype (Fig.

Table 3.5. LD50 values after intracerebral inoculation of individual HG52 pl. 17 plaques following 3 rounds of purification.

Virus \ Dose	10^2	10^3	10^4	LD50 p.f.u./mouse	Virulence class
HG52 pl. 17	1/4*	4/4	ND	2.15×10^2	High
pl. 1	0/4	1/4	2/4	10^4	Intermediate
pl. 2	2/4	4/4	4/4	10^2	High
pl. 3	0/4	0/4	0/4	$>10^4$	Low
pl. 4	0/4	0/4	0/4	$>10^4$	Low
pl. 5	4/4	4/4	4/4	$<10^2$	High
pl. 6	4/4	4/4	4/4	$<10^2$	High

ND = not done
* = number of deaths/number of animals inoculated

3.14). Variation was observed in the size of *Bam*HI *z* and *a'*, the known variable fragments, which span the junctions between Us and TRs/IRs (Davison and Wilkie, 1981). Variation was also observed in the size of *Bam*HI*g* and *x* fragments, the former due to variation in the number of the '*a*' sequences. The relevant *Bam*HI restriction map for HSV-2 strain HG52 is shown in Figure 3.15. It is possible that a mutation which is undetectable e.g. a point mutation or a deletion/insertion of <100bp has occurred either somewhere else in the genome or in the RL1 gene. The latter possibility was subsequently ruled out (section 3.16).

3.12. Virus growth properties *in vitro*

The replication efficiencies of RL1 mutant viruses relative to the parental wild-type HG52 and 2604 were examined in both BHK21/C13 cells and 3T6 mouse embryo fibroblast cells. Confluent cells were infected with either 0.01p.f.u./cell (multi-cycle growth experiment) or 5p.f.u./cell (single-cycle growth experiment), at various times post-infection, plates were harvested, cells sonicated and the progeny virus titrated on BHK21/C13 cells. In BHK21/C13 cells at both low multiplicity of infection (0.01p.f.u./cell) and high multiplicity of infection (5p.f.u./cell), the mutants 2604, 2620, 2621 and 2622 grew well and their growth patterns were similar to that of the wild type virus (Fig. 3.16; Fig. 3.17). Differential growth was observed at low m.o.i. (0.01 p.f.u./cell) in 3T6 cells. This multicycle growth experiment was caried out to test whether the growth differential in confluent 3T6 cells seen between HSV-1 srain 17^+ and the RL1 negative mutant 1716 (Brown *et al.*, 1994a) is mimicked by type2 mutant virus. It can be seen in Figure 3.18 that HG52 showed a 10^4 fold increase in tire by 72h post-infection compared to impaired growth of the deletion mutant 2604. The nutant 2620 with the stop codon in the conserved region was similarly impaired. The nul mutant 2621 showed some evidence of replication by 24h but no subsequent increase in titre. Compared to HG52 and 2604, the mutant 2622 showed intermediate growth with a 10^2 fold increase in titre by 72h post infection. These phenotypes in confluent 3T6 cells are consistent with the growth patterns of HG52 and the RL1 mutants in mouse brain (Fig. 3.13) and their LD_{50} values (Table 3.2).

In 3T6 cells, viruses were also differentiated based on size and morphology of plaques.

Figure 3.14. ***Bam*****HI restriction enzyme profiles of the DNA of the individual plaques of HG52 pl.17**

The genotypes of the individual plaques of HG52 pl.17 were analysed by *Bam*HI restriction enzyme digestion. Cleavage of the genomes of each of the plaques generated fragments of equivalent sizes. Lane 1, HG52 pl.17; Lane 2, pl. 1; Lane 3, pl. 2; Lane 4, pl. 3; Lane 5, pl. 4; Lane 6, pl. 5, Lane 7, pl.6.

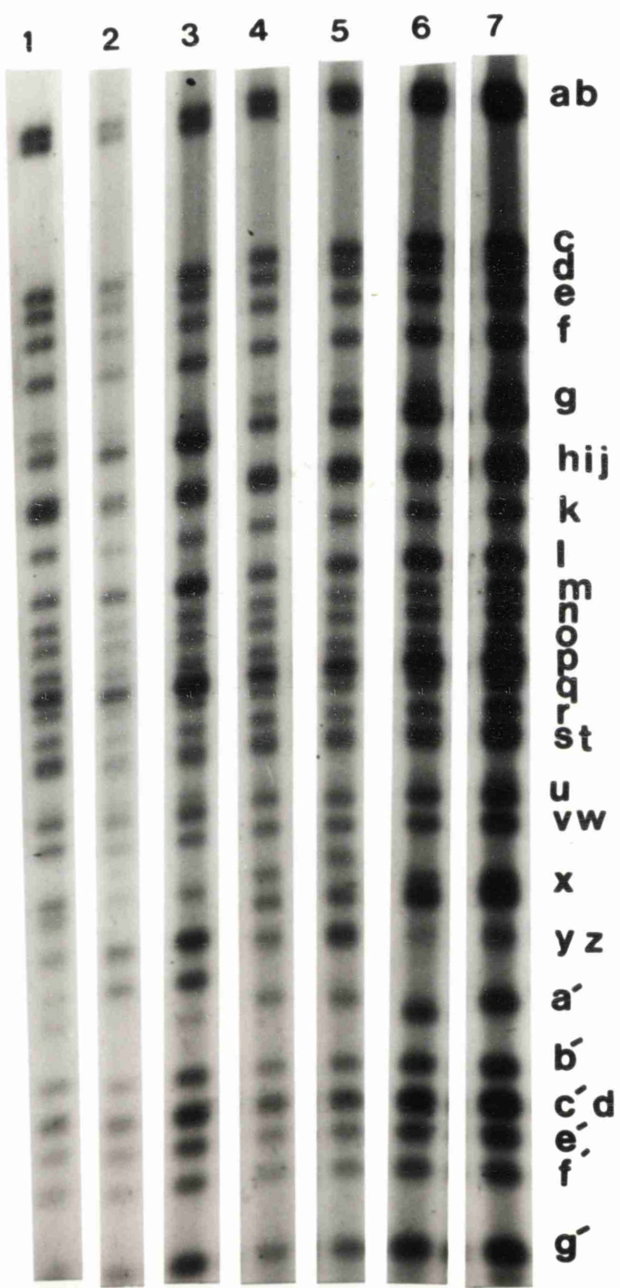
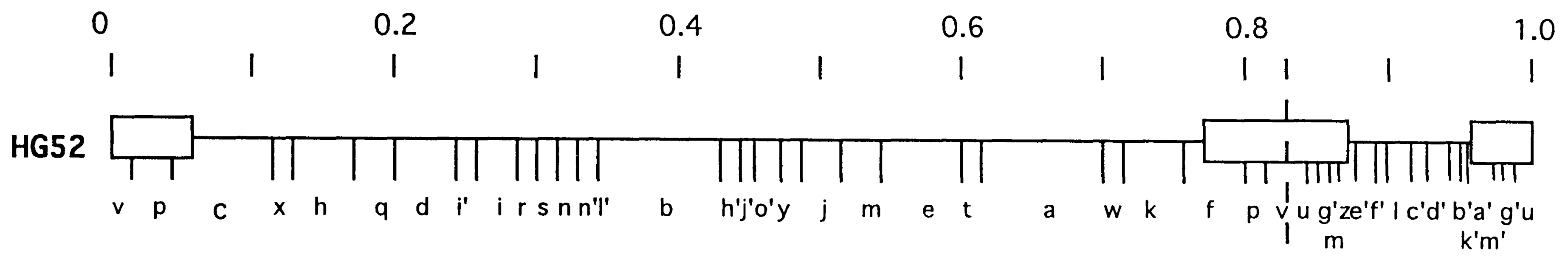

1
2
3
4
5
6
7
ab
c
d
e
f
g
hij
k
l
m
n
o
p
q
r
st
u
vw
x
y z
a´
b´
c´d
e´
f´
g´

Figure 3.15. Position of the *Bam*HI restriction enzyme sites in the HSV-2 strain HG52 genome

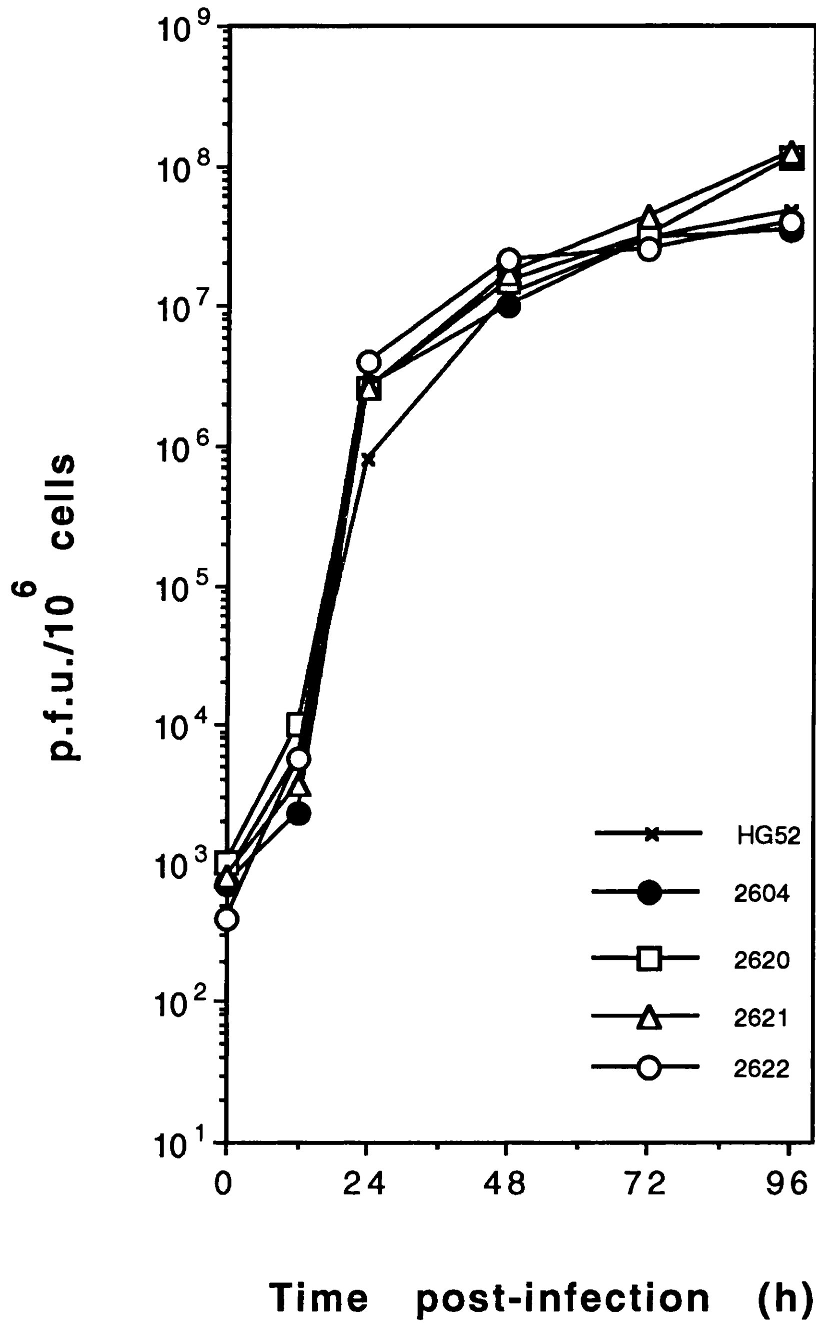
The position of *Bam*HI sites in the HG52 genome in the prototype orientation is illustrated (Wilkie *et al.*, 1978). The dashed vertical line indicates the position of the L-S junction.



g = v + u

Figure 3.16. Multi-cycle growth kinetics of HG52 and RL1 mutant viruses in BHK21/C13 cells

The multi-cycle growth kinetics of 2604, 2620, 2621 and 2622 were compared to HG52 in BHK21/C13 cells. Confluent cells were infected at a m.o.i. of 0.01 p.f.u./cell. At indicated times post-infection (X-axis), cells were harvested, sonicated and progeny virus titrated on BHK21/C13 cells at 37°C. The titres are given as p.f.u./10^6 cells (Y-axis).



p.f.u./10⁶ cells
10⁹
10⁸
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
0
24
48
72
96
Time post-infection (h)
HG52
2604
2620
2621
2622

Figure 3.17. Single-cycle growth kinetics of HG52 and RL1 mutant viruses in BHK21/C13 cells

The single-cycle growth characteristics of 2604, 2620, 2621 and 2622 were compared to the wild-type strain HG52 in BHK21/C13 cells. Confluent cells were infected at a m.o.i. of 5 p.f.u./cell. At indicated times post-infection (X-axis), cells were harvested, sonicated and progeny virus titrated on BHK21/C13 cells at 37°C. The titres are given as p.f.u./10^6 cells (Y-axis).

