The role of glycoprotein C in adsorption of herpes simplex virus type 1 (HSV-1).

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in

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Summary

The work presented in this thesis is a study of the role of gC in adsorption of herpes simplex virus type 1 (HSV-1).

This study used several strains of wild type HSV-1 and their gC negative mutants strains 17, F, SC16 and HFEM. In contrast to published results, comparison of the particle to p.f.u ratios of each wild type strain to their gC negative form showed similar values, differing by two fold at most (Herold *et al.*, 1991).

Initially the ability of radioactively labelled strain 17 wild type and gC negative mutant virions to bind to BHK21/C13 and C6 gliomal cells was examined. There was no difference between either virus or between cell lines.

Subsequently the adsorption kinetics of HSV-1 strain 17 wild type virus and its gC negative mutant were studied on BHK21/C13 cells. Again no differences were observed.

Similarly a study of the rate of penetration of the HSV-1 strain 17 wild type and gC negative viruses demonstrated no difference in the rate of penetration into BHK21/C13 cells.

The adsorption characteristics of the different strains and their gC negative mutants used in this study were compared on BHK21/C13 cells, with no difference being seen between any parental strains, and its gC negative mutant or between HSV-1 strain 17 wild type and any other.

The adsorption kinetics of all strains and their gC negative mutants were compared on Vero cells with no difference being seen.

β-Galactosidase staining of lac Z expressing forms of some of the viral strains (HSV-1 strain 17, its gC negative mutant, SC16-ΔUL44-Z and HFEM-ΔUL44-Z) was used to examine adsorption kinetics on HeLa and 3T6 cells. All viruses displayed similar adsorption kinetics.

In heparin inhibition assays all viruses demonstrated the same pattern of blocking by exogenous heparin although the levels of inhibition differed between strains.

Experiments to determine the intracranial pathogenicity revealed no significant difference in pathogenicity with LD_{50} values of $<10^1$ and $5x10^1$ for the wild type and mutant respectively.

In peripheral pathogenicity and latency studies of the HSV-1 strain 17 viruses a ten fold difference was observed between the wild type and the mutant virus in LD_{50} data; this is not a significant difference. Reactivation frequency was also shown to be similar.

The use of Southern and Western blotting confirmed the structure and gC expression of the HSV-1 strains and their mutants.

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Contents.

Abbreviations

APS	ammonium persulphate
BHK21/C13	baby hamster kidney cells, batch 21 clone 13
BHV	bovine herpesvirus
bp	base pairs
BSA	bovine serum albumin
C	degrees centigrade
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
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DRG	dorsal root ganglia
EDTA	ethylenediaminetetra acetic acid
HCMV	human cytomegalovirus
HS	heparan sulphate
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
k	kilo (10^3)
kbp	kilo base pairs
mg	milligram
mM	millimolar
m.o.i.	multiplicity of infection
M_r	molecular weight
ng	nanogram
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
p.f.u.	plaque forming units
p.i.	post-infection
PRV	pseudorabies virus

RE	restriction enzyme
RNA	ribonucleic acid
r.p.m.	revolution per minute
RT	room temperature
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N,-tetramethylyethylene diamine
Tris	tri (hydroxymethyl) aminomethane
μCi	microcurie
μl	microlitre
μM	micromolar
UV	ultraviolet
V	volts
v/v	volume/volume (ratio)
w/v	weight/volume (ratio)
w/w	weight/weight (ratio)

1. Introduction.

1.1
The herpesviruses.

1.1.1
General properties

Herpes simplex virus (HSV) is a member of the *herpesviridae* family which includes a large number of medically and veterinarily important viruses. They produce a variety of diseases in humans and are widespread in other vertebrates (Minson, 1989). There are more than eighty members of this family (Nahmais *et al.,* 1972) which are all morphologically similar (Fenner, 1976). The virion (120-200 nm in diameter) consists of four structural components: (a) a core made up of a linear double-stranded DNA molecule of 120-240 kbp; (b) an icosohedral capsid 100-125 nm in diameter composed of 162 capsomeres; (c) an amorphous layer termed the tegument which surrounds the capsid - this is often distributed asymmetrically and may be variable in amount; (d) the envelope, a bilayered lipid containing membrane which surrounds the tegument and has surface projections consisting of virus encoded glycoproteins (Wildy *et al.,* 1960; Roizman and Furlong, 1974). The herpesviruses encode around forty 'core' genes which show amino acid sequence homology between all of the herpesviruses and are thought to result from a common evolutionary progenitor (Davison, 1993). These core genes encode structural proteins and proteins involved in virion morphogenesis, nucleotide metabolism and DNA packaging.

All members of the family possess a linear double stranded genome (Roizman, 1982). However, herpesviridae family members are readily distinguished using criteria such as base composition, genome structure, immunological cross-reactivity, biological properties, size and DNA sequence.

1.1.2
Classification.

Based on their biology, the *Herpesviridiae* family can be sub-divided into three groups, the *Alpha, Beta* and *Gammaherpesviriniae* (Roizman, 1982). The distinction between the *Alpha* and *Gamma* subfamilies is based on host range and *in vitro* characteristics, while the differentiation between the *Beta* subfamily and the others is based primarily on the length of the reproductive cycle and slow development of cytopathology in tissue culture.

The *Alphaherpesviriniae* are neurotropic and frequently, but not exclusively, become latent in neurones of the dorsal root ganglia (DRG) of the peripheral nervous system (PNS). They are characterised by their short reproductive cycle and rapid spread of infection in cell culture resulting in mass destruction of susceptible cells. This subfamily can be further subdivided into two genera; the *Simplexviruses* e.g. HSV-1 and 2; and the *Varicelloviruses* e.g. varicella zoster virus (VZV), feline herpes virus (FHV-1) and pseudorabies virus (PRV).
In contrast, the *Betaherpesvirinae* which comprise the cytomegaloviruses have a relatively long reproductive cycle. In tissue culture they produce slowly progressing lytic foci and infected cells frequently become enlarged (cytomegalia) both *in vitro* and *in vivo*. Members of this group can establish latent infections in glands and other tissues. They include human cytomegalovirus (HCMV) and equine herpesvirus 2 (EHV-2).
The final subfamily in this grouping are the *Gammaherpesviriniae*, which are lymphotrophic and specifically infect B and T lymphocytes. In the lymphocyte, infection is frequently arrested either at a pre-lytic stage with persistence and minimal expression of the viral genome, or proceeds to a lytic stage, causing cell death whilst producing complete virions. Examples are Epstein Barr virus (EBV).

1.1.3
Human herpesviruses.

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) are among eight herpes viruses characterised to date that have man as their natural host (HSV-1, HSV-2, VZV, EBV, HCMV, HHV-6, HHV-7 and HHV-8). (See Table 1.1 and Figure 1.1)

HSV-1 and HSV-2.

See 1.1.4

VZV.

The primary disease caused by VZV is known as chickenpox or varicella. The virus enters via the respiratory tract and replicates in the lymph nodes (Minson, 1989; Ginsberg, 1990). Viraemia occurs and after an incubation period of roughly fourteen days, a fever ensues and vesicles form on the skin. In most cases infection occurs before the age of twenty. If the primary infection occurs in adulthood it can be particularly severe.

Table 1.1: The human herpesviruses.

Table showing the human herpesviruses, the group to which they belong, their most common associated illness, the genome size and its GC content.

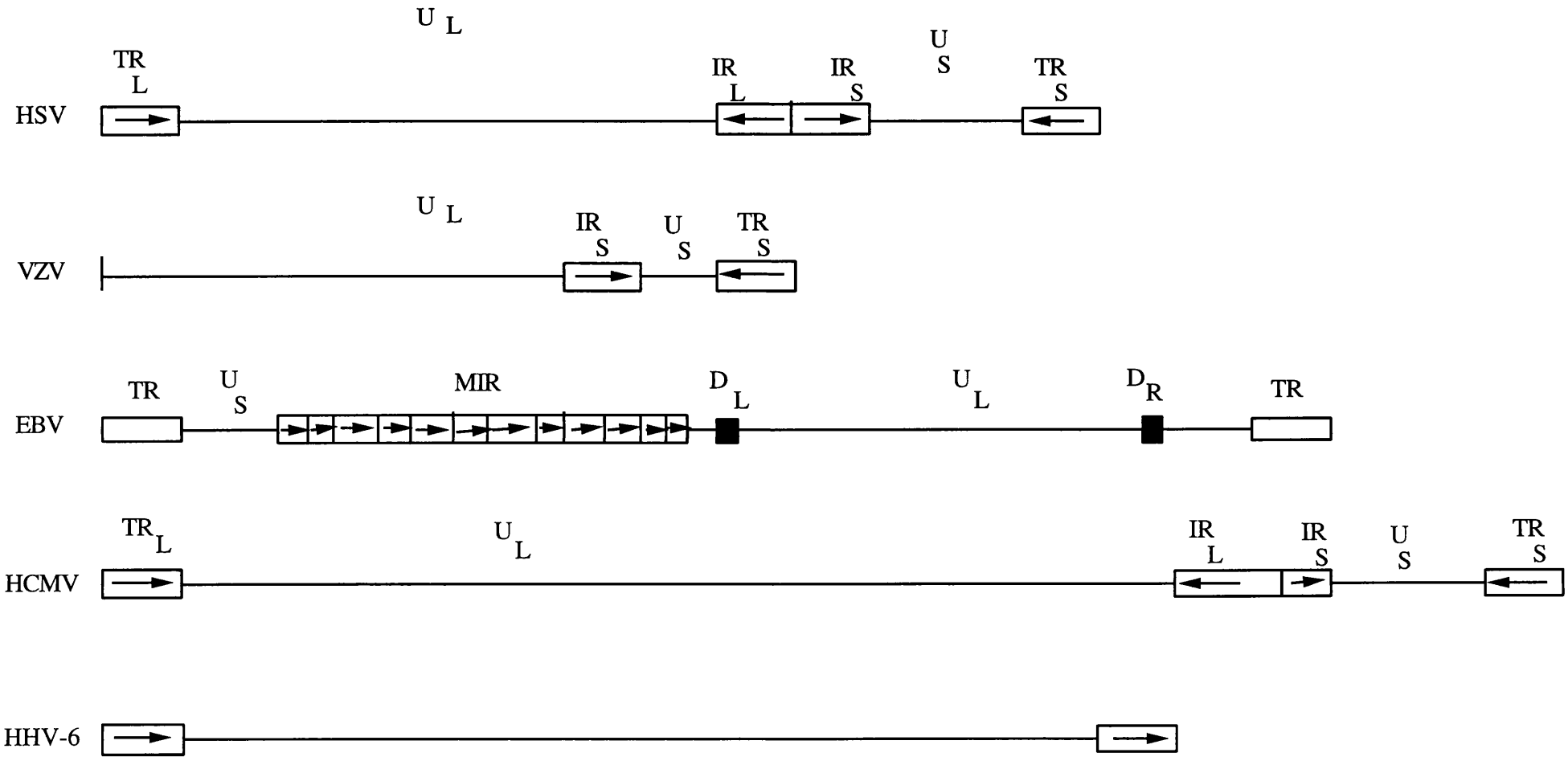
From Subak-Sharpe and Dargan, 1998.

Human herpesviruses	Abbreviation	Herpes group	Associated illness	Genome size Kbp	G+C
herpes simplex virus type 1	HSV-1	alpha	cold sores	152	68.3
herpes simplex virus type 2	HSV-2	alpha	genital lesions	155	70.4
varicella-zoster virus	VZV	alpha	chicken pox/shingles	125	46
Epstein-Barr virus	EBV	gamma	glandular fever, Burkitt's lymphoma, NPC	172	60
cytomegalovirus	HCMV	beta	congenital abnormalities	>229	57
human herpesvirus 6	HHV-6	beta	infant rash exanthium subitum	159	42
human herpesvirus 7	HHV-7	beta	febrile illnesses	145	45
human herpesvirus 8	KSHV, HHV-8	gamma	Kaposi's sarcoma associated	160-170	not sequenced

Figure 1.1: Structures of human herpesvirus genomes.
Repeat regions are represented by open rectangles with the orientation of the repeats indicated by arrows. Unique regions are represented by horozontal lines.

Abbreviations are as follows:

U_L	unique long
U_S	unique short
T/I_{RL}	terminal/internal long repeat
T/I_{RS}	terminal/internal short repeat
D_L	direct repeat left
D_R	direct repeat right
MI_R	major internal repeat.


HSV
TR L
U L
IR L
IR S
U S
TR S
VZV
U L
IR S
U S
TR S
EBV
TR
U S
MIR
D L
U L
D R
TR
HCMV
TR L
U L
IR L
IR S
U S
TR S
HHV-6

During primary infection, VZV establishes latency in sensory ganglia of the spinal or cranial nerves where it persists for the lifetime of the host in the presence of neutralising antibodies. As with HSV, trauma and immune suppression can trigger reactivation. Reactivation leads to shingles (also known as herpeszoster), which manifests itself as vesicles and a severe pain at the distribution of the infected nerve.

HCMV.

HCMV has a prolonged lytic cycle that requires 5-6 days for completion (Smith and DeHaven, 1973). HCMV results in a variety of disorders, which include pneumonitis, encephalitis, neuropathy, retinitis, colitis and vasculitis (Wattre *et al.,* 1995). It has also been implicated in organ transplant rejection (Arbustini *et al.,* 1996; Vogelberg *et al.*, 1996). The virus is able to transform cells in culture and several types of human malignancy have been linked to HCMV.

HCMV is the most common cause of severe intra-uterine infections (Minson, 1989; Ginsberg, 1990; Borucki and Pollard, 1994). HCMV also establishes latent infections.

EBV.

EBV infection occurs via the oral route and the virus replicates in the epithelium of the mouth and salivary ducts (Rickinson *et al.,* 1985; Minson, 1989; Ginsberg, 1990).

Infection usually takes place within the first five years of life and produces a sub-clinical disease. When infection occurs after adolescence approximately fifty percent of patients display the clinical manifestations of the disease known as infectious mononucleosis (IM) or glandular fever. Symptoms include enlarged lymph nodes and spleen, abnormal lymphocytes in the blood and fatigue which can persist for months.

EBV can immortalise B-lymphocytes and the B-lymphocyte is a major site of EBV latency. EBV sequences are commonly found in at least two types of tumours, Burkitt's lymphoma (common in Africa) and nasopharangeal carcinoma (Southern Asia): thus the immortalisation functions may facilitate the development of certain tumours.

HHV-6 and HHV-7.

HHV-6 was first isolated from the blood of patients with lymphoproliferative disorders or AIDS (Salahuddin *et al.*, 1986). Up to ninety percent of the population are asymptomatically infected with HHV-6, although infection in infants sometimes causes a mild skin rash called

exanthum subitum (Yamanishi *et al.*, 1988; Okuna *et al.*, 1989) and acute febrile illness in young children (Pruksanonda *et al.*, 1992; Hall, 1994). HHV-6 can replicate in $CD4^+$ T-lymphocytes and there is evidence that the monocyte is a site of latency.

HHV-7 shares a number of common features with HHV-6 including replication in T-lymphocytes (Frenkel *et al.*, 1990; Berneman *et al.*, 1992; Lusso *et al.*, 1994), a similar genome organisation (Lindqueter and Pellet, 1991; Gompels *et al.*, 1995; Secchiero *et al.*, 1995) and 50-60% nucleotide identity in the genomic regions which have been examined (Berneman *et al.*, 1992). HHV-7 has also been isolated from the blood and saliva of immunosupressed and healthy subjects (Frenkel *et al.*, 1990; Wyatt and Frenkel, 1992). Although HHV-7 has yet to be formally associated with any disease, there is evidence that it may be associated with some cases of exanthium subitum (Portolani *et al.*, 1995).

HHV-8.

Attempts to identify infectious agents associated with Kaposi's sarcoma (KS) lesions suffered by AIDS patients located viral DNA sequences with close homology to regions of the EBV genome (Memar *et al.*, 1995). Sequences of this herpesvirus, HHV-8, are found in peripheral blood mononuclear cells of KS^+ patients: thus the presence of HHV-8 DNA appears to be essential for the aetiology of KS. Sequence analysis suggests that the HHV-8 genome contains sequences which encode a D type cyclin and a number of other genes implicated in growth deregulation which may be relevant to its proposed role as a transforming virus.

1.1.4

Herpes simplex virus infections.

Two HSV serotypes have been identified: type 1 (HSV-1) and type 2 (HSV-2). They have been differentiated by pock size studies on chorioallontoic membranes (Rogers, 1973), neutralisation studies (Nahmias and Dowdle, 1968), monoclonal antibody-based assay and restriction enzyme analysis (Lonsdale *et al.*, 1980; Bergstrom and Trybala, 1996). Serological studies have demonstrated that HSV-1 infections are generally acquired during childhood and that by sixty years of age, up to ninety percent of the population have antibodies to HSV-1 (Nahmias *et al.*, 1970). HSV-2 infections are not usually acquired until puberty and are correlated with sexual activity. Johnson *et al.* (1989) show that the prevalence of HSV-2 antibodies increases with age in the post-adolescent years, an epidemiological pattern typical of a sexually transmitted disease (Kinghorn, 1993).

HSV causes genital infections, conjunctivitis, keratitis and herpetic Whitlow. HSV has a high worldwide prevalence. For example ocular infections affect approximately 500,000 people in the USA each year (Binder, 1977). HSV encephalitis is the most commonly reported viral infection of the CNS, accounting for between 10 and 20% of all viral encephalitises in the USA. Immuno-compromised patients 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 has become less valid, with 30-50% of all genital infections being caused by HSV-1 in some studies (Kalinyak *et al.*, 1977; Smith *et al.*, 1977; Kinghorn, 1993) and 5-20% of oral infections being due to HSV-2. The increased incidence of genital herpes had lead to a corresponding increase in the level of neonatal herpes (Sullivan-Boyali *et al.*, 1983; Whitley *et al.*, 1991).