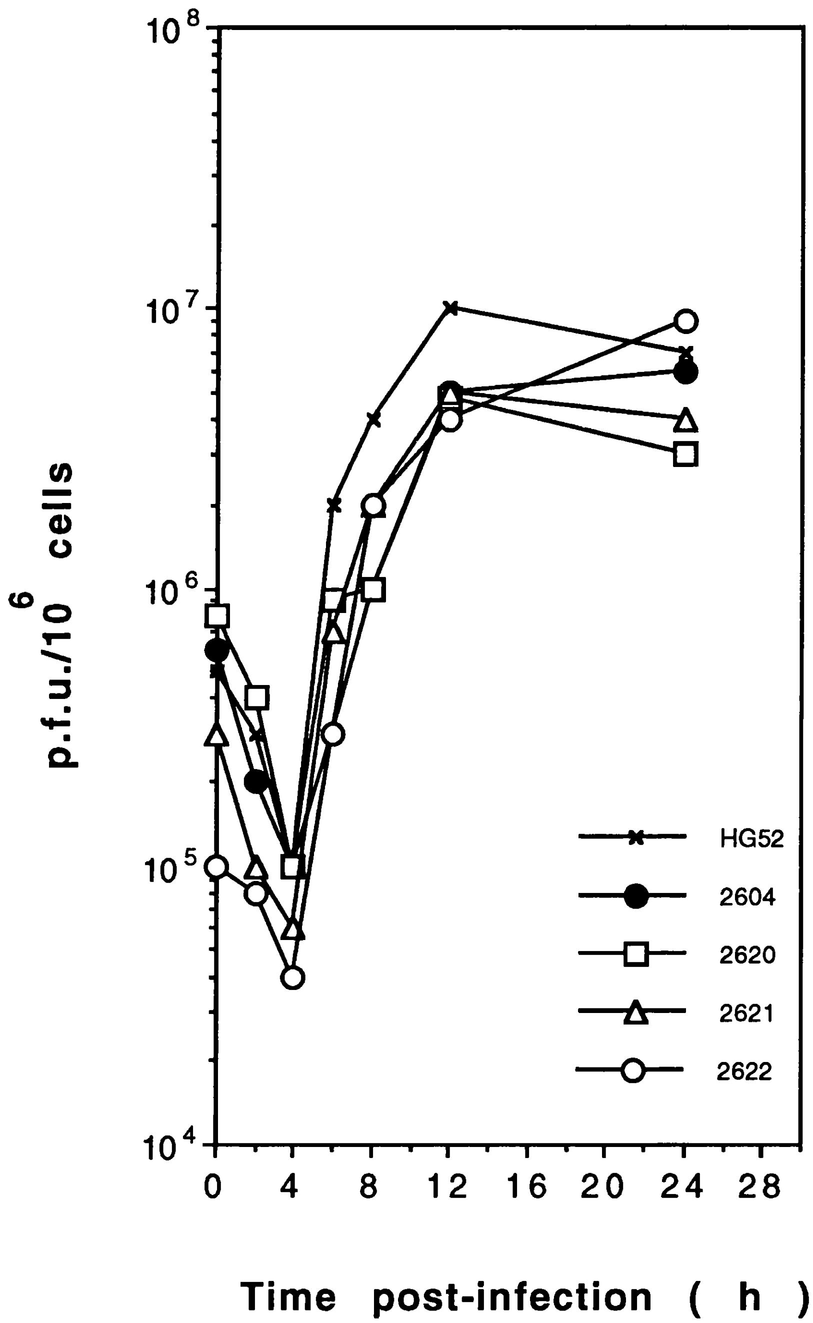
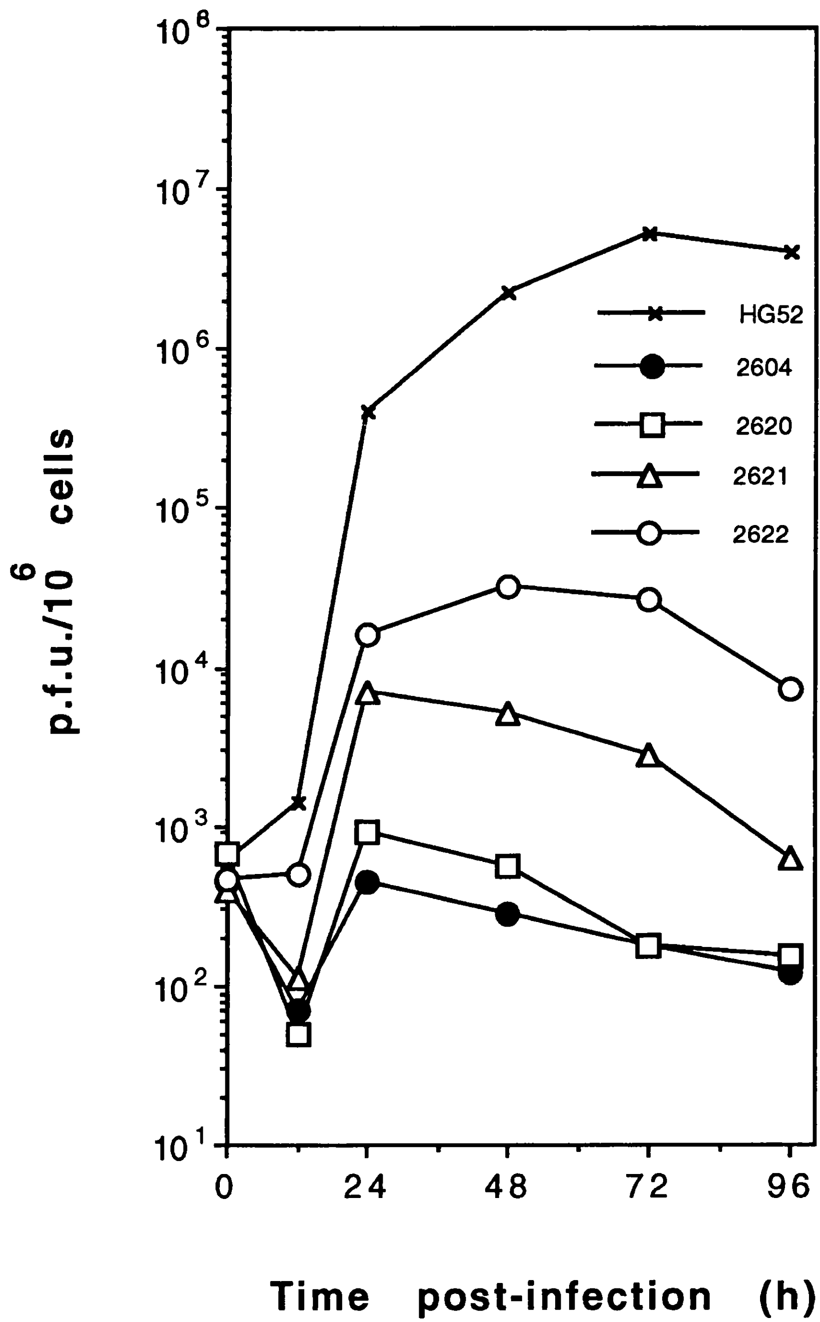

10^8
10^7
10^6
10^5
10^4
p.f.u./10^6 cells
0
4
8
12
16
20
24
28
Time post-infection (h)
HG52
2604
2620
2621
2622

Figure 3.18. Multi-cycle growth kinetics of HG52 and RL1 mutant viruses in 3T6 cells

The multi-cycle growth kinetics of 2620, 2621 and 2622 were compared to HG52 and 2604 in 3T6 cells. Confluent cells were infected at a m.o.i. of 0.01 p.f.u./cell. Cells were harvested at 0, 12, 24, 48, 72 and 96h post-infection. The progeny virus was titrated on BHK21/C13 cells at 37°C. The titres are given as p.f.u./10^6 cells (Y-axis).



10 8
10 7
10 6
10 5
10 4
10 3
10 2
10 1
p.f.u./10 6 cells
HG52
2604
2620
2621
2622
0
24
48
72
96
Time post-infection (h)

HG52 produced large plaques, 2622 produced intermediate plaques, the RL1 negative viruses 2604, 2620, 2621 produced small abortive plaques. In addition to the small plaques, 2621 produced a few large plaques. This was not due to a mixed population of 2621 and HG52, as the *Xba*I genomic profile of the large plaques was identical to that of the small plaques of 2621 (Fig. 3.19). This phenotypic property of 2621 is reflected in its growth pattern and accounts for the one log difference relative to the growth of 2604 (Fig. 3.18). The multicycle growth experiment was repeated several times, the large plaques were observed each time and the same growth pattern of 2621 was obtained. To confirm the plaque morphology of 2621, the virus was reisolated and when the new mutant, N2621 was plated on 3T6 cells, a few large plaques were again observed among the small plaques. The *Xba*I profile of the large plaques of N2621 was identical to that of the small plaques (data not shown).

3. 13. *In vitro* virus growth properties of large and small plaques of the mutant 2621

To determine whether the small and large plaques of 2621 retain their morphology following 3 rounds of purification in 3T6 cells, an aliquot (100μl) of 2621 stock virus ($1x10^9$ p.f.u./ml) was titrated; 6 small and 6 large well isolated plaques were picked and each was transferred to 300μl PBS, sonicated and retitrated on 3T6 cells. Following 48h incubation at 37°C, 2 plaques from each group were picked and subjected to 2 further rounds of purification. After picking plaques, the monolayers were stained to determine the ratio between the large and small plaques on each plate. The large plaques segregated to give an equal proportion of the two plaque sizes. The small plaques gave rise to large plaques at a frequency of 3-18%. Virus stocks of 3 times purified large and small plaques were prepared to determine their replication efficiency in BHK21/C13 and 3T6 cells. The growth patterns of these plaques in 3T6 cells compared to the parental 2621 virus stock, 2604, 2622 and HG52 pl.17 are illustrated in Figure 3.20. Growth of the small plaque stock was more efficient than that of the parental 2621 stock and comparable to 2622 by 48h post-infection, possibly due to adaptation to growth in 3T6 cells. The growth of the large plaque stock of

Figure 3.19. *Xba*I restriction enzyme profiles of the DNA of the small and large plaques of 2621

Autoradiograph of an *Xba* I digest of viral DNA ^{32}P-labelled *in vivo*. Cleavage of the DNA of the small and large plaques of 2621 with *Xba*I generated fragments of equivalent sizes. Lane 1, small plaque of 2621; Lane 2, large plaque of 2621; Lane 3, large plaque of 2621.

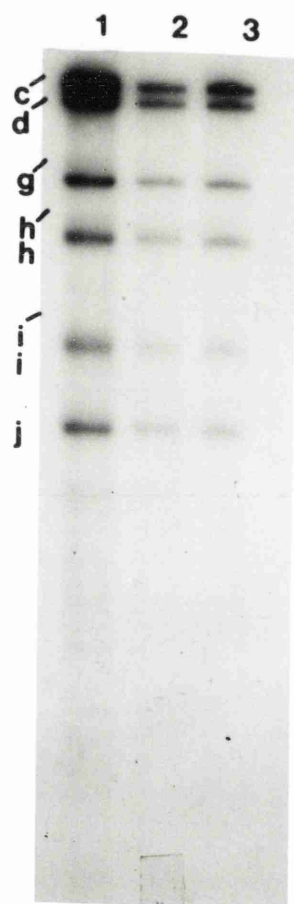
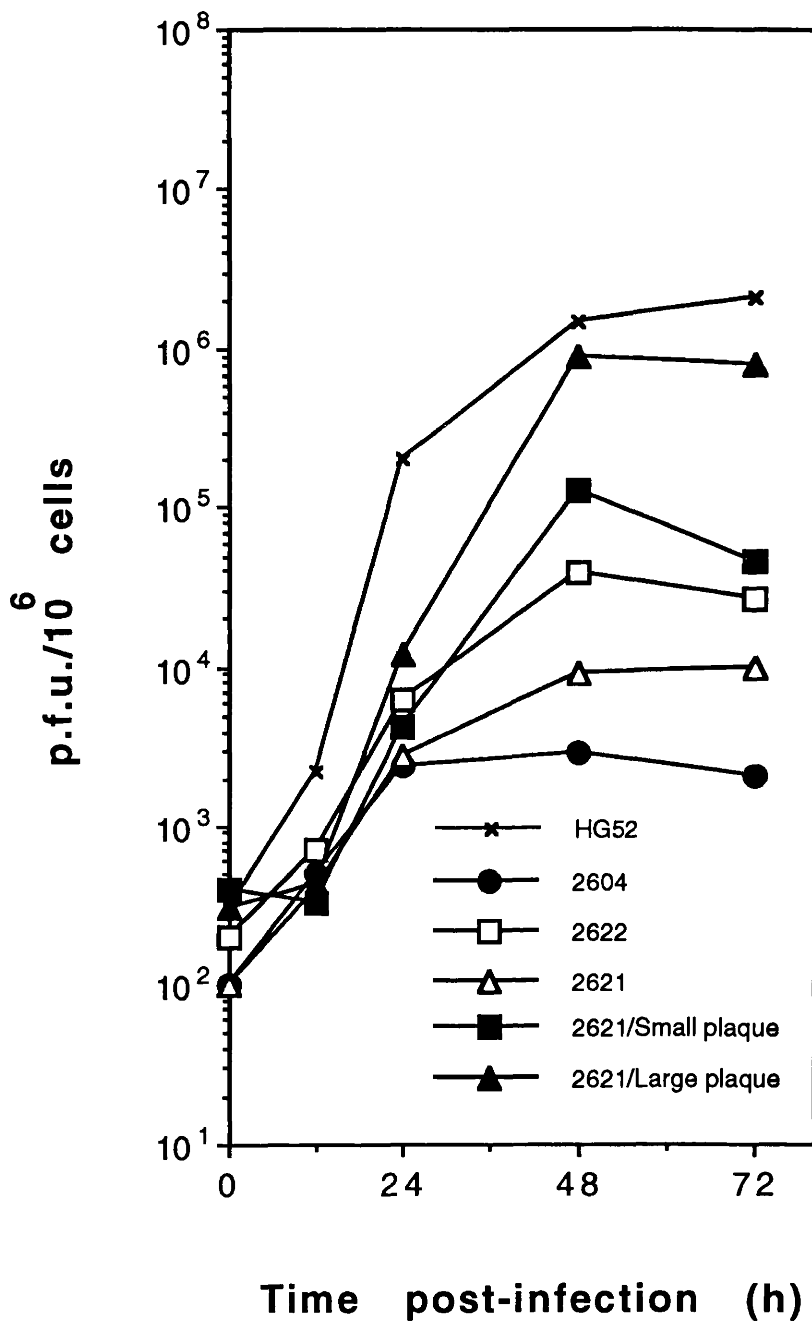