The lifecycle of HSV includes acute, latent and recurrent phases. During acute infection, HSV genes are expressed and the extent of disease is controlled by the cellular and humoral responses of the host. During latent infection, HSV gene expression is transcriptionally silent except for a limited region of the genome encoding the latency-associated transcripts (LATs).

During primary infection, the virus replicates lytically in the peripheral tissues around the site of infection and enters sensory nerves at the termini of axons of sensory neurones. A lifelong latent infection is established in neurones of the sensory ganglia and periodically, various stimuli including stress, exposure to ultra-violet light, hormonal changes and immunosuppression induce reactivation and the production of infectious virus. The reactivated virus then travels back to the tissues around the site of primary infection, where it may again replicate lytically, destroying cells and causing the formation of recurrent lesions.

1.1.5
Pathogenicity of HSV.

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

Various animal models have been used to study HSV pathogenicity, e.g. mouse, guinea pig, rabbit, rat and monkey. Several host factors controlling pathogenicity have been identified; namely, humoral immunity (McKendal *et al.*, 1979) cytotoxic immunity (Nash *et al.*, 1985),

interferons (Lopez, 1985), state of the skin and mucous membranes, age of host and route of inoculation (Sprecher *et al.,* 1986; 1987). Another influencing factor is the body temperature of the host (Thompson and Stevens, 1983a and b). Viral factors affecting pathogenicity are virus strain and serial passage of the virus either *in vivo* (Kaerner *et al.,* 1983) or *in vitro* (Goodman and Stevens, 1986).

1.1.6
Virion structure.

HSV virions are composed of four morphologically distinct structures, the core, an icosohedral nucleocapsid, an amorphous tegument surrounding the capsid and an outer envelope from which protrude the glycoprotein spikes (Dargan, 1986; Rixon, 1993; Beers *et al.,* 1994). The HSV capsid is approximately 125 nm 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; Schrag *et al.,* 1989; Zhu *et al.,* 1994). The capsid is composed of seven proteins: VP5 (UL19), VP19c (UL38), VP23 (UL18), VP24 (UL26 N-terminal) and VP26 (UL35) (Gibson and Roizman, 1972: Cohen *et al.,* 1980).

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 but possess two 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 intermediate 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 surrounded by an amorphous layer known as the tegument (Roizman and Furlong, 1974). The dimensions of the tegument vary between herpesviruses and are thought to be determined, at least in part, by the virus (Nazerian and Witter 1970; McCombs *et al.,* 1971).

The tegument may be more ordered than was previously thought, as specific interactions of various tegument components have been observed (Smibert *et al.,* 1994; Elliott *et al.,* 1995). It contains several viral factors that facilitate infection. Examples of these factors include Vmw65 (Ace *et al.,* 1988, 1989), the virion host shutoff factor (Kwong *et al.,* 1988) and possibly the immediate early (IE) proteins Vmw175 and Vmw110 (Yao and Courtney 1989,1992; Yang and Courtney, 1995).

The tegument is completely enclosed in a trilaminar envelope (Wildy *et al.,* 1960; Darlington and Moss, 1968) of mean diameter 180 nm which has surface spikes 8-10 nm long spaced 5nm apart (Wildy *et al.,* 1960) Use of monoclonal antibodies coupled with immunogold labelling allowed identification of some viral glycoproteins (gB, gC and gD) projecting from the virion envelope (Stannard *et al.,* 1987). To date 12 HSV glycoproteins have been identified: eleven of these glycoproteins have been detected in the envelope, with gK possibly being the only exception.

The glycoproteins are involved in adsorption, penetration, membrane fusion, cell to cell spread, envelopment, viral egress from the cell and prevention of superinfection. They also appear to play a role in evasion of the host immune system. It is likely that the glycoproteins function in envelopment by specifically interacting with virus tegument proteins (Zhu and Courtney, 1994). In addition to the glycoproteins, the envelope contains cell lipids (Asher *et al.,* 1969).

Over half of the eighty plus proteins encoded by HSV-1 are thought to be present in the virion or involved in virion assembly.

1.2
The HSV-1 genome.

Herpesviruses have a large single dsDNA genome which varies in size, GC content and the arrangement of the major repeat regions (Becker *et al.,* 1968; McGeoch, 1989).

The sequence of the HSV-1 strain 17 genome, published in 1988, contains 152,260 residues in each strand (McGeoch *et al.,* 1985, 1986; Perry and McGeoch, 1988). However, the existence of variation in repeat copy number means that this number is not fixed. The genome of HSV-2 has now been completely sequenced (Dolan *et al.,* 1997), and is extremely similar to HSV-1 with a slightly higher GC content, at 68.3% in comparison to 67% (Keiff *et al.,* 1972; Davison and Wilkie, 1983; McGeoch *et al.,* 1988a).

HSV DNA consists of two covalently joined segments, the long (L) and short (S) representing 82% and 18% of the genome respectively i.e. 100% of the entire copy number. Each segment contains a unique region (U_L and U_S respectively) flanked by repeats in opposite orientation to each other, termed the internal (IR) and terminal (TR) inverted repeats; hence the internal repeat in the long region is the IR_L and the terminal repeat TR_L, while those in the short region are the IR_S and TR_S respectively (Sheldrick and Berthelot, 1974; Delius and Clements, 1976). At each of the termini there is a short (250-500 bp) direct repeat termed the *a* sequence. It is present in variable numbers at the long terminus with only one copy being found at the short terminus (Wilkie, 1976; Wagner and Summers, 1978; Roizman

1979; Davison and Wilkie, 1981). There is also at least one copy in an inverted orientation at the L-S junction. Both genomic termini display an overhanging residue leaving a 3' hydroxyl group free (Mocarski and Roizman, 1982). Figure 1.2.

U_S and U_L can invert relative to one another producing equimolar proportions of four isomers (Clements *et al.,* 1976; Wilkie and Cortini, 1976; Roizman, 1979). The four isomers are termed P (prototype), which has been assigned as the prototype to facilitate mapping (Roizman, 1979; McGeoch *et al.,* 1988a); I_L (inversion of the long segment); I_S (inversion of the short segment); and I_{SL} (inversion of both the long and short segments).

1.2.1
The HSV-1 *a* sequence.

The *a* sequence is highly conserved but contains variable numbers of repeat elements. This variation is seen both within (Wagner and Summers, 1978) and between strains (Locker and Frenkel, 1979). Tandem reiterations of the *a* sequence occur 1-5 times at the L-terminus, but only one copy exists at the S-terminus (Wilkie, 1976; Wagner and Summers, 1978).

The HSV-1 strain F *a* sequence has been analysed in depth and is composed of the following elements: a 20 bp direct repeat (DR1), a 65 bp unique sequence (Ub), between 19 and 23 copies of a 12 bp direct repeat (DR2), 2-3 copies of a 37 bp direct repeat (DR4) and a 58 bp unique sequence (Uc) followed by another DR1 (Mocarski and Roizman, 1981). Thus the *a* sequence can be represented as:

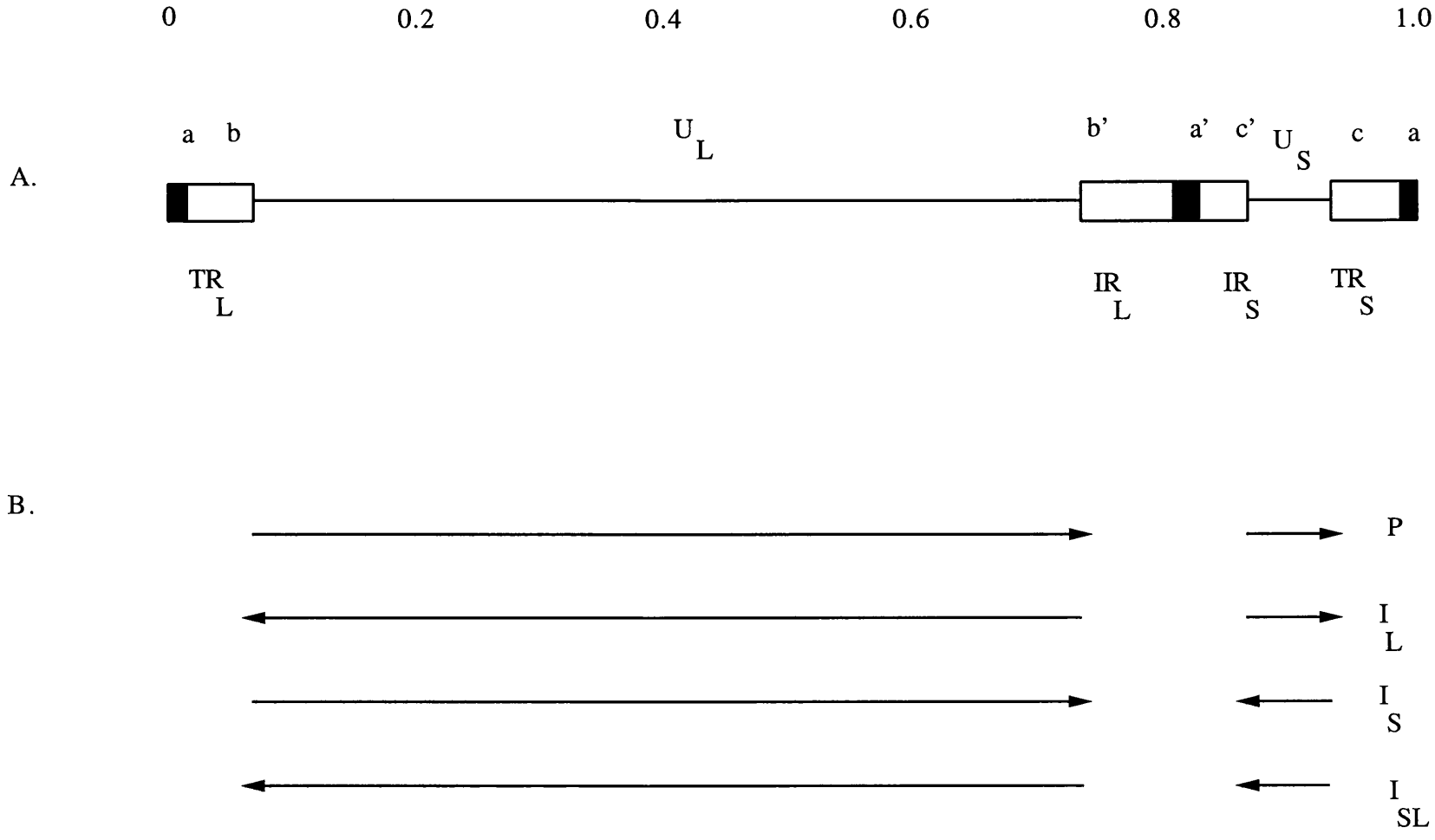
DR1-Ub-DR2n-DR4m-Uc-DR1

Adjacent *a* sequences share the intervening DR1 (Mocarski and Roizman, 1981; see section 1.2.2). The variation in size of the *a* sequence is partly determined by the varying numbers of copies of DR2 and DR4. A complete DR1 is formed on circularisation since the terminal *a* sequence of the L component has a truncated DR1 composed of 18 bp and a one nucleotide 3' overhang, while the *a* sequence of the S-terminal ends with a DR1 consisting of only one bp and one 3' overhang (Mocarski and Roizman, 1982).

It has been demonstrated that deletion of the Ub or Uc domains does not affect the ability of the *a* sequence to mediate genome inversion, whereas deletion of DR4 causes a dramatic reduction in inversion (Chou and Roizman, 1985). It has also been demonstrated that the a terminus is dispensable for isomerisation of the HSV-1 genome (Martin and Weber, 1996).

The *a* sequence mediates genome circularisation, site specific recombination and cleavage and packaging of HSV DNA (Davison and Wilkie, 1983a; Poffenberger and Roizman, 1985).

Figure 1.2: Structure of the HSV-1 genome.
A: the HSV-1 genome is shown, with unique sequences as solid lines (US and UL). The repeats TR_L, IR_L, IR_S and TR_S are illustrated as boxes with their component parts a, b, c and a', b', c' designated, b' and c' are identical to b and c, but in inverse orientation, a' and a are repeat elements in inverse orientation and variable in copy number.
B: below the genome representation, the isomerisation of the HSV-1 genome is illustrated. The four isomers are: P (prototype), I_L (L inverted with pespect to P), I_S (S inverted with respect to P) and I_{SL} (S and L inverted with respect to P).


0
0.2
0.4
0.6
0.8
1.0
A.
a b
U L
b' a' c'
U S
c a
TR L
IR L
IR S
TR S
B.
P
I L
I S
I SL

1.2.2
Cleavage and packaging of DNA.

Capsid assembly and packaging of the viral DNA occurs in the nucleus. Following replication of the HSV-1 genome, the concatameric DNA is cleaved into unit length virus genomes and packaged into pre-formed viral capsids by a tightly coupled mechanism involving amplification of *a* sequences (Deiss and Frenkel, 1986). This produces a packaged viral genome with a free S-terminus containing a DR1 consisting of a single bp and a 1 nt 3' extension, and an L-terminus consisting of between one and five copies of the *a* sequence ending in a DR1 containing 18 bp and a 1 nt 3' extension, so that an intact DR1 is formed when the ends join together.

Two highly conserved regions were identified in the Ub and Uc regions of the *a* sequence, *pac*-1 and *pac*-2 respectively, which appear to be essential for the cleavage/packaging process (Deiss *et al.,* 1986). They were discovered after Stow *et al.* (1983) noted that the ability of a plasmid containing S to be packaged by a helper virus required the presence of an *a* sequence in the plasmid.

Deiss *et al.* (1986) proposed a model for the packaging and cleavage process. The model proposes that a protein binds to an *a* sequence and in turn binds to a site on the capsid. The DNA is then extruded into the capsid until another *a* sequence in identical orientation comes to be packaged, whereupon one of the juxtaposed *a* sequences is cleaved. The cleaved *a* sequence is then repaired and duplicated. Once duplicated, cleavage occurs within the shared DR1 sites of the *a* sequences.

The capsid is enveloped by budding through patches of tegument proteins and immature glycoproteins on the inner nuclear membrane. Newly synthesised precursors of gB, gC and gD of HSV-1 are associated with calnexin, a membrane-bound chaperone in the endoplasmic reticulum (Yamashita *et al.,* 1996). The virion reaches the Golgi where the glycoproteins are glycosylated to their mature form (Darlington and Moss, 1969; Schwartz and Roizman, 1969; Johnson and Spear, 1982). It is unclear whether the envelope present on released virions is derived from the inner nuclear membrane or derived from the Golgi membrane, since two possible pathways of envelopment can be envisaged (Rixon, 1993). There is general agreement that the initial stage involves the capsid budding through the inner nuclear membrane. The differences of opinion are as to the route by which virions leave the perinuclear cisterna. The first pathway predicts that after budding through the inner membrane the envelope is lost by fusion with the outer membrane and that the final virion envelope is formed by budding through the Golgi membrane. Evidence supporting this pathway comes from the betaherpesviruses, where the tegument is a dense structure easily identifiable in electronmicrographs. Particularly clear pictures have been obtained for HHV-6

which suggest that capsids become enveloped by budding through the inner nuclear membrane and are then de-enveloped at the outer nuclear membrane before release into the tegusome. Fully tegumented capsids are then transported to the cytoplasm, where they become enveloped by budding into cytoplasmic vesicles.

In the second pathway, after gaining the envelope from the inner nuclear membrane, the nucleocapsid enters a vesicle derived from the outer nuclear membrane. The vesicle-enclosed virion fuses with the Golgi membrane thus releasing the virion into the Golgi and preserving the envelope derived from the inner nuclear membrane. Evidence for this pathway again comes from EM studies, and no conclusive evidence has been found to support either theory. It is possible that different herpesviruses use different pathways or even that a single herpesvirus may use either pathway in different cell types.

1.2.3
HSV glycoproteins.

Membrane glycoproteins are extremely important to the virus since they mediate its entry into the cell, cell-to-cell spread of infection, cell fusion, envelopment, egress, and immune evasion (section 1.4). They also play an important role in tissue tropism and host range. (See Table 1.2 and Fig 1.3).

HSV-1 encodes at least 12 glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM and gN). All, with the possible exception of gK, are incorporated into the virion envelope and Handler *et al.*(1996a) estimate that the total number of glycoprotein molecules in the envelope to be in excess of 100,000. Of these glycoproteins five, (gB, gH, gL, gM and gN) have been found in every member of the herpesvirus family analysed so far (Mettenleiter, 1994; Spear, 1993) (Table 1.3). The highest degree of conservation is found within the gB homologues, followed by glycoprotein homologues of gM and gH. Glycoproteins G, K and possibly E and I are only found in alphaherpesviruses (Spear, 1993a). Their precise functions have not all been defined, although five of them, gB, gD, gH, gK and gL, have been shown to have an essential role in virus infectivity (Stannard *et al.*, 1996). Glycoprotein C has an important role in binding heparan sulphate (HS) and complement. Glycoprotein K is essential for virus release, and syncytial mutations or mutations in cell to cell spread have been mapped to this gene. Within the virion and in infected cells gE and gI (Dubin *et al.*, 1990; Johnson *et al.*, 1988), as well as gH and gL (Hutchinson *et al.*, 1992; Roop *et al.*, 1993) are heterodimers, and gB (Claesson-Welsh and Spear, 1986) is homodimeric. In addition a proportion of gC can be cross-linked into a heterodimeric

Figure 1.3: Roles of glycoproteins.

1. Adsorption.
2. Penetration.
3. Fc binding receptors, C3b binding receptors.
4. Receptors for other ligands.
5. Envelopment.
6. Egress.
7. Release.
8. Cell fusion.
9. Direct cell-to-cell spread.

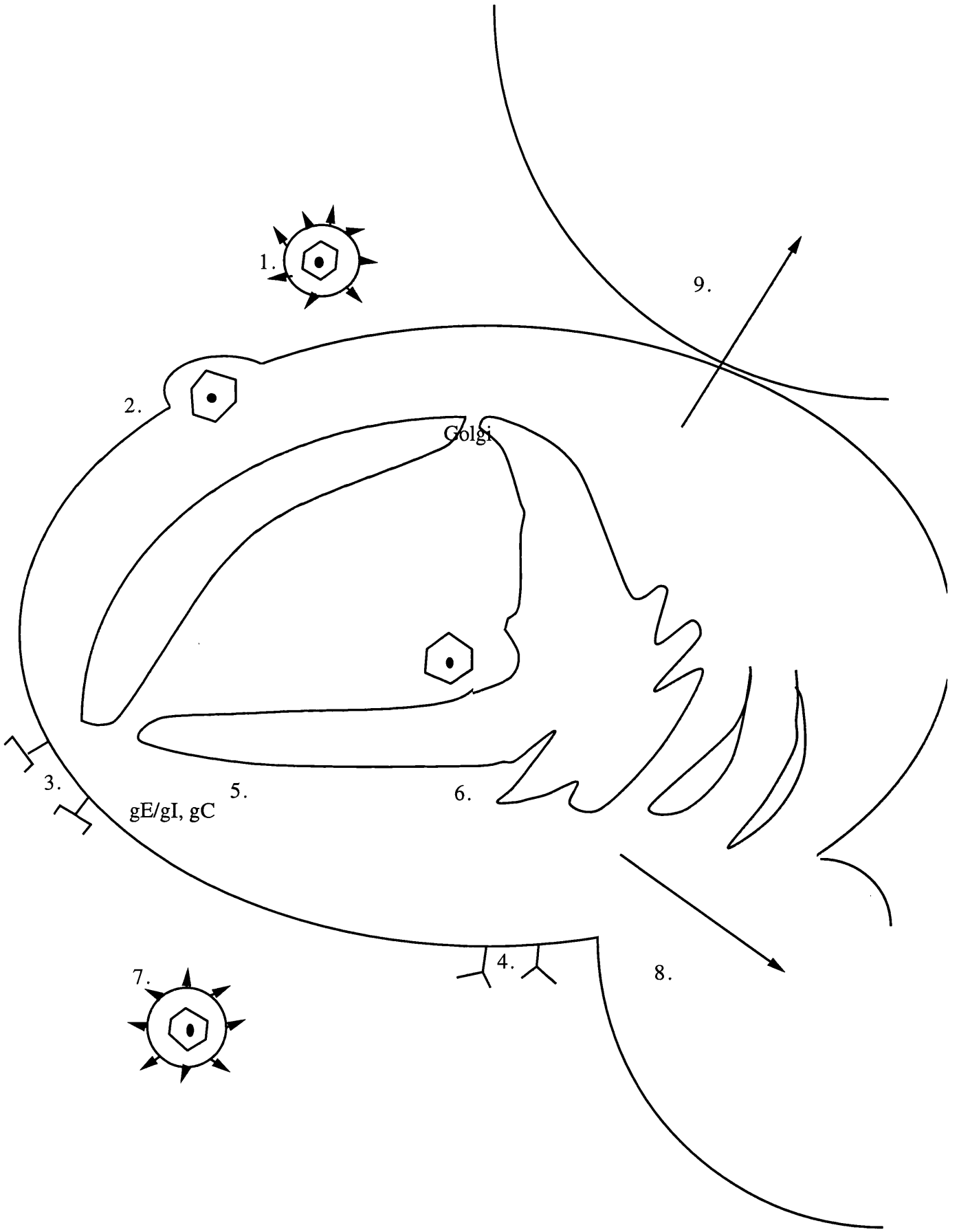

1.
2.
Golgi
3.
gE/gI, gC
5.
6.
4.
7.
8.
9.

Table 1.2: HSV-1 glycoproteins

Gene	Glycoprotein	Role	Requirement
UL1	Glycoprotein L	complexes with gH (UL22)	essential
UL10	Glycoprotein M	virus entry, viral spread	non-essential
UL22	Glycoprotein H	virus entry, complexes with gL (UL1)	essential
UL27	Glycoprotein B	virus entry	essential
UL44	Glycoprotein C	virus entry, binds complement	non-essential
UL49A	Glycoprotein N		non-essential
UL53	Glycoprotein K	*syn* locus, egress	essential
US4	Glycoprotein G		non-essential
US5	Proposed Glycoprotein J		non-essential
US6	Glycoprotein D	role in virus entry	essential
US7	Glycoprotein I	complexes with gE in Fc receptor,	non-essential
US8	Glycoprotein E		non-essential

Table 1.3: Glycoproteins of HSV-1 and homologous proteins in other members of the alphaherpesvirinae.

	HSV-1	PRV	VZV	SVV	MDV	EHV-1	BHV-1
US							
	gD	gD (gp50)	-	-	gD	gD	gD (gIV)
	gE	gE (gI)	gE (gpI)	gE	gE	gE	gE
	gG	gG (gX)	-	-	-	gG	gG
	gI	gI (gp63)	gI (gpIV)	gI	gI	gI	gI
	gJ	-	-	-	-	-	gJ (US5)
UL	gB	gB (gII)	gB (gpII)	?	gB (gpB)	gB (gp14)	gB (gI)
	gC	gC (gIII)	gC (gpV)	?	gC (gpA)	gC/	gC (gIII)
	gH	gH	gH (gpIII)	?	gH	gH	gH
	gK	?	gK	?	?	gL	?
	gL	gL	gL	?	?	gK	?
	gM	?	gM	?	?	gM	?
	gN	gN	gN	?	?	gN	gN

complexed form where gB is cross-linked to gC and gC to gD (Zhu and Courtney, 1988; Handler *et al*, 1996).

Glycoprotein B.

HSV-1 gB is a virion associated glycoprotein that has a role in virus adsorption, penetration, cell-to-cell spread and cell fusion (Navarro *et al.,* 1992); it is also a specific determinant of HSV-1 neuroinvasiveness (Yuhasz and Stevens, 1993).

One of the important features of gB is that it binds HS moieties on the cell surface. It has been shown that dextran sulphate can functionally substitute for cellular glycosaminoglycans to allow initiation of HSV infection and that this dextran sulphate stimulation of infection is primarily mediated by gB (Dyer *et al.,* 1997).

HSV-2 gB also interacts with cell surface glycosaminoglycans during virus attachment (Williams and Strauss, 1997).

Cell surface heparan sulphate proteoglycans also serve as receptors for the binding of HCMV gB (gp UL55), although HCMV can attach to the cell surface in the absence of heparan sulphate proteoglycans via an unidentified nonheparin component (Boyle and Compton, 1998).

In the case of PRV gB (gII), monoclonal antibodies inhibit virus penetration but do not affect adsorption. Glycoprotein C negative PRV infects target cells via a heparan sulphate independent pathway, again suggesting that PRV gB does not productively interact with heparan sulphate (Mettenleiter 1990; Klupp *et al.,* 1997), although it plays an essential role in viral penetration (Byrne *et al.,* 1995).

The conserved nature of gB and its gene in different herpesviruses suggests that different gBs should be readily interchangeable, gB of HSV-1, HSV-2, PRV (although not functionally) and BHV-1 all bind HS. However, complete functional equivalency and interchangeability of the gB gene from different viruses has only been demonstrated for HSV-1 and HSV-2 (Lin *et al.*, 1996). In the case of less related viruses such as PRV and BHV-1 or PRV and HSV-1, unidirectional complementation of the gB polypeptide occurs (Mettenleiter and Spear, 1994; Rauh *et al.*, 1991). Rauh *et al.*, (1991) produced a gB negative PRV mutant that grew normally on PRV gB complementing cells and also on BHV-1 gB expressing cells. The PRV mutants grown on cells expressing BHV-1 gB incorporated BHV-1 gB into their envelopes and became susceptible to neutralization by anti BHV-1 gB monoclonal antibodies. The PRV gB expressing cell lines did not complement a BHV gB mutant, indicating that BHV-1 gB can functionally replace PRV gB but not vice versa. Mettenleiter and Spear (1994), used a PRV gB expressing cell line and demonstrated that it complemented gB

negative HSV-1, however this complementation was again unidirectional since a HSV-1 expressing cell line did not complement gB negative PRV. The functional complementation of PRV gB for HSV-1 gB may be due to the significant homology between the glycoproteins at the amino acid level (Robbins *et al.,* 1987).

Glycoprotein B negative virus cannot enter cells but its infectivity is increased by polyethylene glycol (PEG) treatment which promotes cell fusion (Cai *et al.,* 1988).

HSV-1 gB is one of the four regions of the viral genome that is linked to syncytial mutation. Mutations affecting the cytoplasmic C-terminal domain of gB induce a syncitial phenotype (De Luca *et al.,* 1982; Bzik *et al.,* 1984; Cai *et al.,* 1988).

Glycoprotein C.

HSV-1 gC is not essential for virus production in cell culture, although it has several accessory functions including a role in viral attachment to the cell surface through HS moieties and the ability to bind the C3b component of complement (Padilla *et al.,* 1997; section 1.4.5).

While gC enhances viral entry, it is not required for syncytium formation and has actually been shown to inhibit the fusion of some cultured cells (Manservigi *et al.,* 1977, Novotny *et al.,* 1996). Many syncytial HSV mutants do not express gC, perhaps as a result of this inhibition (Spear 1993).

Purified gC inhibits complement activation. Glycoprotein C-negative mutants are rapidly neutralised in a process mediated by components of the classical component pathway. Glycoprotein C has been shown to mainly protect against antibody independent neutralization which indicates a role for gC early in infection before antibodies have developed (Friedman *et al.,* 1996).

Viruses deleted in PRV gC are impaired in adsorption (Schreurs *et al.,* 1988), penetration (Mettenleiter, 1989) and virus release from infected cells (Schreurs *et al.,* 1988). Mutants of PRV defective in gC are only slightly less virulent in mice and chickens than wild type virus, although mutants defective in both gC and gE are avirulent (Zsak *et al.;* 1992, Kritas *et al.,* 1994a and b).

BHV-1 gC has also been shown to be involved in virus entry (Liang *et al.,* 1991a and b).

Liang *et al..* (1991b) made a recombinant BHV-1 virus that expressed PRV gC in place of BHV-1 gC. This recombinant virus was shown to be as efficient in mediating virus attachment and penetration as the wild type virus. Thus it was concluded that PRV gC and BHV-1 gC share complementary functions.

Glycoprotein D.

Glycoprotein D is a viral envelope glycoprotein that has been studied extensively using immunological, biochemical and genetic approaches. It is essential for viral entry (Willis *et al.,* 1998) and glycoprotein D negative mutants have been shown to be severely compromised in neuronal spread (Dingwell *et al.,* 1995).

Glycoprotein D mediates the stable binding that follows the action of gB and gC by binding gD receptors, (discussed in section 1.4.2) that are limited in number.

Glycoprotein D monoclonal antibodies can neutralise HSV infectivity by blocking penetration of the virus at the cell surface (Fuller and Spear, 1987), due to a block in fusion between the virion envelope and plasma membrane. Neutralised virus binds to cells and accumulates on the surface with reversal of the effect of neutralising antibodies again being achieved by treatment with PEG. Cells expressing HSV gD are resistant to HSV entry as a result of gD mediated interference. HSV strains differ in their sensitivity to this interference, which blocks penetration but not binding. Mutations in gC and gK can confer partial resistance to this gD-mediated interference (Pertel and Spear, 1997).

It is proposed that the role of gD in cell to cell spread may be partially associated with its ability to bind mannose-6-phosphate receptors (MPRs) (Brunetti *et al.*, 1998). MPRs are involved in a well characterised pathway through which lysosomal enzymes are directed to lysosomes via a network of endosomal membranes. HSV can form plaques under conditions prohibiting interaction with MPRs (e.g. using blocking antibodies, ligands and soluble MPR) and thus it was proposed that this interaction functions during egress or cell to cell spread (Brunetti *et al.*, 1995). In their later paper Brunetti *et al.* (1998) report that the virus can be directed to endosomes by both mannose-6-phosphate-dependent and independent mechanisms.

BHV-1 gD is identical to HSV-1 gD in that it is involved in virus entry and direct cell to cell spread. The PRV homologue (gp50) is an envelope glycoprotein, essential for penetration but not for direct cell to cell spread (Peeters *et al.,* 1993; Schroder *et al.,* 1997).

Glycoprotein E.

This glycoprotein was shown to be non-essential by Neidhardt *et al.* (1987). However, gE negative HSV-1, PRV and BHV-1 are less efficient at establishing latency and are therefore less likely to be reactivated than wild type virus. Glycoprotein E is an Fc binding glycoprotein (Bauke and Spear, 1979) and forms a noncovalently linked heterodimer with gI (see section 1.4.4). Glycoprotein E alone acts as a lower affinity IgG receptor binding IgG aggregates but not IgG monomers, while the gE:gI complex acts as a higher affinity receptor,

binding both IgG monomers and aggregates (Dubin *et al.*, 1990). Fc binding activity may prevent complement mediated lysis of infected cells and enveloped virions and may also protect against Fc-facilitated phagocytosis (Bell *et al.*, 1990). The gE:gI complex also facilitates neuron to neuron spread of HSV-1 and its cell to cell spread (Dingwell *et al.* 1995). Chaterjee *et al.* (1989) implicated gE in the induction of cell fusion.

BHV-1 gE has a central role in BHV-1 spread, acting through cell-to-cell spread which is a major mechanism of BHV-1 *in vivo* virulence (Rebordosa *et al.* 1996).

PRV gE and gI are important in virulence and viral spread, and show Fc receptor binding ability for swine IgG but not that of other species (Zuckermann *et al.*, 1988) They form a hetero-oligomer that facilitates the maturation and intracellular transport of both proteins to the plasma membrane of cells, although both gE and gI can reach the cell surface independent of each other's expression (Whealy *et al.*, 1993). PRV gE (gI) plays a role in virus release from infected cells (Mettenleiter *et al.*, 1987). Mutants defective in gE are only slightly less virulent in mice and chickens than wild type virus, while mutants lacking both gE and gC are avirulent. Glycoprotein E of PRV is an accessory protein that promotes cell fusion. Glycoprotein E negative mutants of PRV are deficient in their ability to form syncytia, forming only small plaques on some cell types, these mutants spread mainly by adsorption of released virus to uninfected cells (Zsak *et al.*, 1992). Glycoprotein C mutants are reported to spread mainly by means of direct cell to cell spread, therefore a combination of both mutations results in a defect of adsorption of released virus as well as direct cell to cell spread.

Homologues of gE have been found in all members of the alphaherpesvirinae, indicating an important and conserved role in the biology of the alphaherpesviruses. The homologous proteins appear to have little amino acid identity, but HSV-1, PRV, BHV-1, EHV-1, SVV and VZV share two clusters of closely spaced cysteine residues which are thought to play an important part in the function of gE (Jacobs, 1994).

Glycoprotein G.

HSV-1 gG is the product of the US4 gene (Richman *et al.*, 1986) and has the predicted structure of a class 1 membrane protein. Mutants lacking gG are viable in culture (Weber *et al.*, 1987; Longnecker *et al.*, 1987) and its function is unknown. Balan *et al.* (1994) reported that gG-negative mutants do not have altered particle/infectivity ratios, nor do they show defects in growth or spread in BHK cells.

Glycoprotein H.

Glycoprotein H forms a complex with gL that effects the folding and surface expression of gH. If gH is expressed in the absence of other viral proteins it is antigenically abnormal, is not processed and not expressed at the cell surface. However, when gH is expressed with gL it is antigenically normal, processed normally, and transported to the cell surface. Similar observations are made in the case of gL. This indicates that it is the hetero-oligomer of gH and gL that is incorporated into virions and transported to the cell surface. A 1:1 ratio exists between gH and gL in infected cells, and probably also virions (Hutchinson *et al.*, 1992).

Glycoprotein H is required for membrane fusion but is dispensable for receptor binding. It is conserved in all sequenced herpesviruses and adsorption of gH negative virions to cells blocks superinfection (Forrester *et al.*, 1992). Monoclonal antibodies for gH of HSV-1 can block viral penetration without inhibiting the adsorption of virus to cells (Fuller *et al.*, 1989).

Glycoprotein H may have a role in viral egress from cells, since Buckmaster *et al.* (1984), showed that an anti-gH antibody inhibited plaque formation when added in the overlay following virus adsorption. Mutant viruses with altered gH are retained within cells (Desai *et al.*, 1982): targeting of gH to the RER results in the release of non-infectious virions from infected cells, while infectious virions containing gH are retained in the cytoplasm (Jaychandra *et al.*, 1997).

Glycoprotein H may also be involved in cell fusion, as a gB syn mutant with a mutation in gH does not produce a syncytial phenotype. Glycoprotein H negative virions bind cells while being unable to enter them unless PEG treatment is used (Forrester *et al.* 1992).

Glycoprotein H of PRV is a structural component of the virion and also complexes with gL. PRV mutants lacking gH are not defective in primary or secondary attachment but are not infectious as PRV gH is essential for penetration and propagation of virus in cell culture and in the nervous system of mice (Babic *et al.*, 1996).

Glycoprotein I.

Glycoproteins E and I form a heterodimer which acts as an Fc receptor and also facilitates cell-to-cell spread of virus. Glycoprotein I not required for the infection of cells by extranuclear virus (Dingwell *et al.*, 1995). See glycoprotein E and section 1.4.4.