1
2
3
c
d
g
h
h
i
i
j

Figure 3.20. Multi-cycle growth kinetics of the small and large plaque stocks of the mutant 2621 in 3T6 cells

The multi-cycle growth characteristics of the small and large plaque stocks of the mutant 2621 were compared to the parental stock 2621, 2604, 2622 and HG52 in 3T6 cells. Confluent cells were infected at a m.o.i. of 0.01 p.f.u./cell. Following absorption at 37°C for 1h, the monolayers were washed twice with PBS/foetal calf serum, overlaid with Dulbecco's modified Eagle's medium and incubation continued at 37°C. Plates were harvested at 0, 12, 24, 48 and 72h post-infection. The progeny virus was titrated on BHK21/C13 cells at 37°C. The titres are given as p.f.u./10^6 cells (Y-axis).


p.f.u./10^6 cells
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
24
48
72
Time post-infection (h)
HG52
2604
2622
2621
2621/Small plaque
2621/Large plaque

2621 was comparable to that of HG52 pl.17. Since the virulence phenotype *in vivo* is usually mimicked *in vitro* in 3T6 cells, it was decided to study the virulence efficiency of these individual plaques by intracerebral inoculation of mice [section 3.14]. No differential growth was observed between the small and large plaques stocks of 2621 and the parental 2621 virus stock in BHK21/C13 cells (Fig. 3.21).

3.14. Virulence of the large and small plaques of the mutant 2621

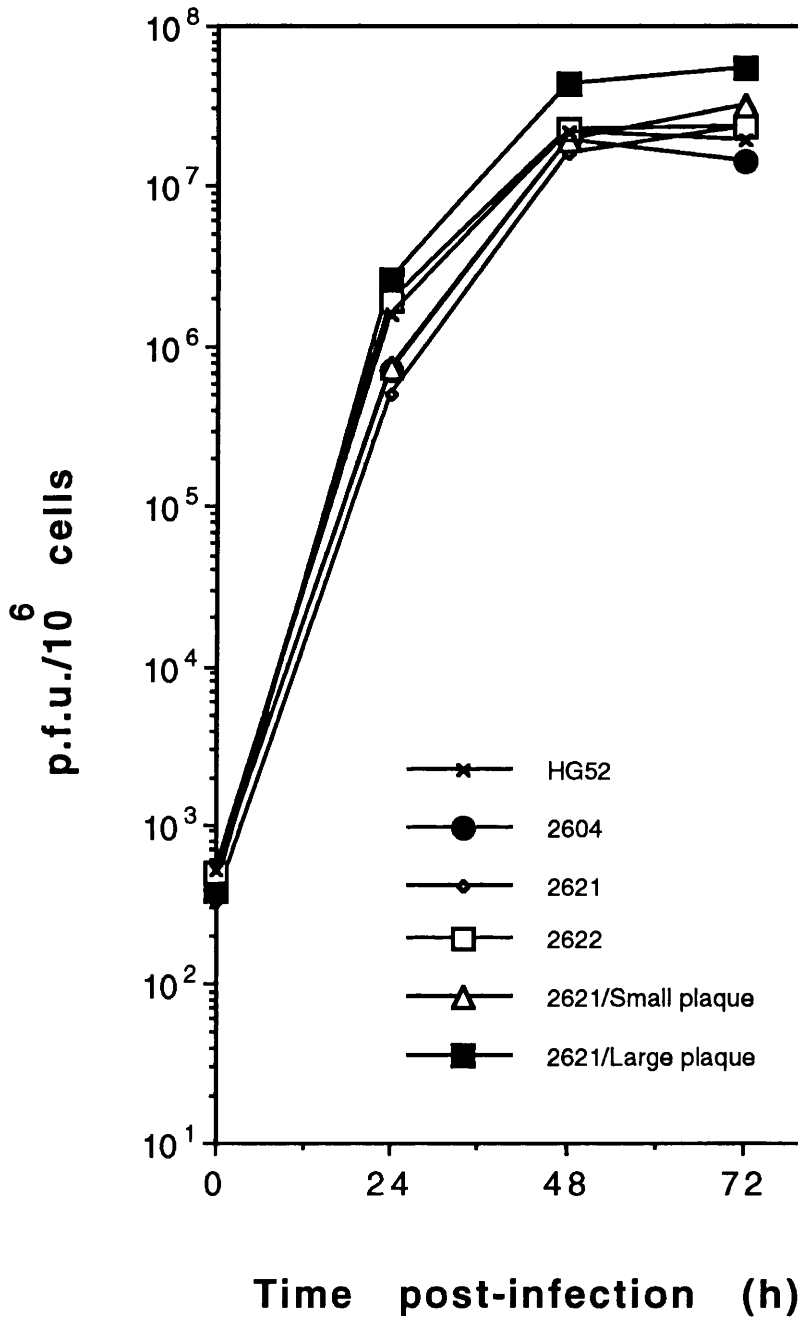
To evaluate the virulence of these plaques, mice were intracerebrally inoculated with HG52 at doses of 10^2-10^3 p.f.u./mouse. The mutants 2604 and 2621 were injected at doses of 10^6 p.f.u./mouse while the mutant 2622 was injected at doses of 10^5-10^6 p.f.u./mouse. The large (2621/L) and the small (2621/S) plaques of 2621 were injected at doses of 10^2-10^6 p.f.u./mouse. The LD_{50} values of these viruses are shown in Table 3.6. No significant difference was seen between the LD_{50} of HG52, 2621 and 2622 and those observed previously (Tables 3.2; 3.4). The 2621/S virus was avirulent and had a LD_{50} value of 10^6 p.f.u./mouse. The 2621/L virus demonstrated an intermediate level of virulence and had a LD_{50} value of $2.15x10^4$ p.f.u./mouse which is within the range of individual plaques of HG52 pl.17.

3.15. Temperature sensitivity

This study showed that the growth of RL1 mutant viruses is identical to that of HG52 in BHK21/C13 cells at 37°C in both single and multicycle growth experiments [section 3.13]. A previous study in our laboratory showed that the variant 2604 is no more impaired in BHK21/C13 cells at 38.5°C than the parental HG52 (Harland and Brown, 1985). Therefore, it was decided to test whether the RL1 mutant viruses 2620, 2621 and 2622 exhibited a *ts* phenotype. The growth of the mutant viruses in BHK21/C13 cells relative to the parental wild-type HG52 and 2604 was compared at 31°C, 37°C and 38.5°C (section 2.2.6). At 24h post-infection, cells were harvested, sonicated and the progeny virus titrated on BHK21/C13 cells. It can be seen in Figure 3.22 that the yield of the mutant viruses were not significantly different from wild-type HG52 and hence do not display *ts* phenotypes.

Figure 3.21. Multi-cycle growth kinetics of the small and large plaque stocks of 2621 in BHK21/C13 cells

Multi-cycle growth experiments of the small and large plaque stocks of 2621 were carried out in BHK21/C13 cells. Cells were infected at m.o.i. of 0.01 p.f.u./cell. Following absorption at 37°C for 1h, the monolayers were washed with PBS/calf serum, overlaid with ETC10 and incubation continued at 37°C. Plates were harvested at 0, 24, 48 and 72h post-infection and titrated on BHK21/C13 cells. The titres are given as p.f.u./10^6 cells (Y-axis). The growth kinetics of these plaques were compared with those of 2621, 2622, 2604 and HG52.



10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
p.f.u./10^6 cells
0
24
48
72
Time post-infection (h)
HG52
2604
2621
2622
2621/Small plaque
2621/Large plaque

Table 3.6. The LD50 values of the large (L) and the small (S) plaques of 2621 following intracerebral inoculation of 3-week old Balb/c mice compared to 2621, HG52 and 2604.

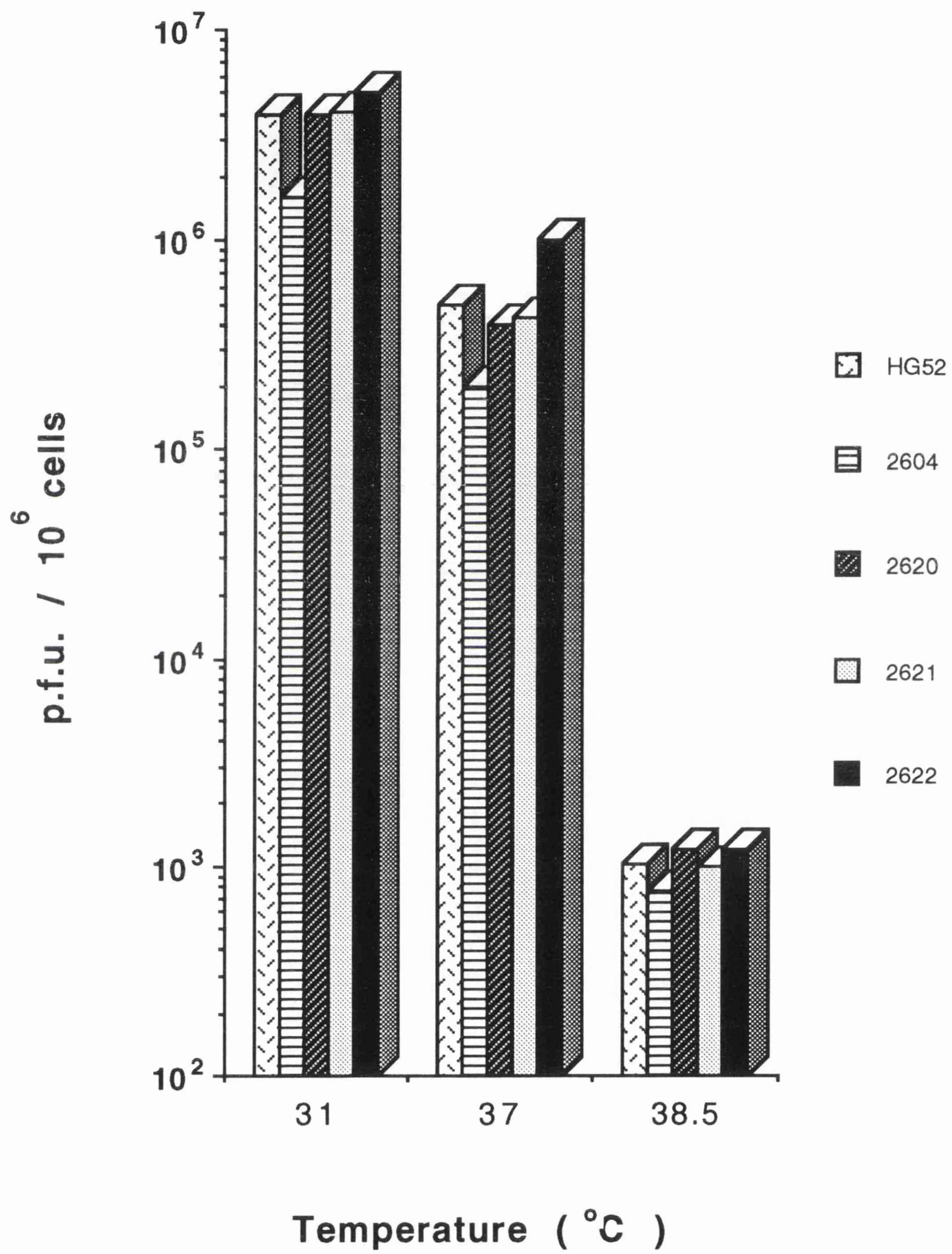
Virus \ Dose	10^2	10^3	10^4	10^5	10^6	LD50 p.f.u./mouse
HG52	0/4*	4/4	ND	ND	ND	3.16×10^2
2604	ND	ND	ND	ND	0/4	$>10^6$
2621	ND	ND	ND	ND	0/4	$>10^6$
2622	ND	ND	ND	0/4	4/4	3.16×10^5
2621/L	0/4	0/4	1/4	4/4	4/4	2.15×10^4
2621/S	0/4	0/4	0/4	0/4	2/4	10^6

ND = not done

* = number of deaths/number of animals inoculated

Figure 3.22. Temperature sensitivity of the mutant viruses

The temperature sensitivity of 2620, 2621 and 2622 was tested on BHK21/C13 cells and compared with HG52 and 2604. Cells were infected at a m.o.i. of 5 p.f.u./cell. Following 24h incubation at 31°C, 37°C and 38.5°C (X-axis), the monolayers were harvested, sonicated and the progeny virus titrated on BHK21/C13 at 37°C. The titres are given as p.f.u./10^6 cells (Y-axis).



10^7
10^6
10^5
10^4
10^3
10^2
p.f.u. / 10^6 cells
31
37
38.5
Temperature (°C)
HG52
2604
2620
2621
2622

The highest virus titres for HG52 and the mutant viruses were observed at 31°C. The titres dropped by 1 log and 3.5 logs with temperatures of 37°C and 38.5°C, respectively.

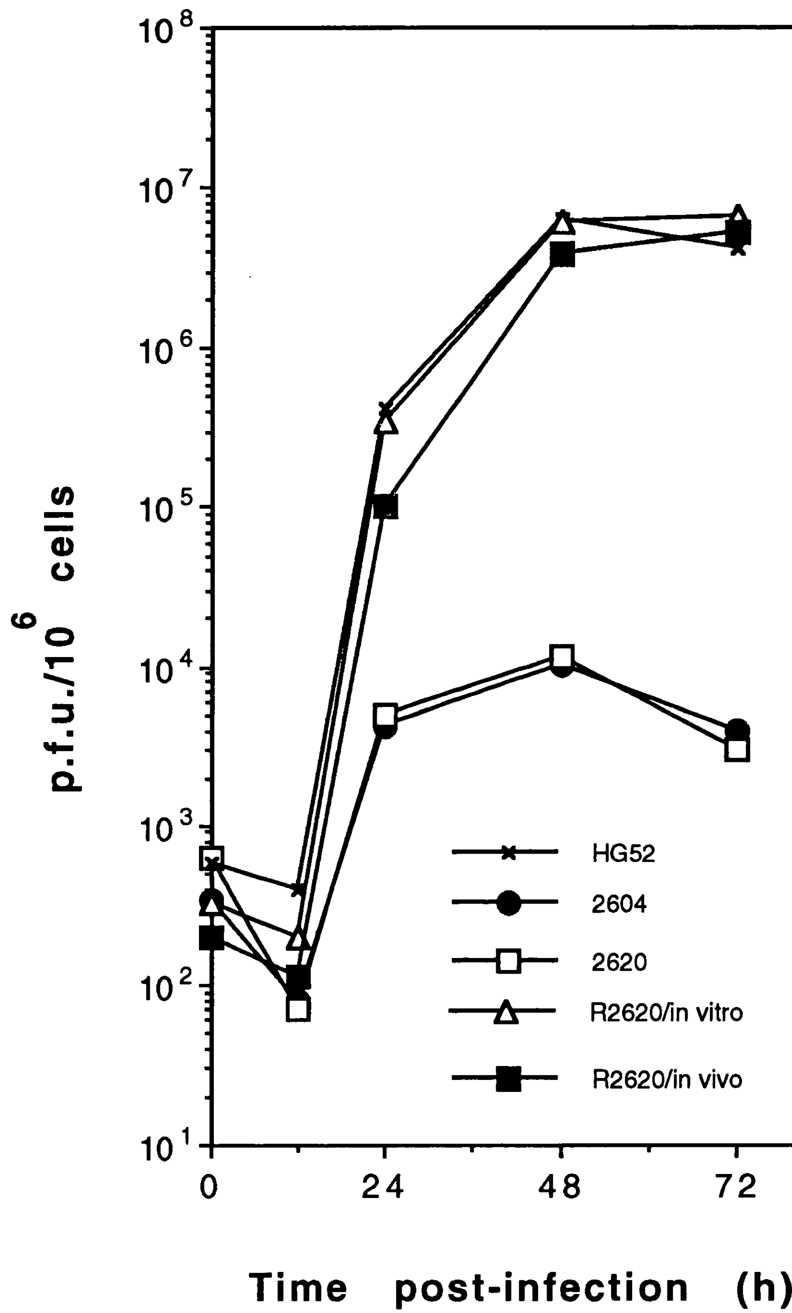
3.16. *In vitro* growth properties of the rescuants and the individual plaques of HG52 pl.17

The rescuants generated *in vitro* (Table 3.2) and the rescuants R2620, R2622/B generated *in vivo* (Table 3.4) did not regain the wild-type level of virulence. To test their phenotype *in vitro*, multicycle growth experiments (m.o.i. 0.01 p.f.u./cell) were carried out in 3T6 cells over a period of 72h and their growth patterns were compared to those of HG52 pl.17 and 2604. The growth patterns of the R2620 rescuants are illustrated in Figure 3.23. As observed previously (Fig. 3.18), the growth of both 2604 and 2620 is impaired in 3T6 cells. The growth patterns of the intermediate virulence R2620 rescuants generated *in vitro* and *in vivo* were identical to that of HG52. Similarly, as can be seen in Figure 3.24, the growth of the intermediate virulence R2621 generated *in vitro* was identical to that of the wild-type and to the highly virulent rescuant R2621/A generated *in vivo*. The growth of the intermediate rescuant R2622 generated *in vitro* was similar to that of the wild-type and to the virulent rescuant R2622 generated *in vivo* (Fig. 3.25). These results rule out a possible mutation in the RL1 gene of rescuants generated *in vitro*.

To evaluate the replication efficiency of the individual plaques of HG52 pl.17 in 3T6 cells, plaque 1 was chosen as a low virulence plaque, while plaques 3 and 5 were chosen as intermediate and high virulence plaques, respectively. It can be seen in Figure 3.26, that the growth patterns of all selected plaque stocks were identical to that of the parental HG52 plaque 17. In comparison, the RL1-deletion mutant 2604 showed some evidence of replication by 48h with no subsequent increase in titre. Using this system as an assay of ICP34.5 expression, the ability of these individual plaques to grow as well as the HG52 pl.17 stock rules out mutations in the RL1 gene which could have accounted for the heterogeneity within the HG52 pl.17 stock as regards LD_{50} values (Table 3.5).

Figure 3.23. Multi-cycle growth kinetics of the 2620 rescuants (R2620) in 3T6 cells

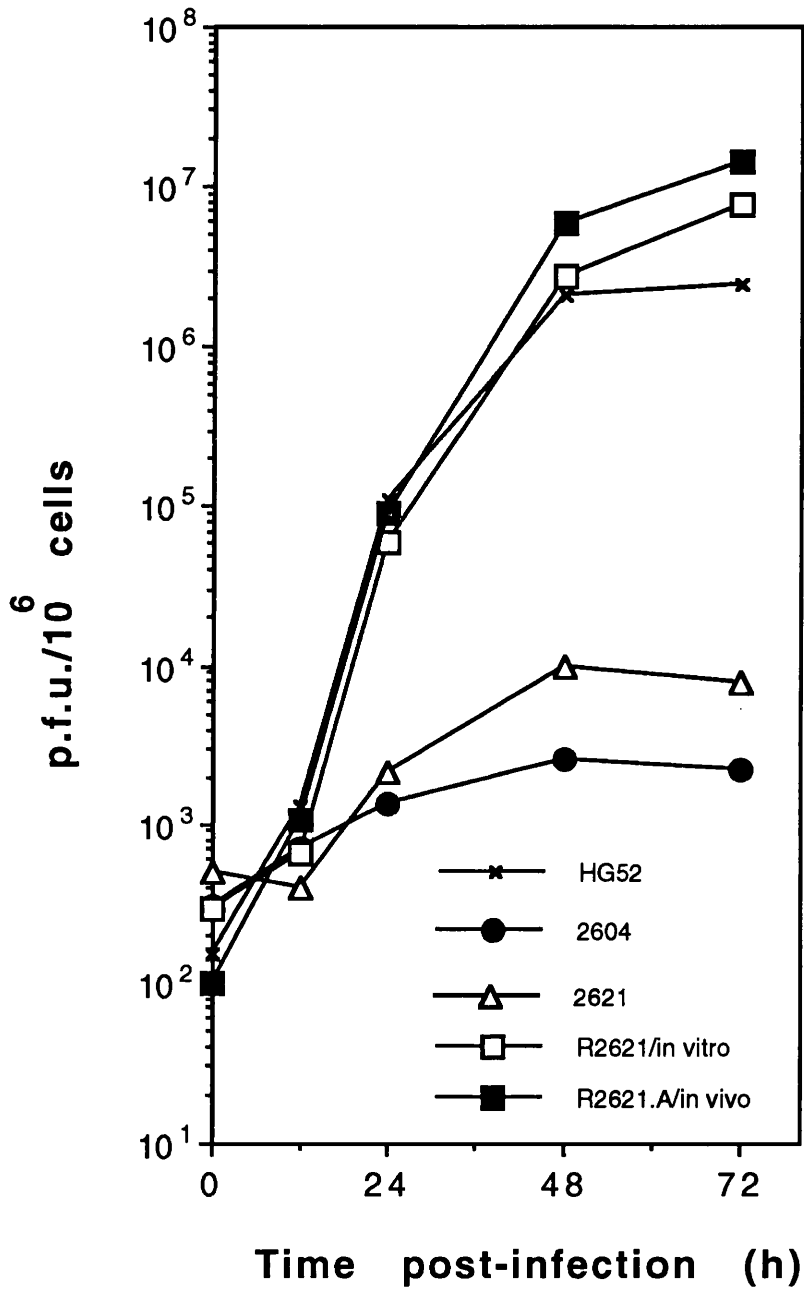
The multi-cycle growth characteristics of R2620 generated *in vitro* and *in vivo* were compared with those of 2620 and HG52 in 3T6 cells. Confluent cells were infected at a m.o.i. of 0.01 p.f.u./cell. After absorption at 37°C for 1h, cells were washed, overlaid with Dulbecco's modified Eagle's medium and incubated at 37°C. At various times post-infection (X-axis), cells were harvested, virus released by sonication and titrated on BHK21/C13 cells at 37°C. The titres are given as p.f.u./10^6 cells (Y-axis).