The fact that gE and gI form a complex makes it difficult to estimate the contribution of either glycoprotein to the complex's biological function as deletion of either component leads to inactivation of the complex.

Glycoprotein J.

No function has yet been ascribed to the putative gJ. The US5 ORF was identified by McGeoch *et al.* (1985) who observed that the predicted translation product contained a N-terminal signal peptide, a potential transmembrane anchor and a single N-glycosylation site. The gene product was named gJ (Roizman and Batterson, 1985), but has not yet been identified. Disruption of the US5 gene did not significantly alter virulence *in vivo* (Weber at al., 1987). Balan *et al.* (1994), showed that disruption of US5 had no effect on particle to p.f.u ratios, or on the growth of the virus in BHK cells and no effect on viral pathogenicity in the mouse ear model.

Glycoprotein K.

Unlike other glycoproteins, gK is apparently not a component of the viral envelope.

The HSV-1 glycoprotein gK (Hutchinson *et al.,* 1992b) is the product of the UL53 gene (Debroy *et al.,* 1985, McGeoch *et al.,* 1988a). It is not absolutely essential for the production of infectious virus in actively replicating cells but is needed for efficient envelopment and transport of infectious virions to the extranuclear space. This glycoprotein is also involved in egress of HSV (Hutchinson *et al.,* 1995b). Glycoprotein K is thought to play an important role in regulating membrane fusion and syncitial mutations arise more frequently in UL53 than any other HSV gene (Ruyechan *et al.,* 1979; Bond and Person, 1984; Pogue-Geile and Spear, 1987; Jaychandra *et al.,* 1997). In contrast to all other HSV glycoproteins described to date, gK accumulates in the perinuclear and nuclear membranes of cells; the other glycoproteins are transported to the surfaces of infected cells (Hutchinson *et al.,* 1995a).

Unlike HSV-1, PRV gK is a structural component of the virion. It is involved in virus release but is not required for virus entry although deletion of gK results in a significant delay in penetration that can be rescued by phenotypic complementation on gK expressing cells (Klupp *et al.*, 1998). PRV gK is required for productive virus replication, and has an important role in egress thought to be the prevention of immediate re-entry of released infectious virions.

Glycoprotein L.

Glycoproteins H and L are dependent on each other for proper processing and expression (Hutchinson *et al.,* 1992a,). Glycoprotein L lacks a transmembrane domain but stably associates with membranes through oligomerisation with gH (Novotny *et al.,* 1996).

Like HSV, the gH:gL complex has a role in penetration of BHV-1 but not its attachment (Littel van den Hurk *et al.,* 1996).

Glycoprotein M.

HSV-1 gM differs from all other cell-associated HSV glycoproteins studied to date in that it is highly hydrophobic (Baines and Roizman, 1993), having eight potential transmembrane regions (McGeoch *et al.*, 1986; MacLean *et al.*, 1993;). There are also two potential N-linked glycosylation sites present. Mutations in the gene have no clear phenotype as yet.

PRV UL10 contains eight hydrophobic regions and a N-glycosylation site. PRV UL10 is not essential for productive replication of PRV in cell culture.

The gM genes of HSV-1 and PRV are not essential for replication *in vitro* or in vivo (Baines and Roizman, 1991, Dijkstra *et al.*, 1996, MacLean *et al.*, 1991, 1993). MacLean *et al.,* (1991) report that the HSV-1 UL10 product is a glycosylated membrane protein required for optimal virus growth *in vitro* and in vivo. PRV mutants grow to lower titres, demonstrate a lag in penetration of cell compared to the wild type (Dijkstra *et al.* 1996) HSV-1 and PRV mutants are impaired for peripheral growth and spread within and/or to the nervous system in mice (Dijkstra *et al.*, 1996, MacLean *et al.,* 1993) They are defective for cell to cell fusion in combination with a gB syn mutant (Davies Poynter *et al.*, 1994), suggesting that gM may play a role in membrane penetration.

In HSV-1 and PRV the deletion of gM had little effect on phenotype, and thus the conservation of gM homologues throughout the herpesviruses is puzzling. Glycoprotein M demonstrates a degree of conservation similar to that for the essential gH and gK homologues (Dijkstra *et al.*, 1996).

Wu *et al.* (1998), reported that BHV-1 gM forms a disulphide-linked heterodimer with gN. They also suggest that since all known gM and gN homologues have similar patterns of hydrophobic domains and cysteines the two proteins may be linked by disulphide bonds in all herpesviruses. Since both gM and gN are conserved in the herpesviruses it may be that this association is a functional complex.

Glycoprotein N.

Glycoprotein N is the product of UL49A, and a γ2 transcript has been mapped to this genomic region (Hall *et al.,* 1982, Barnett *et al.*, 1992). Homologues of HSV-1 UL49.5 have been found in all herpesvirus genomes analysed so far (Jons *et al.,* 1996).

HSV-1 UL49A encodes a small virion protein that is linked by a disulphide bond to a tegument protein with no evidence of glycosylation (Adams *et al.*, 1998).
Glycoprotein N of PRV has homology to that of HSV-1. It is an O-glycosylated structural component of the viral envelope.
Like HSV-1, BHV gN is apparently unglycosylated and is an envelope protein (Liang *et al.*, 1996).

1.3
HSV latency

HSV has evolved a complex interaction with the peripheral nervous system (PNS) whereby it can survive throughout the life of the infected individual in a latent state and thus evade the immune response of the host. Following primary infection and active replication at peripheral sites, virus attaches to the sensory nerve terminals (Vahlne *et al.*, 1978), enters them and travels centripetally via axons to neuronal cell bodies within the sensory ganglia (Cook and Stevens, 1973; McLennan and Derby, 1980). The viral genome is then uncoated, released into the neuronal nucleus and converted into a non-linear form (Rock and Fraser, 1983, 1985; Efstathiou *et al.*, 1986). In the case of HSV-1 these are mainly trigeminal ganglia (Bastian *et al.*, 1972; Baringer and Swoveland, 1973; Efstathiou *et al.*, 1986) and in the case of HSV-2 dorsal root ganglia (DRG) (Baringer, 1974; Galloway *et al.*, 1979,1982). Latent virus has also been recovered from cells of peripheral tissues e.g. human corneas (Shimeld *et al.*, 1982; Cook, 1988) and experimentally infected rabbits (Cook *et al*, 1987) and mice (Openshaw, 1983) as well as the footpads of experimentally infected mice (Al-Saadi et al., 1983). Latency in peripheral tissues is thought to be a secondary sequelae to primary HSV infection (Hill, 1985) and its biological relevance is unknown.

1.3.1
Animal models of latency.

Several animal models have been developed to study HSV latency. The most commonly used is the mouse (Stevens and Cook, 1971; Walz *et al.*, 1974; Al-Saadi *et al.*, 1983; Clements and Subak-Sharpe, 1983, 1988). Other systems used include rabbit (Nesburn, 1967, 1977), guinea pig (Scriba, 1975, 1976) and monkey (Reeves *et al.*, 1976, 1981). Various peripheral injection sites are employed: footpad (Stevens and Cook, 1971; Scriba, 1975), eye (Nesburn *et al.*, 1967), ear pinnae (Hill *et al.*, 1975, 1978, 1980), flank (Underwood and Weed, 1974; Whitby *et al.*, 1987) and vagina (Scriba, 1976). Whilst none

of these models can fully mimic the pathogenesis of HSV infection in humans with regard to inoculation site, input dose, reactivation triggers or immune response, they have provided valuable information on the three basic components of latency - establishment, maintenance and reactivation. Unfortunately, none of the above models provide the ideal system to study all three components in a single host.

The first demonstration that HSV could establish a latent infection in sensory ganglia followed footpad inoculation of mice and the subsequent explantation of DRG that released infectious virus after a period in tissue culture (Stevens and Cook, 1971). This fundamental study provided the basic model of *in vitro* reactivation as a measure of latent infection. As a consequence of this study it was later demonstrated through explantation of human sensory ganglia (post mortem dissection) that HSV resides in a latent state here (Warren *et al.,* 1978). Extensive use has been made of the footpad model (Stevens and Cook, 1971; Walz *et al.,* 1974; Al-Saadi *et al.,* 1983; Clements and Subak-Sharpe, 1983, 1988) and it has allowed the demonstration that HSV can also establish a latent infection within cell populations of the footpad skin (Al-Saadi *et al.,* 1983, 1988) . Infection via the footpad does not provide a system to study reactivation *in vivo* as recurrences are neither spontaneous nor inducible (Hill, 1985).

Another widely used model consists of peripheral inoculation of the eye in both mice (Knotts *et al.,* 1974) and rabbits (Nesburn *et al.,* 1967; Stevens *et al.,* 1972). In the mouse eye model spontaneous or induced reactivation is not reproducible (Harbour *et al.,* 1983; Shimeld *et al.,* 1990) and thus only factors affecting establishment and maintenance *in vivo* can be examined. However in the rabbit, reactivation can be induced at will by iontophoresis of adrenaline into the eye (Nesburn *et al.,* 1977). However, frequent spontaneous recurrent shedding also takes place (Nesburn *et al.,* 1977) and this makes interpretation of reactivating factors difficult.

Another mouse model of HSV latency (Sawtell and Thompson, 1992a) is based on the strong correlation between fever and HSV reactivation in humans (Boak *et al.,* 1934; Roizman and Sears 1990). Latently infected mice are immersed in hot water (42°C) to induce mild hyperthermia, with the rate of change in core temperature being controlled by the depth of immersion of the animal. Using this method virus can be isolated from the trigeminal ganglia of mice within 14 hours post induction: the approximate time required for one round of viral replication in culture. It is not clear if viral shedding occurs at the peripheral inoculation site.

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

While these *in vivo* models have produced valuable information regarding the pathogenesis of HSV infection and the role of viral factors in latency, their use in the investigation of molecular mechanisms controlling latency is limited due to the complexity of the contribution of host factors and immune system to latency, the expense of the models and the fact that only a small proportion of neurons are simultaneously reactivated. *In vitro* latency systems are required in order to help characterise establishment, maintenance and reactivation at the molecular level.

1.3.2
In vitro models.

In the laboratory, latency is defined as the ability to isolate infectious virus from explant cultures (of sensory ganglia) after a period of time in culture, where virus has not been isolated from the explant tissue homogenate immediately post explantation.

In vitro systems can be divided into three types, 1) those using primary cultures from the ganglia of rodents, 2) cell-lines of neuronal origin, 3) non-neuronal tissue culture cells.

Such systems should show the following characteristics: 1) viral genomes should persist in cell nuclei in the absence of infectious virus; 2) viral genomes should be non-linear, as they are *in vivo*; 3) transcription of the genome should be repressed or limited to the LAT region; and 4) latent virus should be capable of reactivation producing infectious virus.

Perhaps the most significant *in vitro* system using primary neuronal cultures involved the treatment of cervical ganglia from neonatal rats with collagenase, followed by dissociation onto a growth surface and treatment with fluorodeoxyuridine (Wilcox and Johnson, 1987).

A system developed by Wigdahl *et al.,* (1983), used isolated rat foetal dorsal root ganglia. C1300 mouse neuroblastoma cells (Nilheden *et al.,* 1985) and HFL cells (O'Neill *et al.,* 1972; Russell and Preston, 1986) are also used as *in vitro* model systems.

1.4
Role of HSV glycoproteins in the HSV lytic cycle

1.4.1
Adsorption

The initial interaction between HSV and cells is the adsorption of the virus to the cell surface and the subsequent fusion of the virion envelope with the plasma membrane, either at the cell surface or in endocytic vesicles (Fan and Sefton, 1978; Marsh, 1984). Early electron

microscopy (EM) studies suggested that both mechanisms of viral entry are used (Morgan *et al.*; 1968; Dales and Silverberg, 1969). However it has since been shown that compounds known to inhibit the endocytic process do not inhibit entry of HSV (Wittels and Spear, 1991). It has also been shown that virus internalised in an endocytic vesicle does not establish a lytic infection in the host cell, most probably because it is degraded in the vesicle (Campadelli-Fiume *et al.*, 1988).

Current opinion favours entry by direct fusion of the virion envelope with the cell membrane, resulting in the release of nucleocapsids into the cytoplasm (Morgan *et al.*, 1968). Immunological data supporting this theory showed that gH neutralising monoclonal antibodies inhibited virus entry but not attachment (Fuller and Spear 1987, Fuller *et al.*, 1989). Virus infectivity was restored when the neutralised virus bound to cells was treated with PEG, a membrane fusing compound. Other evidence supporting the fusion model comes from the work of Para *et al.* (1980) who showed that the virion glycoprotein gE was transferred from the virion envelope to the cell membrane in infected cells prior to DNA synthesis.

HSV specifies at least twelve glycoproteins, gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM and gN (Spear 1976; Marsden *et al.*, 1978; 1984; Baucke and Spear 1979; Buckmaster *et al.*, 1984; Roizman *et al.*, 1984; Spear 1985; Frame *et al.*, 1986; Gompels and Minson, 1986, Longnecker *et al.*, 1987; Johnson and Feenstra 1987; Hutchinson *et al.*, 1992a, b; Ranaswany and Holland, 1992). At least five viral glycoproteins, gB, gD, gH, gC and gL (Fuller *et al.*, 1987; Ligas and Johnson, 1988; Fuller *et al.*, 1989; Hutchinson *et al.*, 1992a, 1992b; Roop *et al.*, 1993) are involved in virus entry into cultured cells. Some glycoproteins cross-link into homodimeric and hetero-oligomeric forms; hetero-oligomers of gB-gC, gC-gD, gD-gB, gH-gL, gC-gL and gD-gL have been found in purified virions (Handler, 1996), the best known HSV glycoprotein htero oligomer is the gE-gI interaction.

It is likely that all of the HSV glycoproteins except gK are present in the virion envelope, although direct evidence is lacking for gL, and gJ. Other proteins which are not glycosylated may also be present in the envelope e.g. the UL34 product which is a membrane-associated phosphoprotein (Purves *et al.*, 1991; 1992) and the UL20 and UL34 gene products, which contain multiple hydrophobic domains (Baines *et al.*, 1991; MacLean *et al.*, 1991).

Immune EM analysis of negatively stained virus preparations revealed that gB, gC and gD each form distinct morphological structures projecting from the virion envelope (Stannard *et al.*, 1987). Although the organisation of the envelope proteins is unknown, their structure and location suggest that they may play important roles in both adsorption and entry of HSV-1 into the host cell.

Heparan sulphate (HS) 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). Observations that virions bind to heparin, a related glycosaminoglycan, and that heparin blocks virus adsorption support this conclusion. Sawitzky *et al.,* 1990 compared the effect of heparin on plaque formation and adsorption of PRV (strain Ka) to the effect on HSV-1 strain HFEM. While demonstrating that both viruses were affected by the addition of heparin they showed that PRV was significantly less sensitive to this treatment than HSV-1 strain HFEM. Enzymatic removal of heparan sulphate from the cell surface significantly reduces the binding of virus to cells and renders them partially 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 all glycosaminoglycans, or defective in sulphation of heparan sulphate (Shieh *et al.,* 1992). Virus adsorbed to cells at 4°C can be inactivated by brief exposure to low pH or soluble heparin, suggesting that HSV-1 binds to HS with low affinity (Huang and Wagner, 1969). In addition, virus bound to the low affinity receptor can be neutralised by HSV-1 gD specific monoclonal antibodies (Highlander *et al.,* 1987).

Glycoprotein C, a non essential protein for virion infectivity, is partly responsible for the interaction of virions with heparan sulphate. It is a viral structural glycoprotein with at least two regions that form a functional domain involved in binding to heparan sulphate (Trybala *et al.,* 1994). The conclusion that gC binds to HS was based on the following findings; gC binds to heparin-sepharose under physiological conditions in affinity chromatography experiments; Herold *et al.* (1991) showed that HSV-1 mutants lacking gC exhibit significant impairment in adsorption and penetration; 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). Plaque-purified gC-negative syncytial mutants are more resistant than HSV-1 (KOS) to heparin inhibition (Pertel and Spear, 1996) due to the fact that syn mutants infect cells via cell to cell spread and thus avoid the need for cell surface attachment. Glycoprotein III, the gC homologue of PRV is also thought to be important for the adsorption of virus to cells and, like HSV-1 gC, is not absolutely essential for virus infectivity (Robbins *et al.,* 1986; Schreurs *et al.,* 1988). The idea that gC makes the initial contact with the cell surface is supported by the work of Stannard *et al.* (1987) who showed that gC formed long slender structures (up to 24 nm in length) projecting from the virion envelope. It has also been suggested that HSV-1 strains exhibit a heparin-resistant phase of attachment, which is determined by gC. Lack of gC delays this heparin resistant attachment phase (Seck *et al.,* 1996).

Neomycin and polylysine aminoglycoside antibiotics have been shown to inhibit the binding of HSV-1 to cells to a much greater extent than HSV-2 (Langeland *et al.,* 1987;1988). Work by Oyan *et al.,* (1993) showed that the region on the HSV genome responsible for the resistance to polycationic drugs maps to the amino-terminal of gC. Since gC of both HSV-1 and HSV-2 bind to heparin, it is possible that the two serotypes may interact differently with cell surface HS, either having different affinities for, or binding to separate epitopes on the HS molecule. This hypothesis is supported by the results of Gerber *et al.,* (1995) and Herold *et al.* (1995, 1996) who suggest that the binding of gC-2 to HS may be more similar to that of gB-1 than gC-1. They also suggest that the different efficiency of binding observed by HSV-1 and HSV-2 to different cell types is due to binding to different features of HS. Carboxyl reduced and 2-3, -O-desulphated heparin selectively inhibits the binding of gC positive viruses (wild type or gB negative) to cells, but has little or no effect on binding of a gC negative virus. O-desulphated heparin compounds also had little or no effect on HSV-1 but inhibited HSV-2 infection via an interaction with gC-2.