10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
p.f.u./10^6 cells
0
24
48
72
Time post-infection (h)
HG52
2604
2620
R2620/in vitro
R2620/in vivo

Figure 3.24. Multi-cycle growth kinetics of the 2621 rescuants (R2621) in 3T6 cells

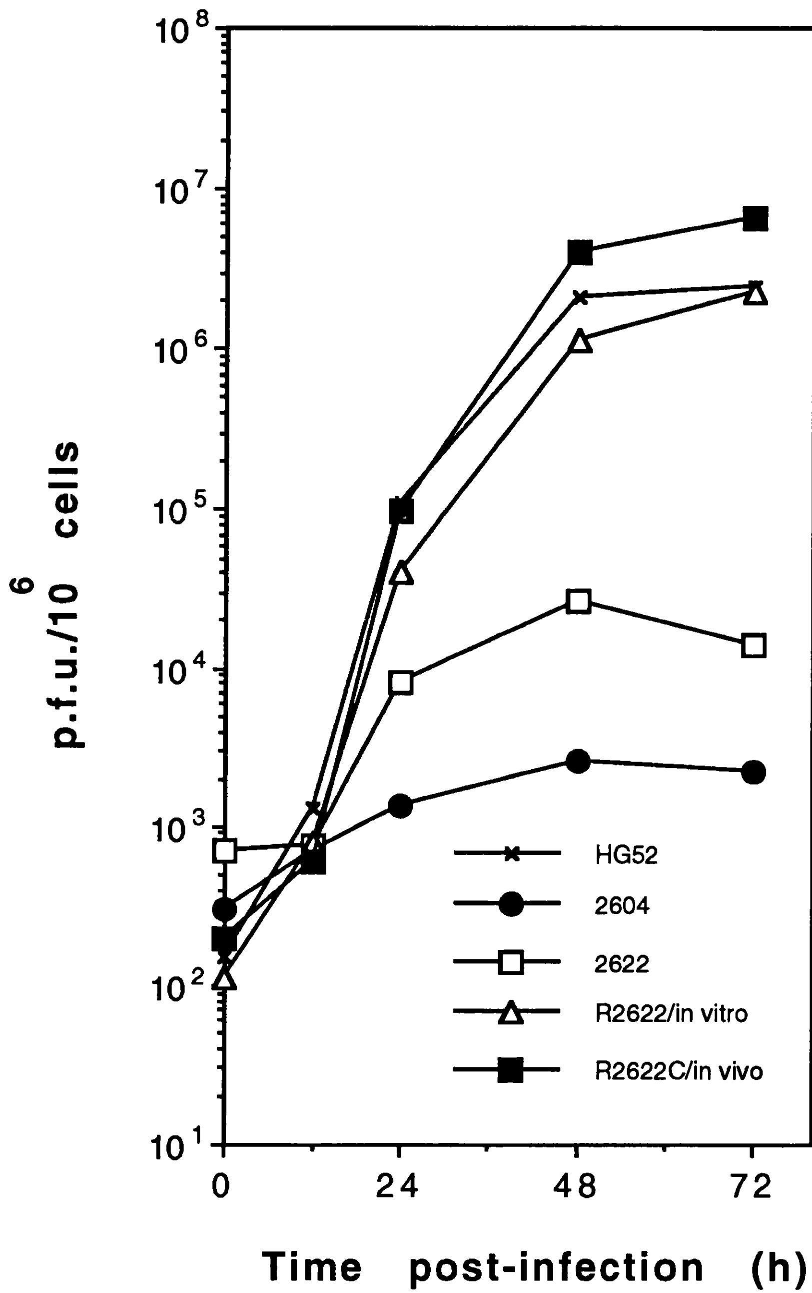
The multi-cycle growth kinetics of R2621 generated *in vitro* and *in vivo* were compared with those of 2621 and HG52 in 3T6 cells. Cells were infected at a m.o.i. of 0.01 p.f.u./cell and incubated at 37°C for 1h. Cells were washed, overlaid with medium and incubated at 37°C. At various times post-infection (X-axis), cells were harvested, sonicated and progeny virus titrated on BHK21/C13 cells at 37°. The titres are given as p.f.u./10^6 cells (Y-axis).



p.f.u./10^6 cells
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
24
48
72
Time post-infection (h)
HG52
2604
2621
R2621/in vitro
R2621.A/in vivo

Figure 3.25. Multi-cycle growth kinetics of the 2622 rescuants (R2622) in 3T6 cells

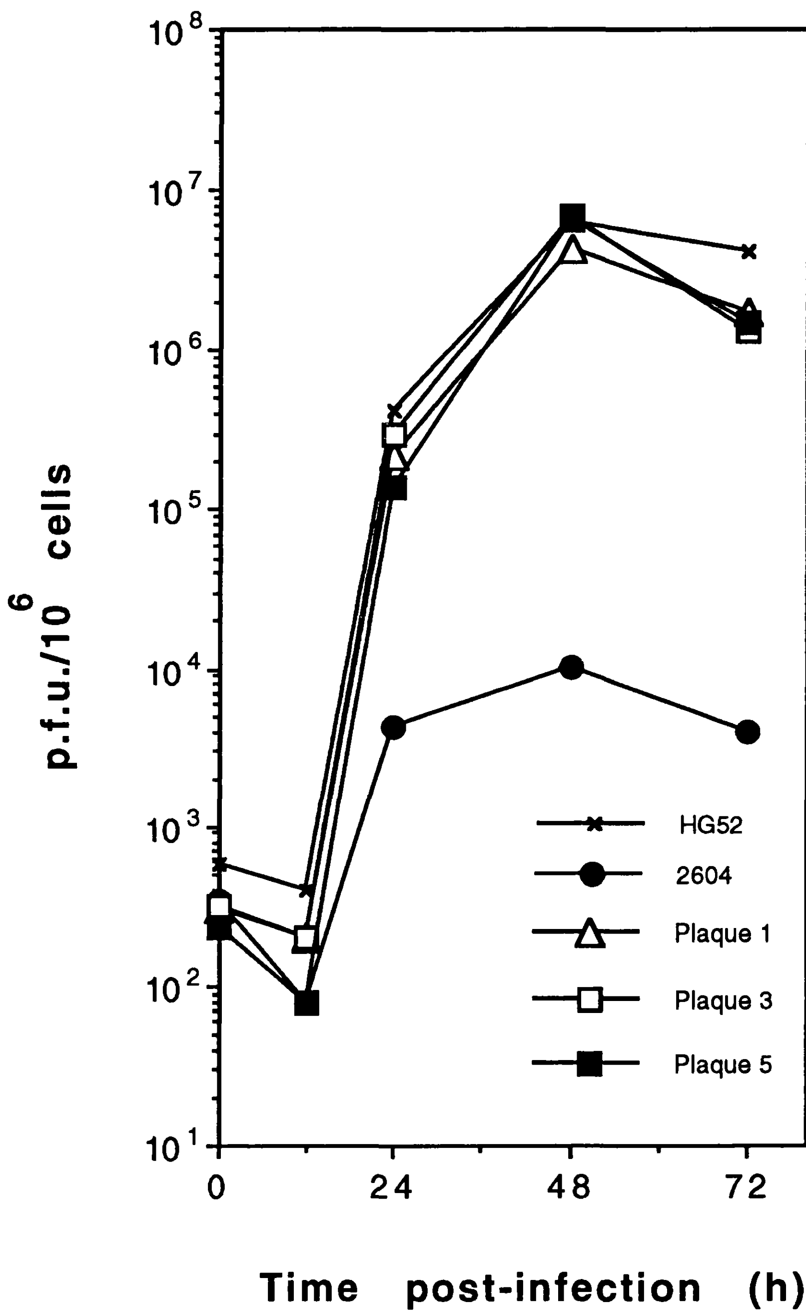
The multi-cycle growth experiment of R2622 generated *in vitro* and *in vivo* was carried out in 3T6 cells. Cells were infected at a m.o.i. of 0.01 p.f.u./cell and incubated at 37°C for 1h. Cells were washed, overlaid with medium and incubated at 37°C. At various times post-infection (X-axis), cells were harvested, sonicated and progeny titrated on BHK21/C13 cells at 37°C. The titres are given as p.f.u./10^6 cells (Y-axis). The growth kinetics of the rescuants were compared with those of 2622 and HG52.



10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
p.f.u./10^6 cells
0
24
48
72
Time post-infection (h)
HG52
2604
2622
R2622/in vitro
R2622C/in vivo

Figure 3.26. Multi-cycle growth kinetics of 3 individual plaque stocks of HG52 pl.17 in 3T6 cells

The growth characteristics of plaques 1, 3 and 5 of HG52 pl.17 were compared with the parental stock and with 2604 in 3T6 cells. Cells were infected at a m.o.i. of 0.01 p.f.u./cell. Following absorption at 37°C for 1h, the monolayers were washed with PBS/foetal calf, overlaid with Dulbecco's modified Eagle's medium and incubated at 37°C. At the indicated times post-infection (X-axis), plates were harvested and progeny virus titrated on BHK21/C13 cells at 37°C. The titres are given as p.f.u./10^6 cells (Y-axis).



10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
p.f.u./10^6 cells
0
24
48
72
Time post-infection (h)
HG52
2604
Plaque 1
Plaque 3
Plaque 5

3.17. cDNA-PCR of RL1 mRNA flanking the predicted intron

To provide evidence that the mRNA of RL1 is spliced, PCR was employed to try to amplify a cDNA product from the HG52 RL1 mRNA, using primers flanking the predicted intron [section 2.2.35(b,c)]. Total RNA and cytoplasmic poly $(A)^+$RNA were prepared as described in sections 2.2.32-34. Three pairs of primers complementary to the DNA sequence flanking the intron (Fig. 3.27; Table 3.7) were designed for reverse transcription and PCR reactions. The first pair of primers (A, B) was selected by the computer program (Oligo; MedProbe A.A); the second (C, D) and the third (E, F) were designed according to the criteria outlined by Dutton *et al* (1993) for amplification of templates with a high G+C content [section 2.2.35(a)]. Each primer pair should produce PCR products of different sizes (Table 3.7). The size of the PCR product would determine whether the reiterated sequence interrupting the predicted RL1 ORF is an intron. An intron would be spliced out and DNA sequencing of the PCR product would determine whether the proposed splicing sites are correct. If there is not an intron, DNA sequencing would define any possible errors in the reiterated sequence or in the flanking regions.

Synthesis of cDNA was carried out as described in section 2.2.35(b) using the antisense primer (B), AMV reverse transcriptase (RT) and total RNA extracted from BHK21/C13 cells infected separately with HG52 and the intronless mutant virus 2622. Amplification was performed with Taq DNA polymerase under the conditions described in section 2.2.35(c). Appropriate positive intron PCR controls (pSB, HG52-DNA), negative intron controls (intronless pSB) and negative controls (mock cells, reagents only) were included in each PCR run. To determine if the desired fragment had been amplified, one-tenth of the PCR product was analysed on a 3% NuSieve agarose gel. As no amplified product was observed, the cDNA/PCR experiment was repeated several times using a new total RNA preparation, primer (B) or random hexanucleotide primer (Promega), AMV-RT or MuLV-RT and Taq DNA Polymerase. Amplification was performed under various conditions. An appropriate fragment could not be detected, possibly due to unsuccessful cDNA synthesis and/or unsuccessful amplification due to the high G+C content of the template. Therefore, it was decided to carry out the cDNA/PCR reactions at higher temperatures in the presence of

Figure 3.27. The location of the primers used to amplify the cDNA product of HG52 mRNA flanking the predicted intron

A schematic diagram of the RL1 gene of HSV-2 strain HG52 illustrating the locations of the primers used in the cDNA/PCR reactions. The nucleotide numbers are from McGeoch *et al.*, 1991.

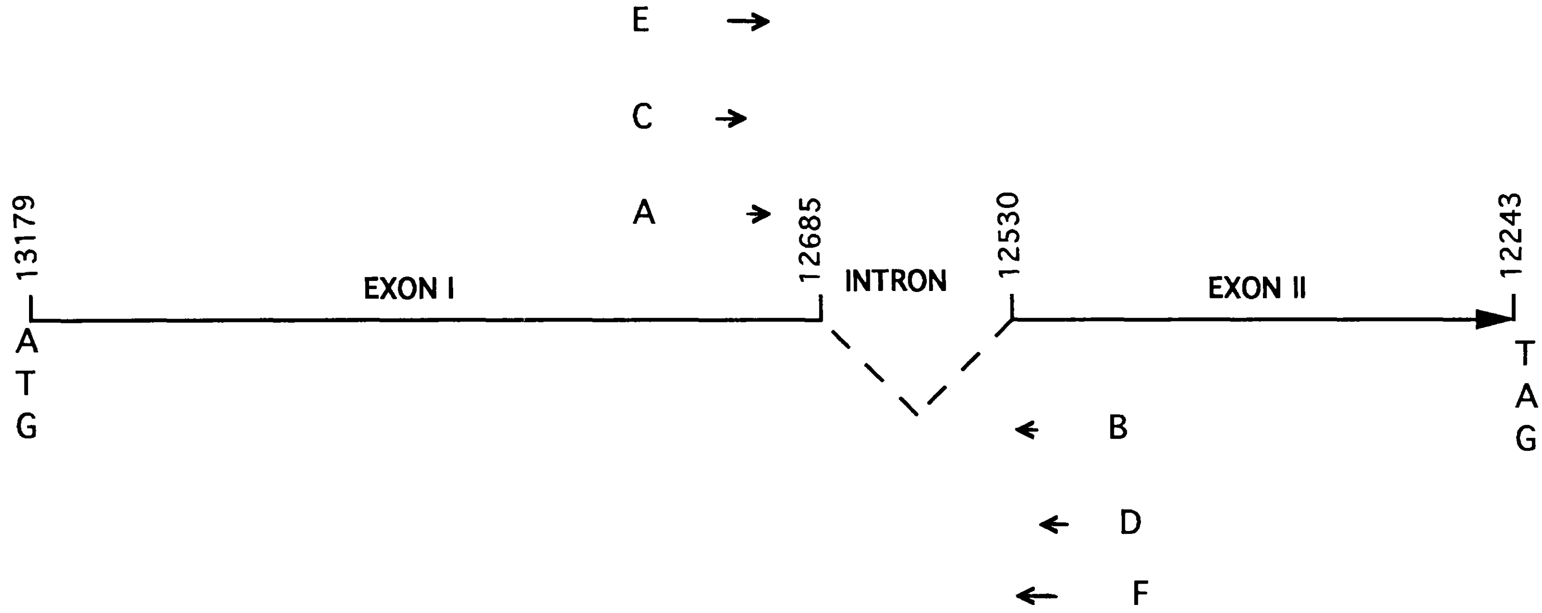

E
C
A
13179
EXON I
12685
INTRON
12530
EXON II
12243
ATG
TAG
B
D
F

Table 3.7. Nucleotide sequence, size and position of the primers used for cDNA/PCR amplification. The size of the PCR products is also indicated. The nucleotide numbers are from McGoech *et al* (1991).

Primer name (position)	Primer size (mer)	Primer sequence	Wallace Temperature	Product size (bp) if the reiterated sequence is an intron and spliced	Product size (bp) if the reiterated sequence is not an intron
A. (12773-12755)	19	5'-TGC GCA CCA CGA CGG AGT A-3'	62 °C		
B. (12511-12527)	17	5'-ACG CGC GGC GAG AAG CA-3'	58 °C	(with A) 109	(with A) 263
C. (12788-12766)	23	5'-ACC TGG CGC TAC GGC TGC GCA CC- 3'	80 °C		
D. (12493-12515)	23	5'- ACC AGA TGG CGC ACC TGC ACG CG-3'	78 °C	(with C) 142	(with C) 296
E.(12788-12754)	35	5'-ACC TGG CGC TAC GGC TGC GCA CCA CGA CGG AGT AC-3'	$\geq$80 °C		
F. (12493-12527)	35	5'-ACC AGA TGG CGC ACC TGC ACG CGC GGC GAG AAG CA-3'	$\geq$80 °C	(with E) 142	(with E) 296

thermostable enzymes. It was thought that the high G+C (hGC) PCR protocol reported by Dutton *et al* (1993) would be the best choice for amplification of the cDNA product. This protocol requires a very heat stable thermal DNA polymerase and primers of Wallace temperatures $\geq$80°C [4°Cx(G+C)+2°Cx(A+T)]. The second and the third pairs of primers (CD,EF) with high Wallace temperatures (Table 3.7) were used separately in cDNA/PCR reactions. These reactions were carried out in the presence of the thermostable Retrotherm-RT, antisense primer, Vent DNA Polymerase and cytoplasmic poly $(A)^+$RNA. To ensure use of intact mRNA in the cDNA reaction, oligo $(dT)_{25}$ dynabeads were used as a rapid method to extract the poly $(A)^+$ RNA [section 2.2.34] from BHK21/C13 cells infected separately with HG52 and 2622. The hGC-PCR amplification protocol was used as described in section 2.2.35(c). Multiple non-specific fragments were seen in the gels. Several attempts were made to optimise the PCR conditions using the positive (pSB) and the negative (intronless pSB) controls but unfortunately all were unsuccessful.

In parallel, another approach (nested PCR) was employed to amplify the target sequence in the positive and the negative PCR controls. The primers (C,D) were used as outer primers in the first round of nested PCR. The amplification was performed with Vent DNA polymerase under the conditions described in section 2.2.35(b). A small aliquot (5µl) of the product was subjected to a second round of amplification under the same conditions in the presence of the inner pair of primers (A,B). Nested PCR was also unsuccessful in obtaining specific amplification.

ADDENDUM

While the text for this thesis was at the final stage of editing, the following results were obtained by Dr. J. Harland:

1. By cDNA-PCR of poly(A^+) RNA using primers on either side of the proposed intron, a cDNA clone corresponding exactly to the predicted spliced mRNA was amplified.

2. During PCR analysis, 2622 RNA was used as a control. Results indicated that the intron was present in non-poly(A^+) RNA. Therefore, 2622 DNA was digested with a range of restriction enzymes in various combinations. Using the pSB plasmid to generate a random primed probe, Southern blot analysis rendered that 2622 had an approximately 150bp deletion partly in RL1 between np. 13037 and np. 13317 and was not deleted between the intron splice sites.

The discussion has thus been written to take account of these findings.

CHAPTER 4

DISCUSSION

CHAPTER 4: DISCUSSION

During the past decade, the pathogenicity of HSV has attracted attention because of the increased incidence of chronically recurring genital disease, neonatal transmission and the severity of symptoms in immunocompromised individuals. An understanding of the molecular basis of HSV pathogenicity is important in intervention therapy such as development of HSV vaccines, antivirals and gene therapy. Several genes have been implicated in HSV virulence [see section 1.7]. A defined virulence gene, designated $\gamma_1$34.5 or RL1 has been identified in the long repeat regions of HSV-1 between RL2 and the 'a' sequence (Chou and Roizman, 1986; Dolan *et al.*, 1992). Its location was consistent with findings which associated virulence following intracerebral inoculation of mice with mutations in sequences located at or near the termini of the long repeat region of the genome (Thompson *et al.*, 1983, 1985, 1986; Javier *et al.*, 1988a; Oakes *et al.*, 1986; Taha *et al.*, 1989a,b; MacLean, A *et al.*, 1991). RL1 is a diploid gene encoding the protein ICP34.5, a 43.5K polypeptide of 263 amino acids in HSV-1 strain F infected cells (Ackermann *et al.*, 1986; Chou and Roizman 1986, 1990). An equivalent polypepetide (37K) of 248 amino acids was also identified in HSV-1 strain 17^+ infected cells (Mckay *et al.*, 1993). The latter group found that the HSV-1(F) polypeptide migrated with a M_r of 39K due to use of different gel systems (McKay *et al.*, 1993).

In our laboratory, the R_L region of the HSV-2 strain HG52 genome between 0-0.02 and 0.81-0.83 m.u. had been found to encode virulence determining sequences. The variants, 2604 and 2616 with 1488bp and 786bp deletions respectively within this region were shown to be avirulent on intracerebral inoculation of Balb/c mice (Taha *et al.*, 1989a,b; Harland and Brown, 1991). Sequence analysis of the long repeat region of HSV-2 strain HG52 indicated that HG52 possesses a RL1 gene equivalent to that of HSV-1. The coding sequence of HG52 RL1 is interrupted by a proposed intron composed of a repeat element of six complete and one partial copy of 19 nucleotides. Each copy contains a stop codon thus all three forward reading frames are blocked.

Removal of the proposed intron should result in the second exon containing an 189bp HSV-1/HSV-2 conserved region, being in frame. RL1 of HG52 is predicted to encode a protein of 261 amino acids. Eighty three percent of a 63 amino acid domain at the C-terminus of the protein is homologous to that of HSV-1 (McGeoch *et al.*, 1991). ICP34.5 has not yet been detected in cells infected with HG52. The majority of HSV genes are unspliced, RL1 of HG52 is unusual in that the predicted splice appears to be serotype specific. Because of these observations it became important to:

(i) confirm the predicted ORF of RL1 in HSV-2 strain HG52 and its role in the virulence of HG52.

(ii) characterise the functional domains of RL1 in HG52 and study the role of the 189bp conserved region in virulence by making truncated versions of ICP34.5.

(iii) determine experimentally whether RL1 of HG52 is spliced.

To confirm the predicted ORF of RL1 in HSV-2 strain HG52, an ICP34.5 null mutant 2621 was constructed with a translational stop codon TAG, 9bp downstream of the RL1 initiation codon ATG [sections 3.3.2+3.4.2]. The stop codon was inserted in a single frame to avoid disrupting any overlapping reading frames and to verify that loss of ICP34.5 alone is responsible for the phenotype of 2621. Unfortunately, two bases downstream of the inserted stop codon were deleted during site-directed mutagenesis which puts RL1 out of frame and disrupts any overlapping reading frame which might exist antisense to the gene. Time constraints did not allow isolation of another ICP34.5 null mutant virus. When this study began, apart from LAT (Zwaagstra *et al.*, 1989), there was no evidence of an antisense gene to RL1. Recently, two transcription units designated ORF P and ORF O were described by Lagunoff and Roizman (1994) antisense to RL1. These transcription units will be mentioned in detail later.

The ICP34.5 null mutant, 2621 was avirulent in mice with *in vivo* and *in vitro* characteristics comparable to those of 2604. On intracerebral inoculation of 3-week old Balb/c mice, 2621 had a $LD_{50} > 10^7$ p.f.u./mouse compared to 3.16×10^6 p.f.u./mouse for 2604 and 10^2 p.f.u./mouse for HG52 (Table 3.2). Similar results have been obtained

when stop codons were inserted at the beginning of the HSV-1 strains 17^+ and F, RL1 genes. The variant 1771 with a single-frame stop codon, 9bp downstream of the RL1 initiation codon was avirulent with a $LD_{50} > 10^6$ p.f.u./mouse compared to <10 p.f.u./mouse for the wild-type HSV-1 strain 17^+ (McKie *et al.*, 1994). A mutant virus of strain F carrying a six-frame stop codon about 90bp from the initiating ATG had a $LD_{50} > 10^7$ p.f.u./mouse compared to 420 p.f.u./mouse for the wild-type (Chou *et al.*, 1990).

The ability of the rescuant R2621 generated by *in vivo* marker rescue to fully restore the wild-type phenotype (Table 3.4) indicated unambiguously that RL1 exists and that it is a virulence determinant of HSV-2 strain HG52. The mechanism by which lack of expression of ICP34.5 results in failure of virus replication in the nervous system is not clear. Infection of cells of neuronal origin (human neuroblastoma SK-N-SH) by HSV-1 mutants incapable of expressing ICP34.5 results in shutoff of cellular protein synthesis. From these studies it was concluded that ICP34.5 is important in precluding shutoff of cellular protein synthesis in order to sustain production of infectious progeny virus (Chou and Roizman, 1992). Similar findings in SK-N-SH cells have been shown for the HSV-1(17^+) mutant 1716 and for the HSV-2 mutant 2604 which grow 2-3 log poorer than their parental-wild types at both high and low multiplicities of infection and shutoff cellular protein synthesis (Dr. A. MacLean, personal communication). This however is not the universal role of ICP34.5 in all non permissive cell types e.g. in confluent 3T6 cells RL1 negative virus fails to grow due to a defect in virus maturation (Brown *et al.*, 1994b). The precise mechanism by which ICP34.5 enables HSV to grow in the central and peripheral nervous systems *in vivo* is not known.

Having confirmed the existence of a HSV-2 RL1 gene and its role in virulence, it became important to determine the functional domains of the gene. Translational stop codons were introduced at unique restriction enzyme sites in exonI at the *Fsp*I site, in the proposed intron at the *Drd*I site and in exonII at the *Pfl*MI and *Rsr*II sites within the conserved region (Fig. 3.3). The mutant 2620 with a six-frame stop codon at the *Rsr*II site was isolated first and its phenotype determined. Insertion of a stop codon at the

*Rsr*II site, 46 codons upstream from the end of RL1 and 13 codons upstream of the 3' end of the conserved region, would result in most of the protein being expressed. It was expected that this mutation might result in a non-functional protein due to disturbance of the 63-residue region conserved between HSV-2(HG52) and HSV-1(17^+). A correponding domain is found in the mouse protein, MyD116 (McGeoch and Barnett, 1991) which was first identified in a myeloid leukaemic cell line induced to differentiate by interleukin 6 (Lord *et al.*, 1990). The conserved domain is also present in the hamster GADD34 gene induced by growth arrest signals and DNA damaging agents (Fornace *et al.*, 1989). A gene with similar homology, LMW23-NL has been identified in African Swine Fever virus. In the conserved domain at the carboxy terminus, LMN23-NL and MyD116, show 78% overall conservation, whereas LMW223-NL and ICP34.5 have 55% overall conservation (Sussman *et al.*, 1992). The conservation of one region within different proteins from diverse sources (Fig. 4.1) suggested that it may represent an important functional domain. On this basis it was expected that disruption of the conserved region at the C-terminus might reduce or abolish the virulence of HG52.

On intracerebral inoculation of 3-week old Balb/c mice, 2620 was as avirulent as 2604 and the null mutant 2621, with a LD_{50} of 2.37×10^6 p.f.u/mouse (Table 3.2). This result indicated that the conserved region and/or the C-terminal domain are essential for ICP34.5 function. It is possible that the 46 amino acids remaining between the inserted stop codon and the 3′ end of RL1 are important in the secondary structure and stability of ICP34.5 and therefore the truncated version of the protein might be degraded directly after its expression. As there is no available antibody which detects HG52 ICP34.5, it has not been possible to analyse this possibility.