An alternative explanation is that, in the presence of drugs which bind to HS, HSV-2 can interact with another cellular component via gC or another viral glycoprotein. The idea that HSV-1 and HSV-2 interact differently with cell surface HS was suggested by WuDunn and Spear (1989) who showed that HSV-1 bound to soluble heparin with much higher affinity than HSV-2.

Although gC is involved in adsorption of virus to cells it is not the only glycoprotein which can mediate this process. Kuhn *et al.,* (1990) showed that gB, gC and gD formed complexes with cell membrane components during the adsorption process, confirming the results obtained by earlier workers who suggested roles for these glycoproteins in viral attachment (Fuller and Spear, 1985; Johnson *et al.,* 1984). HSV mutants that lack gC, gB or gD can still attach to cells, indicating that the function of a missing glycoprotein can be replaced by others; this is not strictly true in the case of gD since productive infection will not occur in the absence of gD or gB. Evidence has been obtained suggesting that the attachment of gC negative virus to the cell surface HS is mediated by gB, and that this mechanism is resistant to neomycin and polylysine (Herold *et al.,* 1991). However, gB negative virions adsorb to cells as efficiently as *wt* virus (Cai *et al.*, 1988; Herold *et al.,* 1991). Whether the cell component described by Kuhn *et al.,* (1990) that binds to gB is HS or another cellular component is not known. Tufaro *et al.,* 1993 showed that cells lacking HS were susceptible to *wt* virus infection; later work (Dyer *et al.,* 1997) showed that HSV-1 gB mediated an interaction with dextran sulphate a glycosaminoglycan analogue.

Following the low affinity attachment of HSV to cell surface HS, tighter binding, presumably via a high affinity receptor occurs, followed by penetration and internalisation; after which

virus is resistant to acid treatment and neutralisation by monoclonal antibodies. Fuller and Lee (1992) speculated that the stable attachment of virus to the cell surface was necessary for fusion of the virion envelope with the cell membrane. Kohn (1979) proposed that high affinity binding involved interactions between a number of viral and cellular components. Evidence supporting mutivalent attachment of virus to cells comes from Rosenthal (1984) who demonstrated that HSV-1 binding to cells rapidly reduced cell surface protein mobility, similar to that caused by the multivalent attachment of other ligands such as antibodies or other cells. The hypothesis that more than one type of HSV attachment can occur is consistent with the ability of herpesviruses to bind to a wide range of cell types from diverse species.

Kaner *et al.,* (1990) suggested that fibroblast growth factor receptor (bFGFR) was a portal of entry for HSV-1 as they found that Chinese Hamster Ovary (CHO) cells expressing a transfected receptor for bFGF bound and internalised greater amounts of radiolabelled HSV than did the bFGF deficient parental cell lines or control cells. Binding was inhibited by bFGF. The drawback with this study was that only one cell line - bovine arterial smooth muscle - was used and several groups (Shieh and Spear, 1991; Muggeridge *et al.,* 1992) have since shown that inhibition by bFGF is restricted to a minority of cell types. Muggeridge *et al.,* (1992) tested several cell lines, but only one showed comparable inhibition by bFGF. Furthermore, they could not find a correlation between the amount of bFGF receptor and the ability of bFGF to block infection, as cell lines expressing low levels of bFGF receptor were permissive for HSV. FGF receptor positive and FGF receptor negative rat myoblasts do not differ in susceptibility to HSV infection (Mirda *et al.,* 1992) and soluble forms of FGF fail to inhibit HSV infection whereas they inhibit basic FGF binding and biological activity. Taken together these results suggest that different cell types harbour different receptors for HSV.

Cell lines expressing gB or gC are not resistant to HSV infection and there is no evidence to date for saturable cell receptors for these viral proteins.

1.4.2
Penetration

Cell fusion is the next stage in virus entry and follows the stable attachment of virus to the cell surface. Studies with *ts* mutants, monoclonal antibody resistant mutants, syncytial mutants and null mutants have suggested that gB functions directly in virus entry and virus induced cell fusion (Sarmiento *et al.,* 1979; Bzik *et al.,* 1984b; Cai *et al.,* 1988; Highlander *et al.,* 1988).

While both gD and gB have been implicated in virion induced cell fusion, it remains to be seen whether the attachment of these proteins to cellular membrane components is a prerequisite to their role in viral penetration. It is possible that gB and gD may interact with other viral components prior to the initiation of cell fusion.

Using biochemical and electron microscopy approaches, the entry process of HSV-1 was examined in detail by Fuller and Lee (1992) who observed discernible changes of the virion envelope and tegument after contact of infectious virus with the cell plasma membrane. Virions rendered non-infectious by anti-gD or anti-gH neutralising antibodies bound to cells but failed to form a visible fusion bridge. On the basis of these investigations, together with earlier findings, a model of HSV entry into cells was proposed, and this is illustrated in Figure 1.4.

According to the proposed model, virion attachment to cell surface HS is mediated by gC and to a lesser extent gB, after which, a more stable attachment of the virus to the cell occurs. The viral glycoprotein thought to be involved in the stable attachment of virus to cells is gD, since bound virions lacking gD are more sensitive to removal by heparin or high ionic strength washes than *wt* virus adsorbed to cells. Moreover, soluble gD can reduce virus attachment when added to cells prior to or during virus adsorption (Fuller and Lee, 1992). The stable attachment of the virion is believed to prime the virus for fusion with the cell membrane, on the basis that gD-minus and gD-neutralised viruses bind to cells but are unable to penetrate the cell membrane (Fuller and Spear, 1987; Highlander *et al.,* 1987; Ligas and Johnson, 1988). The model proposes that gD has two functions in viral entry. Firstly, gD interacts with a cellular receptor, bringing virus and cell closer together. Secondly, gD is believed to interact with another viral component that is directly involved in the fusion initiation event. The idea that gD is bifunctional comes from the observation that antibodies specific to gD, which prevent attachment, do not bind in the same regions as those blocking penetration (Eisenberg *et al.,* 1985). Furthermore, the finding that cell surface gD interferes with superinfection, possibly by interacting with gD or another viral glycoprotein on the virion, is consistent with the hypothesis that gD interaction with another virion component triggers the fusion initiation event. However, it can also be argued that gD mediated inhibition of superinfection results from the sequestering of a cellular receptor by cell surface gD. This idea appears more likely and is supported by the work of Johnson *et al.* (1990) who showed that gD bound to a 170 kDa cellular membrane protein, the mannose 6-phosphate receptor, that is much less abundant than cell surface HS.

The first cellular mediator of HSV entry to be fully determined was herpes virus entry mediator (HVEM), a member of the tumour necrosis factor receptor family, later designated HveA (hepesvirus entry mediator A) (Montgomery *et al.*, 1996; Whitbeck *et al.*, 1997;

Figure 1.4: Model of HSV-1 cell-entry as proposed by Fuller and Lee (1992).

A. Initial attachment of virus to the cell surface.

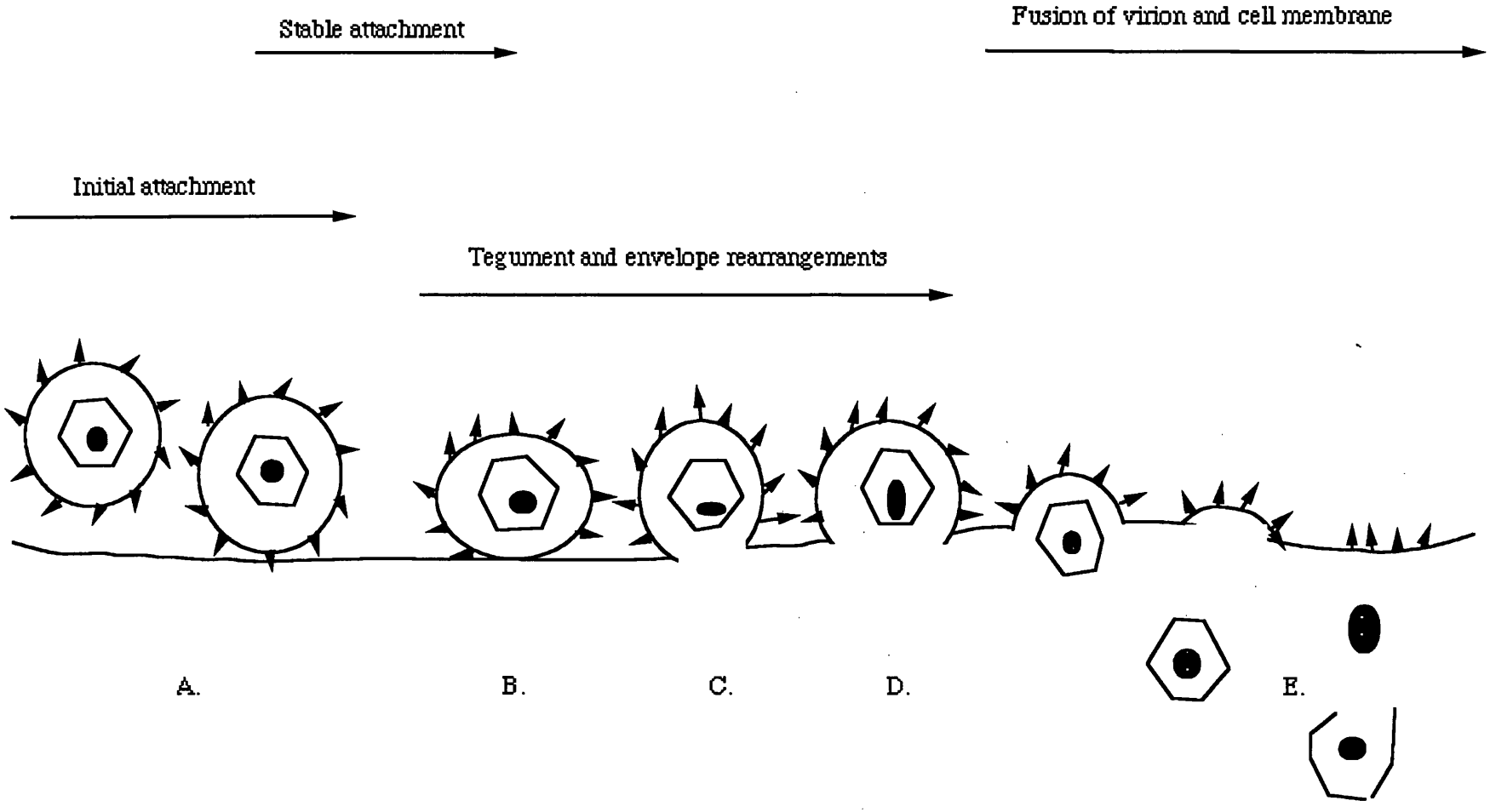
B. Stable attachment.

C. Initiation of fusion bridge.

D. Expansion of fusion bridge.

E. Release of nucleocapsid into the cytoplasm.

Adapted from Fuller and Lee (1992).


Stable attachment
Fusion of virion and cell membrane
Initial attachment
Tegument and envelope rearrangements
A.
B.
C.
D.
E.

Marsters *et al.*, 1997). It is thought that gD binds this receptor since mutations in gD determine the ability of HSV-1 to utilize HveA for entry. HveA has also been implicated in virus-induced cell fusion (Terry-Allison *et al.*, 1998). Hve A is the principal receptor for the entry of HSV-1 into human lymphoid cells, but not other naturally infected cell types (Montgomery *et al.*, 1996). It does not mediate the entry of PRV or HSV-2.

A second mediator of HSV entry identified recently was designated HveB. It was identified as poliovirus receptor-related protein 2, a membrane glycoprotein that is a member of the immunoglobulin superfamily. So far no function and no poliovirus receptor affinity have been reported for this protein. It mediates the entry of HSV-2, PRV and some viable HSV-1 mutants but does not mediate the entry of wild type HSV-1 strains or BHV-1 (Warner *et al.*, 1998).

HveC was also discovered recently (Geraghty *et al.*, 1998). It is poliovirus receptor related protein-1 and mediates the entry of HSV-1 and HSV-2, PRV and BHV-1. It is expressed in human cells of epithelial and neuronal origin making it a suitable candidate for the receptor on epithelial cells. Geraghty *et al.* (1998) also found that the poliovirus receptor (Pvr), designated Pvr-HveD, can mediate the entry of PRV and BHV-1 but not HSV. It is thought that each of these entry proteins may recognise overlapping but distinct structural domains of gD. It has been suggested that receptors for the herpesviruses are heterogeneously distributed on epithelial cells (Sears *et al.*, 1991). However, no quantitative evidence of preferential polarized uptake of *wt* HSV into an epithelial cell has yet been observed (Topp *et al.*, 1997) and likewise, no evidence presently exists for a polarized distribution of gD receptors.

According to the model shown in (Fig.1.4), the formation of a fusion bridge is the next step in virus entry, which may be triggered by changes in the conformation of gD due to receptor interaction. Similarly, formation of a fusion bridge may be mediated in part by conformational changes in gC or gB (or other viral components) which occur after HS interaction. Fuller and Lee (1992) observed visible fusion bridges for virus inactivated by gH specific antibodies and subsequently proposed that gH interacts either directly or indirectly with gD in cell fusion. Glycoprotein H, although essential for virion infectivity, does not appear to form complexes with any cellular component (Kuhn, 1990), and no distinct structures of gH have been detected on the surface of virion envelopes, suggesting that gH is probably not involved in virus attachment (Stannard *et al.*, 1987). Interaction of gH with the cell is thought to follow gD, since virus inactivated by gH neutralising antibodies appeared to be blocked at a later stage than gD inactivated virus (Fuller and Lee, 1992). Viral mutants that are gH negative are frozen at the gD fusion bridge stage and do not become internalised. Furthermore, a high multiplicity of infection (moi) of gH-negative virus bound

to cells inhibited entry of superinfecting wt virus (Fuller and Spear, 1987; Forrester *et al.,* 1992), whereas gD-negative virus had little effect (Johnson and Ligas, 1988). It is possible that gD or gH may independently mediate virion fusion, since both are required for HSV-induced cell fusion (Fuller and Spear, 1987; Highlander *et al.,* 1987; Ligas and Johnson, 1988; Fuller *et al.,* 1989), although the finding that neither gD or gH alone can mediate substantial fusion of most cells, supports the idea that gH and gD and perhaps other viral glycoproteins (i.e. gB and gK) act synergisically to mediate HSV fusion (Campadelli-Fiume *et al.,* 1988b; Butcher *et al.,* 1990; Peeters *et al.,* 1992 a, b). This proposal is supported by reports which show that most or all of the gH in infected cells is complexed with gL and that the correct processing and intracellular transport of gH in infected cells, is dependent on the coexpression of gL (Hutchinson *et al.,* 1992a).

The model also proposes that during initiation of the fusion bridge, rearrangement of the virion envelope and tegument proteins occur (Fig. 1.4), allowing the nucleocapsid to be positioned towards the cell.

The final stage of viral entry, involves the expansion of the fusion bridge and the subsequent release of the nucleocapsid into the cytoplasm (Fig. 1.4). It is likely that gB has an essential role in virus induced cell fusion, since gB-null syncytial virus can cause complete fusion of gB-transformed cell lines, but no fusion of untransformed cells (Cai *et al.,* 1988). Furthermore, the glycoproteins gK and gL, in addition to other viral components, may play important roles in mediating and controlling the changes observed in the virion particle or cell membrane and subsequent release of the nucleocapsid into the cytoplasm (Hutchinson *et al.,* 1992b; Ramaswamy and Holland, 1992).

The role of non-glycosylated proteins in viral attachment and fusion has not been thoroughly addressed. Previous investigations showed that virus attachment can occur when glycosylation is inhibited, suggesting that other viral proteins may be important in virus binding (Svennerholm *et al.,* 1982; Kuhn *et al.,* 1988). Spivack *et al.*, (1982) found that unglycosylated virus was capable of attaching to Vero cells almost as well as control virus, with only a slight decrease in levels of attachment, The rate of adsorption of both viruses was similar. An alternative explanation is that glycosylation is not required for the interaction of viral attachment proteins to the cell receptors. Campadelli-Fiume *et al.*, 1982 reported that the infectivity of HSV-1 does not require the mature form of glycoproteins, but that the precursor forms will suffice.

All this indicates that HSV-1 entry into cells is mediated by a cascade of virus-cell interactions in which each glycoprotein plays a specific role in triggering or signalling changes in the virion which result in membrane fusion and nucleocapsid uncoating.

1.4.3
Cell-to-cell spread.

It is difficult to understand how the glycoproteins are involved in cell to cell spread since many different reports exist that are conflicting or ambiguous.

The process of cell to cell spread is different from that of cell-virion fusion, and even though these processes share some aspects they are not analogous. Glycoproteins H, L, D and B are required both for virion entry and cell to cell spread, whereas gE, gI and gM are only required for cell to cell spread. Glycoproteins G and J are not essential for either process.

This difference in requirement may be due to the difference between the types of membrane involved. Plasma membranes and virion envelopes differ in their lipid and cholesterol composition, as well as in their degree of curvature. It is also thought that the concentration of HSV glycoproteins present in the two membranes are different.

To date four viral genes UL53 (gK) (Debroy *et al.,* 1985; Pogue-Geile and Spear, 1987), UL27 (gB) (Kousoulas *et al.,* 1984; Bzik *et al.,* 1984 a), UL24 (Sanders *et al.,* 1982; Jacobson *et al.,* 1989), and UL20 (Baines *et al.,* 1991) have been associated with the syncytial phenotype. A fifth syncytial locus has been suggested to map within the inverted repeats flanking U_L although the gene product involved has not yet been identified (Romanelli *et al.,* 1991).