Based on this data, the construction of mutant viruses with stop codons in RL1 upstream of the *Rsr*II site (Fig. 3.3) was not pursued. It was decided to rescue the 2620 mutant and also to construct a mutant with a stop codon at the terminus of the conserved region to precisely confirm its role in virulence of HG52. If only the conserved region in exonII plays a role in HG52 virulence, it would be expected that

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HSV-1 strain 17+    VRFSPHVRVRHLV-VWASAARLARRGSWARERADRARFRRRVAEAEAVIGPCLGPEARARALAR
HSV-1 strain F      VRFSPHVRVRHLV-VWASAARLARRGSWARERADRARFRRRVAEAEAVIGPCLGPEARARALAR
HSV-2 strain HG52   VCFSPRVQVRHLV-AWETAARLARRGSWARERADRDFRRRRVAAAEAVIGPCLEPEARARARAR
LMW23-NL            VYFATDDI---LIKVR-EADDIDRKGPWEQAAVDRIRFQRRIADTEKILSAVLLRKKLNPMEHR
MyD116              VHFAEKVTVHFLA-VWAGPAQAARRGPWEQFARDRSRFARRIAQAEEKLGPYLTPDSRARAWAR
GADD34              VHFSENVTVHFLA-VWAGPAQAARRGPWEQLARDRSRFARRIAQAEEKLGPYLTPAFRARAWAR
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Figure 4.1. The conserved carboxy-terminal domains in RL1 of HSV-1 strains 17^+ and F, HSV-2 strain HG52, African Swine Fever Virus ORF LMW23-NL, MyD116 and GADD34 genes.

the mutant would be as virulent as the wild-type. Single-stranded site-directed mutagenesis was used to try to insert at the end of the conserved region the *Xba*I site TCTAGA containing the stop codon TAG. Under various reaction conditions, this was unsuccessful although site-directed mutagenesis had been successful in other regions of RL1 in the presence of G+C rich mutagenic oligonucleotides (Table 3.1). This could be attributed to variations in the G+C content of the regions under mutation.

Interpretation of the degree of attenuation of the mutant 2620 has been complicated by failure to rescue revertants with a wild-type phenotype (Tables 3.2; 3.4). The LD_{50} value of 2620 is 200 fold higher than that of R2620 isolated by *in vitro* marker rescue (Table 3.2) indicating at least a 200 fold attenuation in virulence of 2620. The mutant has been reisolated (N2620) and partially purified.

Recently, Chou and Roizman (1994) mapped the sequence which precludes the total shutoff of protein synthesis in SK-N-SH cells to the carboxyl terminus of RL1 of HSV-1(F). Protein synthesis was shutoff in cells infected with a mutant containing a stop codon at the *Dra*III site within the conserved region, 11 codons downstream of the last PAT repeat. A similar result was obtained when cells were infected with the mutant R3616 from which 1Kb of the coding sequence of RL1 had been deleted. Protein synthesis at levels comparable to those observed in the wild-type infected cells took place in cells infected with all other deletion or insertion mutations in the amino-terminus upstream of the PAT domain

It was predicted from the sequence analysis that the ORF of RL1 is interrupted by an intron flanked by splicing sites and composed of a reiterated sequence with stop codons blocking all 3 forward reading frames (McGeoch *et al.*, 1991). The presence of an intron in RL1 is unusual as the majority of HSV genes are unspliced (McGeoch *et al.*, 1988a) and those which are spliced are conserved between HSV-1 and HSV-2. Five spliced genes have been identified in HSV, these are the immediate early gene RL2 (McGeoch *et al.*, 1988a), US1 and US12 (Watson *et al.*, 1981; Murchie and McGeoch, 1982; Rixon and Clements, 1982) in addition to the latency transcript LAT (Farell *et al.*, 1991; McGeoch *et al.*, 1991) and UL15 (Costa *et al.*, 1985; Dolan *et al.*, 1991).

Some of the HSV spliced genes possess homologues in other herpesviruses. Both the organization and the amino acid sequence of the HSV-1 UL15 gene are highly conserved in varicella-zoster virus (Davison and Scott, 1986), human cytomegalovirus (Chee *et al.*, 1990), human herpesvirus 6 (Lawrence *et al.*, 1990), Epstein-Barr virus (Baer *et al.*, 1984; Costa *et al.*, 1985) and channel catfish virus (Davison, 1992).

A novel promoter upstream and in the opposite orientation to the RL2(α0) promoter within the LAT region of the HSV-1 genome was identified by Bohenzky *et al* (1993). Two transcripts of 0.9Kb and 4Kb, antisense to RL1 have been detected by Nothern blotting. The 5′ ends of these transcripts have not been mapped, but they are present in cells infected with HSV-1 mutants lacking the LAT promoter and are therefore not processed forms of LAT. Moreover, a family of five L/S junction-spanning transcripts (L/STs), antisense to RL1, ranging in size from 2.3 to > 9.5Kb has been reported by Yeh and Schaffer (1993). L/STs are 5′ coterminal transcripts expressed in abundance in cells of neural and non-neural origin infected only with Vmw175 null mutant virus. Their expression is regulated by the bidirectional RL2 (α0) promoter described by Bohenzky *et al* (1993). Recently, Lagunoff and Roizman (1994) reported the presence of two ORFs, O and P with 173 and 248 codons respectively, antisense to RL1 of HSV-1 strain F. ORF P is quasi-totally antisense to RL1 with only 8 codons not antisense and 23 codons of RL1 not antisense to ORF P. The sequence of ORF P is well conserved among HSV-1 strains F, 17^+, CVG and MGH10. The number of the triplet Ala-Gly-Val (AGV) repeats, corresponding to the Ala-Thr-Pro repeats of RL1, is variable among strains. ORF P is expressed and encodes a protein with a M_r of 30,000 which is in agreement with its predicted M_r of 28,000. An equivalent to ORF P has not been identified yet in HSV-2(HG52). No protein product corresponding to ORF O has yet been identified. In a subsequent study, Bohenzky *et al* (1995) reported that the novel antisense promoter can be divided into two α X (an IE) and β X (an early) promoters, ORF P is driven by αX promoter while the ORF O is driven by βX promoter. Alignment of the ORF P sequence in HSV-1(F) to that of HG52 RL1 shows little or no homology except in the first amino acids of ORF P which correspond to the RL1

conserved region (Lagunoff and Roizman, 1994). Downstream sequences beginning with the repeat triplet AGV in HSV-1 showed little or no homology to the published HG52 sequence (McGeoch *et al.*, 1991). It was suggested that the homology of the P protein could be improved by assuming a HG52 RL1 sequencing error resulting in frame shifts. It was important therefore to provide evidence that RL1 is spliced and if spliced, to determine whether the intron is functional. The strategy employed to achieve this objective was: (i) amplification of a reverse-transcribed cDNA product of RL1 mRNA using primers flanking the predicted intron [section 3.17]. (ii) deletion of the predicted intron at the proposed splicing sites to study its role in virulence [section 3.4.3]. (iii) it was also intended to insert a stop codon in the intron and to this end a stop codon was inserted at the *Drd*I site within the proposed intron in the pSB phagemid (Fig. 3.3).

To amplify the cDNA product for RL1 mRNA, PCR was carried out on either the total or cytoplasmic poly $(A)^{+}$RNA with three pairs of primers flanking the predicted intron (Table 3.7, Fig. 3.27). This approach was unsuccessful possibly due to poor quality of RNA obtained from infected cells or to unsuccessful cDNA synthesis.

Construction of the 2622 mutant using the intronless pSB phagemid made it unnecessary to construct a mutant with a stop codon at the *Drd*I site. Intracerebral inoculation of 2622 resulted in intermediate virulence for mice with a LD_{50} of 10^5 p.f.u./mouse compared to $<10^2$ p.f.u./mouse for HG52 and 3.16×10^6 p.f.u./mouse for 2604 (Table 3.2). Restoring wild-type virulence by *in vivo* marker rescue (R2622/C) (Table 3.5) confirmed the 2622 phenotype. It was concluded that either: (i) The predicted intron in HG52 is functional and required for full expression of RL1. (ii) The proposed splicing sites of the intron are not correct. (iii) The deleted DNA sequence was not an intron but a functional domain of RL1. (iv) There was a secondary mutation in RL1.

While this thesis was being written, Dr. A. MacLean (personal communication) re-sequenced the predicted intron and the flanking region of RL1 and ruled out an error in the published sequence (McGeoch *et al.*, 1991). Subsequently, cDNA-PCR was

employed by Dr. J. Harland (personal communication) and the size of the PCR product demonstrated that RL1 is spliced and sequence analysis confirmed that the proposed splicing sites are correct. Reanalysis of 2622 subsequently revealed a deletion within RL1 which did not encompass the intron (Dr. J. Harland, personal communication). The intron was precisely deleted in the plasmid and hence reisolation of an intronless mutant is now underway. The role of the RL1 intron *in vivo* and *in vitro* in 3T6 cells will require to be reassessed.

To confirm the phenotype of the mutants 2620, 2621 and 2622, wild-type rescuants were constructed *in vitro* and *in vivo*. Rescuants were generated *in vitro* by transfection of BHK/C13 cells with intact mutant DNA and the linearised pXB phagemid. The same transfection mix was used for the *in vivo* marker rescue experiments. In addition two other transfection mixes containing the *Bam*HIg fragment derived either from the pAT-*Bam*HIg plasmid or from HG52 DNA were used (Table 3.3). Signs of encephalitis were an indication of growth and spread of wild-type rescuants in mouse brains which were then removed and rescuants isolated and purified. The virulence of rescuants generated *in vitro* and *in vivo* was compared to HG52 following intracerebral inoculation of Balb/c mice. The rescuants were injected at doses of 10^2-10^5 p.f.u./mouse, as it was anticipated that the rescuants would be as virulent as HG52 or 10-200 fold less virulent. Virulence differences between rescuants and wild-type virus have been shown previously e.g. the LD_{50} values of *ts* 1207 rescuants were 60-200 fold higher than that of the parental wild-type virus (Cameron *et al.*, 1988) and those of the intertypic recombinant RE6 were 50 fold less virulent than the wild type parents (Thompson *et al.*, 1983b).

Apart from R2620, rescuants generated by *in vivo* marker rescue regained wild-type virulence (Table 3.4). It was concluded that the virulence phenotype of the mutants 2621 and 2622 was due to the specific RL1 mutations. The R2622 rescuants regained virulence with LD_{50} values ranging from 3.16×10^2-10^4 p.f.u./mouse. These LD_{50} values are within the range of the individual plaque stocks of HG52 pl.17 (Table 3.5). Rescuants generated by *in vitro* marker rescue failed to return to the wild-type HG52

phenotype and demonstrated an intermediate level of virulence (Table 3.2). The possibility that a mutation in the pXB *Xho*I fragment might be responsible for the intermediate phenotype was ruled out as this fragment restored virulence to the rescuants of 2621 and 2622 (R2622/C) generated by *in vivo* marker rescue (Table 3.4). A secondary mutation in the genome of the rescuants generated *in vitro* is the most likely explanation. Virulence is multigenically controlled both in HSV [section 1.7] and other viruses e.g. pseudorabies virus (Lomniczi *et al.*, 1984), myxoviruses (Rott, 1979) and bunyaviruses (Rozhon *et al.*, 1981). A secondary mutation could be attributed to inherent heterogeneity in the parental stock and/or passage of the viruses during three rounds of purification. Heterogeneity in this context means that viruses arising from individual genomes have different virulence potentials. Taha *et al* (1989a) transfected BHK21/C13 cells with a DNA stock of HG52 pl.17 and isolated 5 well separated progeny plaques. The virus stocks from the 5 plaques were intracerebrally inoculated into mice and their LD_{50} values ranged from 10^2 to $> 10^4$ p.f.u./mouse. It was found that individual plaques derived from high virulence stocks demonstrated either a high or intermediate level of virulence. Individual plaques derived from intermediate and low virulence stocks remained stable as low or intermediate virulence. The basis of this virulence variation is not clear, although Stroop *et al* (1994) attributed the avirulent phenotype of one of these individual plaques to deficiency in TK, a gene involved in HSV virulence [section 1.7.2.1].

In this study, six individual plaques of pl.17 were picked randomly and subjected to three rounds of purification which included seven passages in BHK21/C13 cells at 37°C. The individual plaques demonstrated a range of LD_{50} values and were categorised as: virulent with a $LD_{50} \leq 10^2$ p.f.u./mouse, intermediate with a $LD_{50} = 10^4$ p.f.u./mouse and avirulent with a $LD_{50} > 10^4$ p.f.u./mouse (Table 3.5). Serial passage could have an effect on the stability of the virulence phenotype of these individual plaques. It could be concluded that the highly virulent individual plaques No. 2, 5, 6 with LD_{50}s $\leq 10^2$p.f.u/mouse originated from a pl.17 genome with a high virulence capacity. The avirulent phenotype of plaques No. 3 and 4 (LD_{50}s $> 10^4$p.f.u/mouse)

may be due to a secondary mutation in other virulence genes arising during passage The same applies to the intermediate virulence plaque No. 1 with a LD_{50} of 10^4p.f.u./mouse. Assigning phenotype to a specific mutation in RL1 requires rescuants which return to full virulence.