1.4.4
Virulence.

Since HSV-1 glycoproteins play an important role in the early stages of the virus life cycle *in vitro* (adsorption and penetration) it is expected that they are also relevant *in vivo*. It was believed that gC had no role as a virulence determinant in the mouse model as gC negative mutants of both HSV-1 and HSV-2 remained highly virulent following intravaginal (Johnson *et al.,* 1986), intracerebral and footpad inoculation (Dix *et al.,* 1983; Sunstrum *et al.,* 1988). However, Lubinski *et al* . (1998) demonstrated that gC is a virulence factor when examined in their guinea pig model. These workers claim that in previous studies the virus did not actually encounter complement *in vivo*. They showed that in C3 deficient guinea pigs gC negative viruses reached significantly higher titres than in complement intact animals behaving similarly to wild type or rescued virus. They concluded that gC has a 10-100 fold effect on virulence, while wild type virus has a near-total protection against complement conferred by gC.

Monoclonal resistant (mar-) gB or gD mutants have no significant alteration in pathogenicity (Kumel *et al.,* 1985). Meignier *et al.* (1988) demonstrated that a gE negative mutant was not significantly impaired in neurovirulence following intracranial inoculation of mice, with a LD_{50} less than 100 fold higher than that of the parental virus.

Both HSV-1 Ang and HSV-1 KOS are completely non-neurovirulent following inoculation on the mouse footpad (Kumel *et al.,* 1983; Kaerner *et al.,* 1983; Thompson *et al.,* 1986) although both are neurovirulent following intracerebral inoculation. Following passage in the mouse brain, variants of HSV-1 Ang arise (Ang path) which are capable of causing lethal neurological disease following footpad inoculation. This increase in neurovirulence (as defined by the ability of the virus to spread to the CNS following peripheral inoculation) is associated with a single base change in the gD ORF (Izumi and Stevens, 1990). In HSV-1 KOS, two loci are required for complete restoration of a neuroinvasive phenotype (Yuhaz and Stevens, 1993): one within a region of the genome encoding the genes UL10, UL11, UL12, UL13 and parts of UL9 and UL15 has not yet been fully characterised, whereas the other is localised to gB; as with HSV-1 Ang, a single base pair change in a glycoprotein greatly affects neuroinvasineness. gB and gD play a role in virus adsorption and penetration, and thus the decrease in neuroinvasiveness associated with these amino acid substitutions may be due to a decreased affinity of the mutant glycoproteins for their cellular receptors, leading to less efficient spread in the nervous system.

Two amino acid substitutions in the C-terminal region of gB have been shown to produce a virus which is still virulent following footpad inoculation but produces unusual clinical symptoms (Goodman and Engel, 1991; Engel *et al.,* 1993). Unlike inoculation with the wild type virus, HSV-1 strain 17+, an acute inflammatory response occurs in inoculated footpads with subsequent death of the inoculated animal without hindlimb paralysis. This is possibly due to the alterations in gB affecting antigen presentation, and thus affecting the host response to infection.

1.4.5

Fc receptor.

Receptors which have affinity for the Fc domain of immunoglobulin G (IgG) are expressed on the surfaces of cells infected with a number of human herpesviruses (Johnson *et al.,* 1988). HSV-1 infected cells and the virion envelope express such receptors (McTaggart *et al,*. 1978; Westmoreland and Watkins, 1974). A group of electrophoretically similar HSV-1 polypeptides that bound IgG columns with high affinity were identified by Bauke and Spear (1979). They were thought to be derived by post-translational modification of gE (Cross *et*

al., 1987). However, Johnson and Feenestra (1987) subsequently discovered a novel HSV-1 induced polypeptide that was structurally distinct from gE and was provisionally named gp70. This polypeptide could be detected on the surfaces of infected cells and was co-precipitated using rabbit or human IgG or anti-gE antibodies. It was later mapped to the US7 open reading frame and named gI (Johnson *et al.*, 1988; McGeoch *et al.*, 1988a). The gE-gI hetero-oligomer also facilitates cell-to-cell spread of virus in epithelial and neuronal cells (Dingwell *et al.*,1995).

Expression of gE alone is sufficient for induction of Fc activity, while gI alone produces no detectable binding (Bell *et al.*, 1990; Dublin *et al.*, 1990). Coexpression of gI and gE gives better binding than expression of gE alone, suggesting that HSV-1 has the potential to induce two Fc receptors - one a low affinity receptor composed of gE alone, and a second high affinity receptor composed of gE and gI. gE appears to be the Fc receptor for complexed IgG, while gE and gI form the Fc receptor for monomeric IgG (Dublin *et al.*, 1990; Basu *et al.*, 1997).

It is not clear what physiological role these Fc receptors play in the replicative cycles of herpesviruses since studies to define their biological relevance have been hampered by the fact that gE null mutants are severely attenuated *in vivo* due to the defect in cell to cell spread. However it has been suggested that they influence events in the infected cell or act to reduce immune cytolysis of virus-infected cells. Fc receptors may function by coating the virus infected cells with IgG so as to reduce exposure of viral antigens to immune effector cells, or they may bind oligopeptides that share some structural homology with immunoglobulin Fc receptors, e.g. histocompatability antigens (Johnson *et al.*, 1988).

The domain of gE involved in IgG binding is however distinct from the domain involved in mediating cell-to-cell spread (Weeks *et al.*, 1997). Nagashunmugan *et al.*, (1998) constructed a mutant virus that was only altered at a small region within the gE IgG Fc binding domain and thus conserved other functions mediated by gE. This mutant displayed a significant reduction in its ability to cause disease and was highly sensitive to human anti-HSV IgG.

1.4.6
C3b binding.

HSV-1 infected mammalian cells express receptors for the C3b component of complement in culture (Cines *et al.*, 1982). These receptors are not present following HSV-2 infection. It has been shown that gC-1 but not gC-2 functions as a C3b receptor when expressed on cells (Friedman *et al.*, 1984). There is a 28 amino acid insert in gC-1 compared to gC-2 (Frink *et*

al., 1983; Swain *et al.,* 1985) that may explain the functional differences between the two glycoproteins.

Kostavasil *et al.,* (1997) used a baculovirus expression system to show that both gC-1 and gC-2 bind C3, C3b and C3c, but not to C3d. This suggests that: 1) the binding sites for these proteins are found in the C3c region of C3 and 2) that gC, in contrast to the other C3-binding proteins, interacts with native C3.

gC-1 but not gC-2 inhibits the binding of C5 and properdin to C3b and also inhibits the alternative pathway-mediated lysis of rabbit erythrocytes (Kostavasil *et al.,* 1997; Hung *et al.*, 1994). Inhibition of alternative pathway-mediated lysis and properdin binding to C3b, but not of C5 binding to C3b, requires the transmembrane segment of gC-1. These roles of gC inhibit activation of the complement cascade thus protecting HSV-1 from complement mediated neutralisation (Gerber *et al.*, 1995; Hidaka *et al.*, 1991). There may also be a strain dependent effect on the level of binding with HSV-1 strain KOS exhibiting much less C3b binding than most other HSV-1 isolates (Freidman *et al.*, 1986).

gC-1 demonstrates dose-dependent acceleration of decay in the activity of C3 convertase of the alternative pathway, and can block the effects of complement via this mechanism (Fries *et al.,* 1986). Thus gC-1 can protect against complement mediated cytolysis (Harris *et al.,* 1990) by inhibition of the alternative pathway. In the absence of gC-1, complement neutralisation is rapid and is mediated by components of the classical complement pathway (Friedman *et al.,* 1995). Glycoprotein C mainly protects against antibody-independent complement neutralisation, suggesting that it has a role early in infection, before antibodies develop. Friedman *et al.* (1986) observe that once antibody binds to gC it alters the ability of this glycoprotein to interact with C3b. Thus appears that once the host develops an antibody response the C3b binding will be reduced and thus the selective advantage offered by the presence of gC is only valid before anti-gC antibody is made.

2. Materials.

2.1
Cell culture media.

Eagle's medium (Gibco), supplemented with 10% new-born calf serum (Gibco), 100 µg/ml streptomycin (Gibco), 100 units/ml penicillin and tryptose phosphate. This is subsequently referred to as ETC10.

A variation on this basic growth media was:

EMC10 Eagles medium containing 1% carboxymethylcellulose and 10% new-born calf serum.

Dulbecco's Modified Eagle's medium (Gibco) supplemented with 10% foetal calf serum (Gibco), 200mM glutamine (Gibco), 100 µg/ml streptomycin (Gibco) 100 units/ml penicillin was used for the growth of HeLa, 3T6, Vero and C6 glioma cells. This is subsequently referred to as DMEM10.

2.2
Cells.

BHK21/C13 (Mcpherson and Stoker, 1962), C6 gliomal cells (Hill, 1968), HeLa (Griffiths, 1953), 3T6 (Harris and Sutherland, 1965) and Vero Thornton, 1963).

2.3
Viruses.

The wild type HSV-1 strains used in this study were HSV-1 strain 17+ (Brown *et al.,* 1973), HSV-1 strain F (Ejercito *et al.,* 1968), HSV-1 SC16 (Hill *et al.,* 1975) and HSV-1 HFEM (Halliburton *et al.,* 1980).

The gC- mutants of these viruses were designated strain 17 gC negative, R6012, SC16-ΔUL44-Z and HFEM-ΔUL44-Z respectively. (Cunningham and Davison 1993; Sears *et al.,* 1991; Griffiths *et al.,* 1998). Both the SC16 mutant and the HFEM mutant contained lac Z expression cassettes in the UL44 gene (Fig. 3.3).

HSV-1 strain 17 wild type and gC negative lac Z expressing viruses (with lacZ in the UL43 locus) were called 17+ lacZ and 17gC negative lacZ. (Figure 3.2).

2.4

β-galactosidase stain.

PBS A containing 5mM $K_4Fe(CN)_6$, 5mM $K_3Fe(CN)_6.3H_2O$, 2mM $MgCl_2$, 1mg/ml X-gal. X-gal was dissolved in dimethyl formamide (DMF) at a concentration of 20mg/ml and added just before use.

2.5

Antibodies.

Mouse monoclonal antibodies against gC were supplied by Dr. Anne Cross, Institute of Virology, and used at a dilution of 1/1000.

2.6

Enzymes.

Restriction enzymes were supplied by Boehringer Mannheim or New England Biolabs and used in the appropriate manufacturer's buffer

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2.7

Radioisotopes.

^{32}P dCTP was supplied by New England Biolabs.

2.8

Chemicals.

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

APS and TEMED	Bio-Rad Laboratories
Boric acid and glycerol	PROLAB
Ficoll	Pharmacia, Upsalla, Sweden
Gluteraldehyde	Agar Aids, Cambridge, England
Proteinase K	Boehringer Mannheim, Germany

2.9

Commonly used buffers and solutions.

Alkaline lysis solution I	10mM EDTA, 50mM glucose, 25mM Tris-Cl pH 8.0 and 5mg/ml lysozyme added prior to use.
Alkaline lysis solution II	200mM NaOH, 1%(w/v)SDS.
Alkaline lysis solution III	5M potassium acetate, pH4.8.
Chloroform:isoamylalcohol	24:1 mixture of chloroform and isoamylalcohol
Gel soak I	600mM NaCl, 200mM 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
Phenol chloroform (1:1)	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.
RE stop	100mM EDTA, 10% (w/v) Ficoll 400, 0.25% (w/v) bromophenol blue, 5x TBE.
Saturated phenol	Phenol saturated by mixing 1:1 with phenol saturation buffer, (10mM Tris-HCl, pH7.5, 10mM EDTA, 100mM NaCl).
SSC	153mM trisodium citrate, 150mM NaCl.
TBE	2mM EDTA, 89mM boric acid, 89mM Tris-HCl pH8.0.
Trypsin	0.25% (w/v) Difco trypsin dissolved in Tris-saline.
Trypsin-Versene	1 volume trypsin and 4 volumes versene.
Versene	0.6mM EDTA dissolved in PBS containing 0.002% (w/v) phenol red.
X-gal	20mg/ml in dimethylformamide.

2.10
Miscellaneous.

Hybond™_ N hybridization transfer membranes	Amersham
Hybond™_ECL hybridization transfer membranes	Amersham
Giemsa stain	BDH
XS-1 film	Kodak

3. Methods.

3.1
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). This gave a yield of approximately $1x10^8$ cells per bottle. Confluent cells were harvested by washing the monolayers twice with 25ml of trypsin-versene, and resuspending the detached cells in 20ml of ETC10. Cells from one bottle could then be split 1/10 to seed a further ten roller bottles.

The 3T6, HeLa, Vero and C6 cells were grown in flasks in DMEM and harvested as described above, with a split of 1/4.

For some experiments cells 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.

3.2
Preparation of virus stock.

Virus stocks were prepared as described by Brown *et al*. (1973). Confluent roller bottles of BHK21/C13 cells were infected with 0.001 p.f.u./cell (assuming 1 x 10^8 cells/bottle) of HSV in 20ml of ETC10 and incubated at 31°C for 3-4 days, until c.p.e. was complete, when the cells were shaken into the medium. The cells were pelleted in 250ml plastic falcon tubes by spinning at 2K for 10 minutes at 4°C in a Fison's Coolspin. The supernatant and cell pellet were separated and two individual virus stocks prepared:

Supernatant stock: The supernatant was poured into 250ml centrifuge bottles and spun at 12K for 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 1ml 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.

3.3
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 45min-1h, to allow adsorption of the virus to the cells, before overlaying with 5ml EMC10%. Plates were incubated at 37°C for 2 days or 31°C for three days. Monolayers were fixed and stained with Giemsa at RT. After washing, plaques were counted on a dissecting microscope and virus titres calculated as p.f.u./ml.

3.4
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 stocks, a small aliquot was streaked onto BHI plates in duplicate and the plates sealed with parafilm and incubated at RT. Bacterial contamination was detected by plating onto blood agar and incubating at 37°C. If no colonies were visible after 7 days incubation, the stocks were considered sterile.

3.5
Growth, labelling and purification of HSV-1 Virions.

Virions were labelled and purified essentially by the method of Szilagyi & Cunningham (1991). Monolayers of BHK21/C13 cells in roller bottles were infected with HSV-1 at a moi of 0.01 p.f.u/cell and incubated at 31°C for 3 days. To label the virion proteins, monolayers were infected with HSV-1 at a moi of 5 p.f.u/cell in medium containing one-fifth the normal amount of methionine/2% calf serum and incubated at 37°C for 4 h, 25uCi/ml of ^{35}S-methionine added and labelled for 16-20 h at 37°C. Once the c.p.e. was complete the supernatant was removed and cell debris pelleted by low speed centrifugation (1,000g for 10 min at 4°C). Virus particles from the clarified medium were pelleted by centrifugation at 23,000 g for 2 h at 4°C. The pellet was gently resuspended in 1 ml of modified medium (culture medium without phenol red or calf serum) and layered onto a 35-ml preformed gradient of 5 to 15% ficoll 400 suspended in this medium. After centrifugation in a swing-out rotor (26,000g for 2 h at 4°C in an AH629 cellulose nitrate tube), the virion bands were

withdrawn by puncturing the tube with a broad needle. These virions were diluted and pelleted by centrifugation (21,000 r.p.m. for 2 h at 4°C in an AH269 tube). The virion pellet was gently resusupended in 100µl of modified medium and either used immediately or stored at -70°C.

3.6

Purification of β-galactosidase expressing viruses.

Serial 10 fold dilutions of virus were prepared and 100µl of each dilution used to infect a BHK21/C13 monolayer in a 35mm diameter tissue culture dish. After adsorption for 1hr the inocula were removed, the cells overlaid with 3ml of EMC10 per dish and incubated at 37°C until plaques were visible under a low magnification. The overlay was removed and replaced with an X-gal overlay (EMC10 with 100µg/ml X-gal) and incubated at 37°C until blue plaques were visible.

Blue plaques were isolated in a volume of 20µl with a micropipette, transferred to 1ml PBS/calf serum, bath sonicated and subjected to plaque purifications until 100% of plaques were stained blue by the X-gal overlay.

Plaque isolates were screened by Southern blotting to ensure the correct DNA structure at the region of modification.

3.7

Assay for rate of virus adsorption

Monolayers of cells ($4x10^6$) in 50 mm petri dishes were precooled for 1 hr at 4°C and inoculated with virus at a multiplicity of infection (m.o.i.) of 300 p.f.u/plate and left at 4°C for various lengths of time up to 4 hr. At each timepoint, the appropriate plates were washed three times with PBS/calf serum and overlaid with EMC10. The plates were incubated at 37°C for 2 days after which time they were fixed and stained. Plaques were counted, and the % of virus adsorbed at each time-point determined, relative to the final timepoint (4 hr) which represented 100% adsorption. Each timepoint was performed in triplicate.

3.8

Assay for rate of virus penetration

Virus penetration was assessed by determining the rate at which adsorbed virus became resistant to inactivation by a low-pH citrate buffer. The method used was essentially as described by Huang and Wagner (1964) and Highlander *et al.*, (1987). Confluent BHK21/C13 cells ($4x10^6$) in 50 mm petri dishes were separately infected with 300 p.f.u per plate of either wild-type or mutant virus and allowed to adsorb for 2h at 4°C. The unbound virus was removed by washing four times with PBS and the cells overlaid with Eagle's medium and moved to 37°C to allow viral penetration to proceed. At selected timepoints after the temperature shift, plates were either treated with 1 ml of citrate buffer (40 mM citric acid, 10mM KCl, 135mM NaCl , pH 3.0) or 1 ml of PBS for 5 min as a control. The monolayers were washed three times with PBS overlaid with EMC10 and incubated at 37°C for 2 days. The plaques were visualised with Giemsa stain and counted. Each timepoint was performed in triplicate. The amount of penetrated virus at each timepoint was expressed as % of p.f.u on the citrate buffer treated plate compared to PBS control.