It is noteworthy that heterogeneity and virulence attenuation by passage is not restricted to HG52. Recently it has been observed in HSV-1 strain 17^+ (Dr. A. MacLean, personal communication). Moreover, it is known that both virulent and avirulent stocks of HSV-1 strain KOS exist (Dix *et al.*, 1983). The effect of passage in tissue culture on HSV virulence is similar to findings reported for poliovirus. The three attenuated strains of poliovirus which are used as oral vaccines were derived from wild-type virulent strains by extensive passage in monkey tissue (Sabin, 1955). Point mutations were observed scattered over the entire length of the genome of the attenuated strains, but those in the 5' non-coding region of the genomic RNA contributed to the attenuation of the parental strain (Skinner *et al.*, 1989; Kawamura *et al.*, 1989)

In contrast, Thompson and Stevens (1983a) reported that the capacity of HG52 isolates to replicate in mouse brain is increased following serial passage at 37.5°C. The Glasgow elite stock of HG52 showed a LD_{50} of 10^4 p.f.u./mouse following 7 serial passages in rabbit skin monolayers at 31°C. Passage at 37.5°C resulted in at least a 10,000 fold increase in virulence. Five isolates of the elite stock of HG52 were purified twice at 31°C and the progeny serially passaged 4-5 times at 31°C or 37.5°C. Following intracranial inoculation of 4-6 week-old Swiss Webster mice, it was found that all five isolates passaged at 31°C retained the avirulent phenotype, while those passaged at 37.5°C became virulent. Possibly passage at 37.5°C resembles conditions required for *in vivo* growth. Goodman and Stevens (1986) demonstrated that passage of HSV-1 strain 17^+ *in vitro* in chick embryo fibroblasts selected for a virus capable of killing chick embryos indicating that *in vitro* passage of HSV potentiated rather than attenuated virulence. The increase in virulence might be due to adaptation to species. Multiple brain passage has been shown to increase the virulence of the avirulent HSV-1/HSV-2 recombinant R13-1 and the avirulent HSV-1 strain ANG. Javier *et al* (1988a)

reported that virulence of R13-1 increased 25-60 fold after two passages through mouse brains. This finding was unexpected as the avirulent phenotype of the recombinants RE6 (Thompson *et al.*, 1983b) and RS6 (Javier *et al.*, 1987) was extremely stable following seven and eight passages, respectively in mouse brains. Kaerner *et al* (1983) reported that HSV-1 ANG is avirulent for DBA/2J mice upon intraperitoneal or intravaginal inoculation but virulent upon intracerebral inoculation. The virulent variant HSV-1 ANG path was generated following 12-15 serial passages of HSV-1 ANG in mouse brain . In mixed infections both the avirulent and the virulent viruses replicate at the primary site of infection and spread to various organs, but only HSV-1 ANG path could be recovered from the spinal cords and brains of mice. The restriction endonuclease pattern of the virulent Ang isolates differed from those of the avirulent strain. It was not clear whether these changes were related to the virulence phenotype observed.

In this study the rescuants generated *in vitro* (Table 3.2) and the rescuants R2620, R2622/B generated *in vivo* (Table 3.4) did not return to the wild-type phenotype. This may have been due to a point mutation in the RL1 gene of these rescuants. A possibility which could also apply to the low and the intermediate virulence plaques of HG52 pl.17 (Table 3.5). As there is no available antibody which detects HG52 ICP34.5 we were unable to look at expression of the protein in these variants. However, the 3T6 mouse embryo fibroblast selection system described by Brown *et al* (1994a) is a good *in vitro* model for distinguishing RL1 mutant virus from wild-type. Confluent 3T6 cells are permissive for wild-type HSV-1 strain 17^+ and non-permissive for the RL1-negative mutants 1716 and 1771 (Brown *et al.*, 1994a). The same was observed for HSV-2 HG52 and the RL1 negative mutant viruses 2604 (Brown *et al.*, 1994a). It has been shown for HSV-1 that the inability of 1716 to grow in 3T6 cells is not due to an inability to adsorb to and penetrate cells but due to an inability to spread from the initially infected cells (Brown *et al.*, 1994b). Analysis by electron microscopy showed that 90% of virus particles are confined to the nuclei of 1716 infected cells. It is clear therefore that in certain cell types and under certain cell states failure to express

ICP34.5 results in a defect in virus maturation and egress from infected cells (Brown *et al.*, 1994b).

In 3T6 cells, 2620 and 2621 behaved as 2604 (Fig. 3.18). It would be expected therefore that a defect in RL1 of the individual plaques of HG52 pl.17 or the rescuants would result in impaired growth in 3T6 cells. Multicycle growth experiments for the individual plaques of HG52 and the rescuants were carried out in 3T6 cells (Figs. 3.23, 3.24, 3.25, 3.26) over a period of 72h. The growth patterns of the selected individual plaques of HG52 pl.17 regardless of their LD_{50} values (Table 3.5) were identical to that of HG52. Similarly, the growth kinetics of the rescuants generated *in vitro* were identical to those of HG52 pl.17 and the rescuants generated *in vivo*. This strongly suggests that RL1 of the individual plaques and the rescuants generated *in vitro* is intact and expressed. As HSV virulence is multigenetically controlled [section 1.7], point mutations in any gene involved in HSV virulence might contribute to the intermediate phenotype of these variants.

The *in vivo* characterisation of the RL1 mutants involved studying their replication capacity in mouse brain. It was found that the parental virus HG52 pl.17 replicated to high titre within 120h (Fig. 3.13). The avirulent mutants 2620 and 2621 replicated poorly with kinetics similar to that of 2604. Virus multiplication was blocked and there was no detectable virus by 96h post-inoculation. It was concluded that the avirulent phenotypes of 2620 and 2621 (Table 3.2) was due to their inability to replicate in mouse brain. The expression of viral antigens in brains inoculated with 2620 and 2621 was not tested as a previous study by Taha *et al* (1990) showed that following infection with 2604, HSV antigens were located only at the site of inoculation, while following infection with HG52, HSV antigens were widely distributed in neurons and supporting brain cells. It would be expected that 2620 and 2621 would demonstrate a similar result in immunohistochemical analysis. Growth of the mutant 2622 was intermediate with respect to 2604 and HG52 and the virus was cleared from the brain by 120h post-inoculation (Fig. 3.13). The growth kinetics of 2622 are consistent with its LD_{50} value (Table 3.2).

All RL1 mutants exhibited no temperature sensitivity (Fig. 3.22) greater than that of HG52. Similar results were demonstrated by Taha *et al* (1989a) for HG52 and 2604 at 38.5°C in single cycle growth experiments using mouse embryo fibroblast 3T3 cells. Differential replication of HG52 and the RL1 mutants was not observed in BHK21/C13 cells at either low or high multiplicities of infection (Fig. 3.16, 3.17). A consistent finding in our laboratory is that all RL1 variants of HSV-1 strain 17^+ (MacLean, A *et al.*, 1991) and HSV-2 strain HG52 (Taha *et al.*, 1989a; Harland and Brown, 1991) are not impaired in BHK21/C13 cells. Electron microscopical analysis of BHK21/C13 cells infected separately with HSV-1(17^+) and the avirulent deletion mutant 1716 revealed that virus particles were equally distributed between the nucleus and the cytoplasm of infected cells. However, delamination of the nuclear membrane and a significant number of particles trapped between the nuclear lamellae were observed in 50% of BHK21/C13 cells infected with 1716. It appears that lack of ICP34.5 expression may result in blockage of one of the two pathways proposed by Rixon (1993) for egress of HSV from the nuclei to the extracellular space (Fig. 1.3). Trapping of virus between the nuclear lamellae might be due to overloading of one pathway which compensates for the other being blocked (Brown *et al.*, 1994b).

Differential growth was observed at low m.o.i. (0.01 p.f.u./cell) in confluent 3T6 mouse embryo fibroblasts (Fig. 3.18) in which the mutants 2604, 2620 and 2621 mimicked the *in vivo* phenotype (Table 3.2 ; Fig. 3.13) by failing to replicate and producing abortive plaques; wild-type HG52 is not impaired and produces large plaques. The mutant 2622 showed intermediate growth and produced intermediate sized plaques.

In 3T6 cells, 2621 produces plaques of two morphologies, small and large. The existence of large plaques in the 2621 stock was reflected in its growth pattern relative to 2604 (Fig. 3.18). This phenotypic property of 2621 was confirmed by reisolation of a new mutant virus N2621 which also predominantly produced small plaques plus a few large plaques. The small and large plaques could not be completely segregated following three rounds of purification and their *Xba*I genomic profiles were identical (Fig. 3.19). At present, there is no explanation for this property of 2621 especially as it

has not been observed for the RL1 mutants of HSV-1 strain 17+ and other HG52 mutants. The large plaques imply expression of ICP34.5 indicating that a second site reversion might have occurred which could not be detected by genomic digestion with *Xba*I (Fig. 3.19). Because there is no available antibody which detects HG52 ICP34.5 we were not able to test this hypothesis. When the replication efficiency of the small and large plaques of 2621 was tested in BHK/C13 cells, no differential growth was observed (Fig 3.21). Differential growth was observed in 3T6 cells where large plaque growth was comparable to HG52 and growth of the small plaques was greater than the parental 2621 (Fig. 3.20). Possibly this is due to adaptation of the small plaques to grow in 3T6 cells as a result of passage in these cells. *In vivo*, the small plaque virus of 2621 was avirulent and had a LD_{50} of 10^6. The large plaque virus demonstrated an intermediate level of virulence with respect to HG52 (Table 3.6) with a LD_{50} of 2.15×10^4, at least 100 fold more virulent than the parental 2621. This intermediate phenotype might be due to virulence attenuation as a result of passage in 3T6 cells as demonstrated before for the parental stock HG52 Pl.17.

The results presented in this thesis demonstrate the existence of an ORF encoding the HSV-2(HG52) RL1 as predicted by McGeoch *et al* (1991). Like HSV-1, RL1 determines HG52 virulence, hence insertion of a stop codon in the predicted ORF, 9bp downstream of the initiation codon was shown to abolish the virulence of HG52. It has now been shown that the mature mRNA of RL1 is spliced. Introduction of a stop codon at the *Rsr*II site within the conserved region of RL1 yielded a virus whose characterisation was complicated possibly due to a secondary mutation in the genome of the isolated mutant. The LD_{50} value of this mutant was 200 fold less than that of the revertant isolated by *in vitro* marker rescue. Virulence heterogeneity in HG52 pl.17 was demonstrated in this study with LD_{50} values ranging from $\leq 10^2$ to $\geq 10^4$ p.f.u./mouse. This fact emphasises the necessity of obtaining rescuants with a totally virulent phenotype to confirm that the phenotype of mutants is due to a specific mutation.

Unfortunately the mutant 2622 which was thought to be intronless turned out to have a

deletion in another part of RL1 partly within exon I with the intron remaining intact. Not only was this spontaneous deletion approximately the same size as the intron but it was in the same fragment chosen for analysis. At the time there was no reason to suppose that the deleted sequence was other than the intron. When it became obvious from the PCR analysis that 2622 still contained the RL1 intron, a range of double restriction enzyme digests were carried out. The genotype of 2622 has now been resolved and the intronless plasmid which has been sequenced and shown to be correct will be used to generate a new mutant. The mutant 2622 has however demonstrated that sequences upstream of the intron interfere with RL1 function but do not completely abolish virulence.

Future prospects:

1. Purification and characterization of the new mutant of 2620 (N2620) with a stop codon at the *Rsr*II site within the conserved region of RL1 to confirm the role of this region in HG52 virulence.

2. Insertion of an in-frame stop codon at the end of the conserved region to determine the precise role of this region in HG52 virulence.

3. Identification of the HSV-2 RL1 encoded protein (ICP34.5). For this purpose, an antiserum directed against the HG52 ICP34.5 expressed protein or possibly against the HSV-1(17$^+$) expressed protein could be used. It has been shown previously, that anisera raised against synthetic oligonucleotides representing different regions of HSV-1(17$^+$) RL1 including the conserved region were in general unsuccessful in identifying ICP34.5 in HSV-1 (17$^+$) infected cells and HSV-2 (HG52) infected cells (McKay *et al.*, 1993). The only successful peptide used to generate an antiserum was the (PAT)10 repeat which is not conserved in HG52. A polyclonal antiserum raised against the partially purified ICP34.5 of HSV-1(17$^+$) expressed in the *E. coli* pET system failed to detect HSV-2(HG52) ICP34.5 by either immunoprecipitation or Western blotting. This could be due to a low level of ICP34.5 expression and/or low titre of antisera due to low purity of ICP34.5 used to generate the polyclonal antibody. A higher level and higher

purity of ICP34.5 may be required to get a more sensitive antiserum (McKie *et al.*, 1994). The baculovirus and the glutathione S-transferase-fusion (GST) expression systems are currently being used to express the HSV-1(17^+) RL1 gene.

4. Reisolation of a mutant in which the RL1 intron has been deleted and characterisation of the mutant *in vivo* and *in vitro*.

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