3.9

Heparin inhibition.

Three hundred p.f.u of virus were adsorbed to 100% confluent BHK21/C13 monolayers at 4°C for 3hr in the presence of increasing concentrations of heparin. The inoculum was removed, and the monolayers washed three times with PBS/calf serum before overlaying with EMC10 and incubating at 37°C for 2 days. The number of plaques was counted and expressed as a % of the value obtained with no heparin. The experiment was performed in triplicate with each heparin concentration.

3.10

Staining of tissue culture monolayers for β-galactosidase.

The medium was removed from cell monolayers in tissue culture dishes and the cells washed with PBS A. The cells were fixed by overlaying with PBS A/Gluteraldehyde/Formaldehyde and incubated at RT for 15 minutes. This solution was removed, and after two washes with PBS-A, 1ml β-galactosidase stain was added and the monolayer incubated at 37°C until blue

foci were visible. The β-galactosidase stain was removed, the monolayer washed with distilled water and blue foci counted under a dissection microscope.

3.11
Binding of radioactive virions.

Monolayers of BHK21/C13 cells ($4x10^6$) in 50mm petri dishes were precooled for 1hr at 4°C and inoculated with radioactively labelled Ficoll banded virus at a moi of $5x10^2$ particles/cell, and left for various lengths of time up to 4hr. At various timepoints, the inoculum was removed and placed in a scintillation vial. The cells were then lysed with 100µl 2.5% (w/v) SDS for at least 10 minutes at RT and the lysate transferred to a fresh scintillation vial. The radioactivity was counted and the percentage of virus bound at each timepoint calculated relative to the total counts (bound and unbound).

3.12
In vivo studies of latency.

As described by Clements and Subak-Sharpe (1983, 1988), 4 week old Balb/C mice were inoculated in the right rear footpad with various doses of virus in 25µl PBS/calf serum. After six weeks, all surviving mice were killed, dissected and the ten ipsilateral dorsal root ganglia supplying the lower limb of each mouse were separately cultured in microtitre plates. They were screened every second day for the presence of infectious virus by transferring the culture supernatant to control BHK21/C13 cells which were incubated for two days at 37°C, before staining and examination for the presence of virus plaques or cpe. Results were expressed as the percentage of the total ganglia reactivating each day.

3.13
Pathogenicity.

Three week old Balb/C mice were anaesthatised and 0.025ml of the appropriate virus dilution in PBS/calf serum injected into the central region of the left cerebral hemisphere. Groups of 6 or 7 mice were inoculated with a single dilution of each virus stock (between 10^1 and 10^4 p.f.u/animal). Mice were observed daily for 21 days after inoculation and their clinical states

recorded. The 50% lethal dose value (LD50) was calculated according to the formula of Reed and Muench (1938).

3.14
Phenol-chloroform extraction of DNA.

If the digestion mixture was less than 100µl, it was increased to this volume using dH_2O. An equal volume of phenol:chloroform was added, the mixture shaken vigorously, and spun at 13000g for 2 minutes in a microfuge. The top aqueous layer was transferred to a separate eppendorf tube and the bottom layer back extracted with an equal volume of dH_2O. An equal volume of chloroform was added to the aqueous layer and the mixture mixed and spun at 13000g for 2 minutes in a microfuge. The top layer was removed and the sample 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 vacuum desiccator, before resuspending in an appropriate volume of dH_2O.

3.15
Preparation of small amounts of virus infected cell DNA for Southern blotting

BHK21/C13 cell monolayers ($5x10^5$ cells) on 24 well linbro trays were infected at a moi of 5 p.f.u/cell at 37 °C. After 48 h the supernatant was removed and the cells lysed by incubating with 750ul of lysis buffer (0.6% (w/v) SDS,10mM EDTA, 10mM Tris.HCl pH 7.4) containing 500µg/ml protease, for a minimum of 4 h at 37°C. Infected cell DNA was extracted twice with an equal volume of phenol and once with chloroform, precipitated by the addition of 2 volumes of ethanol and dried in a vacuum desiccator. The DNA was dissolved in 200ul of water containing 50µg/ml RNase A. Restriction enzyme digestions were usually carried out on 5% of the total sample (10µl).

3.16
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 3.14.

3.17
Agarose gel electrophoresis.

Restriction endonuclease digestions of viral or plasmid DNA were analysed on 0.4-1.5% (w/v) agarose gels. Agarose was boiled in 200ml 1xTBE buffer. When cool, ethidium bromide was added to the agarose solution to a final concentration of 0.5µg/ml before pouring the solutions onto glass plates (16.5x26.5cm) whose edges had been sealed with gel tape and onto which 12-26 tooth combs had been placed. The gels were allowed to set at RT and 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).

3.18
Southern blotting.

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

The gel was placed in 500ml Gel Soak I (see section 2.19) for 1hr, rinsed with deionised water and transferred to Gel Soak II for 1hr. After rinsing as before, it was transferred to 500ml 10xSSC for a further 1hr. The gel was then ready for blotting onto either one or two sheets simultaneously of Hybond N nylon membrane (Amersham). For each transfer one sheet of nylon membrane and five sheets of Whatmann 3MM paper, cut to the gel size, were required.

A pile of "Hi-Dry" towels was placed on the bench and topped by three sheets of dry 3MM filter paper, followed by two sheets soaked in 10xSSC. The nylon membrane, which had also been soaked in 10xSSC was placed on top of the filter paper followed by the gel, ensuring that there were no air bubbles between the gel and the nylon membrane. A glass plate and heavy weight were placed on top and left overnight, 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 in reverse on top of the gel.
The DNA was cross linked to the nylon membrane using a UV Stratalinker, and hybridized to the labelled probe in a sealed bag containing 20ml hybridization buffer overnight at 65°C.
The nylon membrane was washed twice for 30 minutes with 1 litre 2xSSC, 0.1% (w/v) SDS before drying and setting up for autoradiography against Kodak XS-1 film.

3.19
Western blotting.

SDS-PAGE was carried out using slab gels cast vertically in a sandwich consisting of two glass plates separated by 1.5mm perspex spacers. A 60ml gel mix was prepared using a running gel buffer (RGB) which contained 375mM Tris-Cl, pH 8.9 and 0.1%(w/v) SDS. Polymerisation was achieved by the addition of 0.006% (w/v) ammonium persulphate (APS), and 0.004% (v/v) N,N,N',N', tetramethylenediamine (TEMED), just prior to pouring. A thin layer of butan-2-ol (3.5ml), was poured on top to exclude air and produce a level surface. Prior to adding the stacking gel, the butan-2-ol was removed and the surface of the running gel rinsed with distilled water.
The stacking gel was composed of 5% acrylamide, crosslinked with the same ratio of N,N'-methylenebisacrylamide used in the resolving gel, in stacking gel buffer (SGB), composed of 0.11M Tris HCl, pH 6.7 and 0.1% (w/v) SDS. APS and TEMED were added to the gel just before pouring and a Teflon comb was used to form the wells.
Infected cell proteins were harvested into boiling mix (50mM Tris Cl pH6.7, 2% SDS, 700mM 2-mercaptoethanol, 10% glycerol, bromophenol blue). Samples were boiled for five minutes before loading on the gel, and run overnight at 8mA. Once separated, the proteins were transferred onto nitrocellulose using a Bio-Rad Transblot apparatus. The foam pads, sheets of Whatmann 3MM paper and the nitrocellulose used for the transfer were pre-soaked in transfer buffer (192mM glycine, 25 mM Tris.HCl pH8.3, 20% methanol). The gel was placed on top of the nitrocellulose, three sheets of 3MM paper and a foam pad. Three more sheets of 3MM paper and another foam pad were placed on top and the gel sandwich was held together in a plastic folder that was closed and placed in the transfer kit, ensuring that the

gel was towards the cathode and the nitrocellolose to the anode. Transfer took place for a minimum of three hours at 250mA and when complete the nitrocellulose was removed, placed in a plastic tub and blocked by washing twice for 30 minutes using 2% dried milk in PBS, 0.05% Tween 20 (PBST). The nitrocellulose was then washed for 10 minutes three times in PBST, before addition of the primary antibody diluted appropriately in PBST, 1% BSA and incubated for 2 hr at 37°C or overnight at 4°C or RT. The appropriate second antibody was added as a horseradish peroxidase (HRP) conjugate at a 1/1000 dilution in PBST, 1% BSA and incubated at 37° for 1 hr, the nitrocellulose was washed three times in PBS. 0.05% Tween 20. Bound antibodies were detected using the ECL system (Amersham). The nitrocellulose was incubated with equal volumes of reagents 1 and 2 for one minute at RT. The detection reagents were drained off and the nitrocellulose sheet covered in cling film before exposure to film for between 5 seconds and 60 minutes.

4. Results.

4.1
Aims and background.

The entry of HSV into cells requires a cascade of events to occur at the cell surface; these can be divided into two distinct phases: adsorption and penetration (see sections 1.4.1 and 1.4.2).

The adsorption of HSV-1 to host cells in culture is mainly dependent on cell surface heparan sulphate or related glycosaminoglycans. Heparan sulphate is a ubiquitous glycosaminoglycan found on proteoglycans in cell plasma membranes, extracellular matrices and basement membranes. It is structurally and chemically similar to heparin, although heparin is more highly sulphated. Although glycoprotein C (gC) of HSV-1 and its homologues in other alphaherpesviruses is dispensable for infectivity it is thought to play an important role in the initial adsorption of the virus to the host cell, because (1) gC is a heparin binding protein, (2) anti-gC antibodies inhibit the binding of virions to cells and (3) virions which lack gC bind to cells with a reduced efficiency (Fuller and Spear, 1985).

Glycoprotein B also binds to heparin and virions lacking both gC and gB bind cells with lower efficiency than virions lacking gC alone (Herold *et al.* 1994; Laquerre *et al.*, 1998). This data led to the proposal that the weak binding activity of gB mediates the initial interaction of HSV-1 virions with cell surface glycosaminoglycans when gC is absent. The idea of gC as the main agent in the primary adsorption mechanism is supported by studies on PRV and BHV-1. The gC homologues of these viruses are heparin binding proteins and mutants lacking gC bind to cells with reduced efficiency.

This initial and dispensable binding of HSV-1 gC to cell surface glycans is thought to be followed by a stable, non-dispensable binding reaction involving other viral membrane proteins. Glycoprotein D is the most likely candidate as soluble gD will block infection (Johnson 1990), while gD negative virions fail to block infection by superinfecting virus (Johnson and Ligas, 1988) and several gD receptors have recently been identified in different cell types.

These findings led to the consensus view that the binding of alphaherpesviruses to host cells is achieved by the initial interaction of gC and gB with cell surface proteoglycans, followed by a stable binding of gD to saturable receptors. Penetration is thought to be mediated by gB and the gH:gL heterodimer. It appears that other glycoproteins play no significant or essential role in this process: virions lacking several non-essential glycoproteins show normal

particle:p.f.u ratios (Balan *et al.,* 1994) and the expression of gD, gB and gH:gL in infected cells is sufficient to induce cell-cell fusion (Turner *et al.,* 1998). See section 1.4.2

The initial aim of this project was to alter the binding of HSV-1 to different cell types through the deletion of gC and the heparan sulphate binding region of gB. Deletions of these binding regions should decrease the binding of virus to cells and allow for the insertion of targeting molecules to specify and alter the cell types infected. Published data at the time indicated that there should be a difference in cell entry between wild type virus and gC$^-$ virus. The results obtained during my study did not correlate with this and as a result of this observation the project moved to look at the role of gC in various strains of HSV-1 in a range of different cell lines.

Due to the large numbers of figures they are presented in appendix I.

4.2
Viruses.

Wild type HSV-1 strain 17 (Brown *et al.,* 1973) was used, along with a gC negative mutant constructed by Cunningham and Davison (1993). The mutant contains a stop codon in the gC (UL44) ORF (Fig. 1), which results in expression of a truncated form of gC lacking the membrane spanning domain and therefore produces virions devoid of gC. LacZ expressing forms of these viruses, HSV-1 strain 17 lacZ and gC negative lacZ, were also used. The lacZ expression cassette driven by the HCMV IE promoter was inserted into the *Nsi*I site in UL43 (np 94911) (Fig. 2) by Dr. Alistair McGregor.

Three other wild type HSV-1 strains were used, F (Ejercito *et al.,* 1968), SC16 a low passage isolate (Hill *et al.,* 1975); and HSV-1 strain HFEM a high passage syncytial strain (Halliburton *et al.,* 1980).

R6012 is a gC negative form of HSV-1 strain F (Fig. 3). (Sears *et al.,* 1991)

SC16-ΔUL44-Z and HFEM-ΔUL44-Z were constructed as described by Griffiths *et al.,* 1998 (Fig. 4) with the UL44 gene disrupted by insertion of a lacZ expression cassette (Forrester *et al.,* 1992).

4.3
Particle to p.f.u ratio.

Herold *et al.,* (1991) showed that KOS gC negative virions demonstrated delayed adsorption kinetics and had a lower specific infectivity as measured by a higher particle to p.f.u ratio.

The viruses used in this study, both wild type and mutant, had their particle to p.f.u ratio measured. In all cases the wild type parent and its gC negative mutant had similar particle to p.f.u ratios (Table 4.1), differing by two fold at most. This is in contrast to the defect in the specific infectivity of the gC negative virus used by Herold *et al.* (1991).
Since the particle to p.f.u ratios of the viruses used were all within normal limits, some doubt was cast on the idea that gC has an important role in the infection process through binding cell surface proteoglycans.

4.4
Binding of radioactively labelled virions to BHK21/C13 and C6 gliomal cells.

Radiolabelled virions of HSV-1 strain 17 and its gC negative mutant were purified (see methods section 3.5) and binding to BHK21/C13 and C6 glioma cells carried out. This method extends the particle to p.f.u ratio measurements looking only at deficits in binding and does not rely on virus infectivity. It allows us to look at absolute levels of adsorption (See section 3.11 for method). The main problem in this assay is the high background level of radioactivity. Herold *et al.* (1991) recorded that significantly fewer KOS gC negative virions bound to Hep-2 cells than wild type or gB negative virions. However the results obtained in this case did not agree with that finding.
There was no difference between the ability of HSV-1 strain 17 wild type to bind to BHK21/C13 and C6 cells (Fig. 5). Similarly the gC negative virus bound equally well to both cell lines in the binding of the gC negative virus (Fig. 6). Both HSV-1 strain 17 wild type and its gC negative mutant bind equally well on both cell lines (Fig. 7 and 8), in the case of these figures the values shown are values obtained when the level of background binding (binding at t=0) is removed. This allows for differences in background radioactivity of different virus preparations.

4.5
Adsorption kinetics of HSV-1 strain 17 wild type virus and its gC negative mutant to BHK21/C13 cells.

Assays were carried out as described in section 3.7, in order to examine the kinetics of viral adsorption to the cell surface. Graphs in the following sections show how adsorption varies over time. Initially adsorption is rapid and as time progresses the rate of adsorption slows as it approaches a maximum. Values are expressed as a percentage of the final adsorption at

t240. Thus all graphs have a final adsorption of 100% and no account is made of the absolute level of adsorption.

In order to further examine whether the HSV-1 strain 17 gC negative mutant was impaired in adsorption we looked at the adsorption kinetics of this virus as compared to the wild type on BHK21/C13 cells. Initial experiments indicated that the HSV-1 strain 17 gC negative mutant was not impaired for adsorption to BHK21/C13 cells. This result differed from published data (Herold 1991, Trybala 1994) which showed a lag in viral adsorption of gC negative virions. In order to confirm our observations, the experiments were repeated and subsequent experiments confirmed that there was no difference between the gC negative virus and its wild type parent.

Figure 9 shows the results of these experiments. Values shown represent the average of three experiments with the standard deviation indicated. The viruses show t50 values (the time taken to reach 50% adsorption levels) of 61 and 42 minutes for the wild type and mutant respectively (see table 2).

Adsorption experiments were also carried out using strain 17 wild type and the strain 17 gC negative viruses containing a lacZ expression cassette in the UL43 locus (Fig. 10). UL43 is a non-essential gene with no role in adsorption (MacLean *et al.,* 1991; C. MacLean - personal communication). The results indicate no difference in the adsorption pattern observed for the lacZ gC negative mutant and the lacZ wild type (Fig. 10, Table 2). In a comparison of all four strain 17 viruses no significant difference was observed (Fig. 11, Table 2).

4.6

Rate of penetration of HSV-1 strain 17 wild type and its gC negative mutant into BHK21/C13 cells.

Following the observation that there was no difference between the viruses in adsorption as judged by specific infectivity, radioactively labelled virion binding and kinetics, the rate of penetration of the viruses was examined. This was assessed by determining the rate at which adsorbed virus became resistant to inactivation by a low-pH citrate buffer (section. 3.8). Herold *et al.* (1991) concluded that there was a lag in penetration of KOS gC negative virions. In contrast, the results presented here in Figure 12 show that HSV-1 strain 17 and its gC negative mutant displayed no difference in the rate of penetration into BHK21/C13 cells. They displayed similar t50 values (time taken for 50% of the total virions to penetrate the cell) at 14.1 and 12.8 minutes respectively.

4.7

Intracranial pathogenicity.

Experiments to determine the pathogenicity of the strain 17 gC negative mutant as compared to its parent, HSV-1 strain 17 were carried out in three week old female Balb/C mice following intracranial inoculation (section 3.13). Tenfold serial dilutions of strain 17 wild type and the gC negative mutant were inoculated into the left cerebral hemisphere of the mice. Deaths from encephalitis were scored up to 21 days post inoculation and the results are shown in Table 4.3. The viruses displayed no significant difference in pathogenicity with LD_{50} values of $<10^1$ and $5x10^1$ respectively.

4.8

Peripheral pathogenicity and latency studies of HSV-1 strain 17 and its gC negative mutant.

The ability of the HSV-1 gC negative mutant to reactivate from latency was examined using the mouse footpad model (see section 3.12). Groups of four week old female Balb/C mice were inoculated in the right rear footpad with varying doses of wild type strain 17 and gC negative strain 17 and monitored daily for signs of illness or death. Six weeks post inoculation, surviving mice were sacrificed and the ten ipsilateral ganglia supplying the footpad removed and assayed for reactivation as described in section 3.12. The LD_{50} data following footpad inoculation are summarised in Table 4.4.

HSV-1 strain 17 has a LD_{50} of 10^5 p.f.u/mouse compared to the gC negative mutant at 10^6. This ten fold difference is not significant. The reactvation frequency is shown in Figure 13, and shows that the two viruses behave similarly.

4.9

Analysis of the structures of the virus DNAs used in this study.

To confirm the genotype of the viruses, DNA was prepared from all of the viruses used in this study and analysed by Southern blotting. The DNA was digested with *BamHI* (section 3.16), Southern blotted (section. 3.18) and probed with radiolabelled *KpnId* (np 90383-103012).

Figure 14 is a diagrammatic representation of the structure and np of the relevant sites for each virus. Figure 15 is the autoradiogram produced, showing the expected profiles for each virus.

All viruses contain bands of 8055, 3866 and 717kbp with the 3866 and 8055kb bands being fainter since the probe only spans part of their length.
The lanes containing wild type strain 17 (lane9), SC16 (lane 4), HFEM (lane 3), F (lane 11) and the mutant strain 17 gC negative viruses (lanes 6 and 7) show a band of 6640kb, the fragment spanning UL44 and UL43.
As expected in lane 10 containing R6012 the 6640 band is reduced due to the 1372bp deletion in UL44, producing a band of 5267bp.
The lanes containing SC16-ΔUL44-Z (lane 4) and HFEM-ΔUL44-Z (lane 2) have bands of 5.1kb and 600bp.
The lacZ expressing strain 17 viruses (lanes 6 and 7) have bands of 3.3kb and 7.3kb.

4.10
Analysis of gC expression of the wild type HSV-1 strains and their gC negative mutants.

All of the viruses used in this project were analysed by Western blotting (section 3.19) in order to determine if they did or did not produce gC. All produced the expected pattern with HSV-1 strain 17 wild type, strain 17 wild type lacZ expressing virus, SC16, HFEM and F all expressing gC; while the HSV-1 strain 17 gC negative mutant, the lacZ expressing form of the mutant, SC16-ΔUL44-Z, HFEM-ΔUL44-Z and R6012 did not produce glycoprotein C (Fig. 16).
The marker lane shows the 66 and 46kD markers. There is a 46kD cellular band which is detected non-specifically by the antibody. A gC band is observed in all the wild type lanes. Another band is visible below gC in the wild type virus lanes, this is a precursor form of gC (pgC). Due to differences in the rate of progression of infection, HSV-1 F shows more pgC than gC, and two precursor bands are visible. The lanes containing gC negative mutants do not display any bands corresponding to gC or pgC.

4.11
Adsorption characteristics of other HSV-1 strains and their gC negative counterparts on BHK21/C13 cells.

As stated in section 4.5, these experiments were initially carried out using HSV-1 strain 17 alone. It was shown that there was no difference between HSV-1 strain 17 wild type and gC negative virus (Fig. 9), or between lacZ expressing variants of these viruses (Fig. 10).

Due to the difference between the published and our observed data we obtained more gC negative viruses in order to see if this was a strain dependent event. Work which was later published (see discussion) agreed that there was no difference in the rate of adsorption of two of the gC negative viruses used here (SC16 and HFEM) as compared to the wild type parents (Griffiths *et al.,* 1998).

No difference was observed between HSV-1 strains SC16 and SC16-ΔUL44-Z (Fig. 17), HFEM and HFEM-ΔUL44-Z (Fig. 18) or HSV-1 strain F and R6012 (Fig. 19).

A comparison was also made between each virus and the HSV-1 strain 17 wild type. Again no difference in adsorption characteristics was observed. HSV-1 strain 17 wild type shows the same pattern of adsorption as SC16 (Fig. 20), SC16-ΔUL44-Z (Fig. 21), HFEM (Fig. 22) , HFEM-ΔUL44-Z (fig. 23), F (Fig. 24) and R6012 (Fig. 25).

The t50 values were very similar and are shown in Table 2.

4.12

Adsorption characteristics of all HSV-1 viruses and their gC negative counterparts on Vero cells.

Following the results obtained on BHK21/C13 cells it was decided to look at the different viruses (HSV-1 strain 17, SC16, HFEM, F and their gC negative mutants) in various cell lines. Vero cells were chosen since they are permissive for HSV and produce visible plaques. The observations made were similar to those made on BHK21/C13 cells. HSV-1 strain 17 wild type and gC negative viruses showed the same pattern of adsorption (Fig. 26), as did their lacZ expressing variants (Fig. 27). SC16/SC16-ΔUL44-Z (Fig. 28), HFEM/HFEM-ΔUL44-Z (Fig. 29) and F/R6012 (Fig. 30) were also indistinguishable in their adsorption characteristics.

As before SC16 and its gC negative form SC16-ΔUL44-Z, HFEM and HFEM-ΔUL44-Z, F and R6012 were compared to the HSV-1 strain 17 wild type. No differences were observed as can bee seen in the following figures SC16 (Fig. 31), SC16-ΔUL44-Z (Fig. 32), HFEM (Fig 33), HFEM-ΔUL44-Z (Fig. 34), F (Fig. 35) and R6012 (Fig. 36).

4.13

Comparison of adsorption characteristics as detected by Giemsa/β-galactosidase staining.

We wanted to determine the adsorption levels of the viruses (both wild type and gC negative) in cells that did not produce countable plaques by Giemsa staining (HeLa, section 4.14 and 3T6, section 4.15). We therefore decided to measure plaquing ability by lacZ expression and β-galactosidase staining.

Prior to doing this we needed to ensure that using the two forms of staining (β-galactosidase/Giemsa) gave similar results. An adsorption experiment was performed in duplicate with HSV-1 strain 17 wild type, lacZ expressing strain 17, the strain 17 gC negative mutant and gC negative lacZ; one set being stained with Giemsa (the non-lacZ viruses, 17+ and gC-) and the other with X-gal (the lacZ expressing viruses, 17+lacZ and gC- lacZ). Identical results were obtained in terms of number of plaques using the two methods of staining (Fig. 37). As reported in section 4.5 both viruses showed similar adsorption rates.

4.14

Adsorption profile of HSV-1 strain 17 wild type and the lacZ expressing gC negative mutants on HeLa cells.

We proceeded to look at the adsorption rates of all lacZ expressing viruses on HeLa cells. Comparisons were made between strain 17 wild type lacZ and gC negative lacZ (Fig. 38). Comparisons were also made between strain 17 lacZ and SC16-ΔUL44-Z (Fig. 39), and HFEM-ΔUL44-Z (Fig. 40). We had previously shown in BHK21/C13 cells (section 4.5 and 4.11) that strain 17 wild type displayed the same level of adsorption as the wild type SC16 and HFEM viruses, and therefore we felt that it could be used in place of these viruses on non-plaquing cell lines to provide a guide as to the behaviours of parental viruses.
No analysis of HSV-1 strain F or R6012 were possible as lacZ expressing versions of these viruses were not available.
In all cases the adsorption kinetics of the different viruses were similar to HSV-1 strain 17 lacZ.

4.15

Adsorption profile of HSV-1 strain 17 wild type and the lacZ expressing gC negative mutants on 3T6 cells.

As in section 4.14 it was not possible to examine the adsorption of HSV-1 strain F or its gC negative mutant R6012 on this cell line because of the lack of availability of lacZ expressing versions.

Following the same comparisons as in section 4.14 we found that the HSV-1 strain 17 lacZ expressing viruses (17+ and gC negative) produced a similar adsorption pattern (Fig. 41). Figure 42 shows the comparison between SC16-ΔUL44-Z and strain 17 lacZ, while figure 43 shows that there was also no difference between strain 17 lacZ and HFEM-ΔUL44-Z.

4.16

Heparin inhibition assays.

HSV displays reduced binding to cell surfaces that have been enzymatically stripped of heparan sulphate or to mutant cells that do not produce heparan sulphate (see discussion - section 5). Reductions in levels of cell surface heparan sulphate produce a reduction in viral infectivity that is accompanied by a reduction in viral adsorption. HSV virions can bind to immobilised heparin and soluble heparin can block the binding of virions to cells (WuDunn and Spear, 1989). This is assumed to be due to blocking heparan sulphate-binding sites on the virion surface. It has also been shown that heparin binding proteins such as platelet factor 4 and fibroblast growth factor (FGF) can inhibit HSV adsorption and plaque formation, probably through competition for receptor sites associated with the cell surface heparan sulphate. The addition of exogenous heparin can block the adsorption process but addition of the other glycosaminoglycans can not do this.

Exogenous heparin was used in order to inhibit the binding of virus to cells. Glycoprotein C negative mutants should be less affected by the interference than wild type viruses (Herold, 1995). A possible explanation for this is that since gC negative virions are deficient in a receptor binding route; the attachment to heparan sulphate proteoglycans is no longer available for blocking by exogenous heparin.

A comparison was made of the ability of heparin to block the adsorption of the HSV-1 strains 17, F, SC16 HFEM and their gC negative derivatives. All of the parental viruses were shown to be sensitive to varying concentrations of heparin, although the dose response is not identical and on the whole the gC negative mutants are less sensitive to heparin than their parental viruses.

Initially the experiment was carried out using HSV-1 strain 17 viruses only (Fig. 44). All viruses were inhibited in binding by heparin (Fig. 45).
Later experiments using all viruses showed that they all followed the same pattern of blocking by heparin (Fig. 46).

As expected, SC16-ΔUL44-Z was less affected by the addition of heparin than SC16 (Fig. 47). HFEM-ΔUL44-Z appeared to be less affected by the application of exogenous heparin than HFEM (Fig. 48), however it is not certain if this difference is significant.. This was also the case with HSV-1 strain F, were R6012 appeared to be less affected by exogenous heparin than the wild type F (Fig. 49).
All of the gC negative viruses were compared to each other (Fig. 50, see scetion 3.9 for method), and they showed differing levels of inhibition in adsorption, SC16-ΔUL44-Z was the least affected and the HSV-1 strain 17 gC negative lac Z expressing virus appeared to be most inhibited in binding.

5. Discussion.

As discussed in chapter one the entry of herpes simplex virus (HSV) into host cells involves a cascade of interactions between several virion envelope components and molecules on the cell surface. The entry of the virus can be broken down into two parts: adsorption (section 1.4.1) where the virus attaches to the cell surface membrane; and penetration (section 1.4.2) where the nucleocapsid enters the cytoplasm. Attachment of the virus is the first step in the entry process and is one of the major determinants of cell and tissue tropism. Glycoproteins B and C have been shown to be involved in the initial attachment phase when positively charged residues interact with negatively charged heparan sulphate moieties. This initial attachment facilitates a second, stronger attachment where gD binds a cellular receptor (see section 1.2.3) to form a fusion bridge. Following attachment the virus penetrates the cell by fusion of the virus envelope with the cell plasma membrane. Virus penetration is a highly complex process involving the cooperative activities of multiple viral glycoproteins (gD, gH/gL and gB).

There are several lines of evidence identifying heparan sulphate as the initial receptor for HSV infection. Firstly, heparan sulphate proteoglycans are found on the surface of most vertebrate cell types, including those that are susceptible to HSV infection (Gerber *et al.,* 1995a and b, Herold *et al.,* 1991; Lycke *et al.,* 1991; Subramanian *et al.,* 1994; WuDunn and Spear, 1989). Secondly, enzymatic removal of heparan sulphate from the cell surface or the use of cell lines selected to be lacking in heparan sulphate makes the cells at least partially resistant to HSV infection through a reduction in viral attachment (Banfield *et al.,* 1995; Grunheid *et al.,* 1993; Shieh *et al.,* 1992). Finally, heparin inhibits viral infection by blocking the heparan sulphate binding domain on the virus envelope (Herold *et al.,* 1995, 1996, Shieh and Spear, 1994). Both gC and gB bind heparin-sepharose individually and can be eluted with soluble heparin (Herold *et al.,* 1991). In general during infection with herpesviruses, more that one envelope protein plays a critical function in virus adsorption to cellular receptors. Kuhn *et al.* (1990) showed that the interactions of gC and gB with the cell surface occur independently and do not involve complex formation. Thus both gC and gB must be considered to be the principal mediators of HSV-1 attachment. Campadelli-Fiume *et al.* (1990) presented evidence that there are at least two pathways for viral attachment to cells; one mediated by gC with the other independent of gC and possibly these pathways make different contributions in different strains of virus or in different cells.

Trybala *et al.,* (1993) could not exclude the use of a heparan sulphate-binding factor as a substitute for gC in gC deficient virions. Glycoprotein C may also interact with a specific receptor other than heparan sulphate, possibly complement receptor 1 (CR1) (Sears *et al.,*

1991). It has been shown that certain modified heparin components differentiate the binding of HSV-1 gC and gB with cell surface heparan sulphate (Herold *et al.,* 1995) and this difference may therefore play a role in cell tropism and binding ability.

Herold *et al.* (1996) provided evidence supporting the idea that the envelope glycoproteins of HSV-1 and HSV-2 interact with different affinities for different structural features of heparin. This may also be true between HSV-1 strains explaining the different observations regarding the role of gC in viral binding (see this thesis and Griffiths *et al.,* 1998).

During the course of this project it was found that gC negative viruses displayed no discernible phenotype on a range of cells (Griffiths *et al.,* 1998); a result that contrasts with other reports. These previous studies assume that gC plays a principal role in the adsorption of virus to cells. This assumption arises from the finding that significantly fewer gC negative virions than wild type virions bind to cells under conditions approaching saturation of available sites on the cell surface. (Herold *et al.,* 1991).

It was previously reported that virions which lack gC bind to cells with reduced efficiency (Fuller and Spear, 1985; Langeland *et al.,* 1990; Herold et al., 1991; Trybala *et al.,* 1994).

In the results section of the thesis presented here, a study is described which was aimed at examining the adsorption kinetics of several HSV-1 strains (17+, F, SC16, and HFEM) lacking gC on a range of cell types (BHK, 3T6, HeLa and Vero). Prior to this work, and that by Griffiths (Griffiths *et al.,* 1998), studies of the role of gC in non-polarised cells were limited to the KOS strain (Langeland *et al.,* 1990; Herold *et al.,* 1991, 1994; Trybala *et al.,* 1994).

The study presented here suggests that deletion of gC does not significantly affect the binding of wild type HSV-1 of the strains used to the cell lines examined. However, gC is a heparin-binding glycoprotein and therefore has a role in viral binding, possibly this role is more significant on other cell lines or in different strains or under specific conditions.

Glycoprotein C negative virions were indistinguishable from their wild type parents in their adsorption characteristics, and all viruses used had particle to p.f.u ratios that were within the normal range. An investigation into the inhibition of virus adsorption by exogenous heparin is also described. Some strains adsorbed more efficiently in the presence of exogenous heparin. The heparin inhibition data are not simple to interpret since heparin binds weakly to gB as well as binding to gC and thus gB may have a role in triggering the fusion reaction (Shieh and Spear, 1994). It is however difficult to countenance the idea that gC has an important role in adsorption with the observation that gC negative mutants are of higher specific infectivity than wild type viruses in the presence of heparin.

One possible consideration is that all the wild type viruses used in the study lacked a functional gC and as result were defective before mutagenesis of the glycoprotein. This

suggestion is however most unlikely since a range of viral strains from different sources were used. All wild type viruses were gC positive by Western blotting while all mutant viruses did not express gC as judged by this method; additionally the profile produced by Southern blotting detected no obvious deletion.

These observations suggest that under the conditions tested (virus strains and cell types) the role of gC in the adsorption process can be substituted for by gB and that the binding of heparin to gC causes a decrease in the infectivity of the virion. Cell surface glcosaminoglycans are required for HSV-1 infection and wild type viruses mediate their binding to them via gB and gC. A possible explanation of the higher specific infectivity of virus in the presence of heparin is that the binding of exogenous heparin to gC sterically inhibits adsorption through gB.

A deletion mutant lacking both gC and gB coding sequences was demonstrated to be substantially impaired in binding (Herold *et al.,* 1994), suggesting a role for gB in heparan sulphate binding and adsorption. This mutant did however retain some residual attachment ability, indicating either that other viral glycoproteins have limited heparan sulphate binding activity or that additional receptors are recognised in the absence of heparan sulphate binding. The paper published by Laquerre *et al.,* (1998) reports a decreased capacity for attachment of a gB mutant deleted in the heparan sulphate binding domain. This report contrasts with previous studies showing gB negative mutants to have the same level of binding as wild type virus (Cai *et al.,* 1988).

Laquerre *et al.,* 1998 follow on from these observations and examine the role of gB in adsorption. They state that gB contributes less to viral binding of heparan sulphate than gC does. In their report, even when the heparan sulphate binding region of gB and all of gC are deleted there remains a twenty percent residual binding activity. This residual binding was also seen using wild type KOS on sog9 cells; a cell line lacking glycosaminoglycans, indicating that it is heperan sulphate independent.

They also show that the heparan sulphate recognition sequence of gB differs from earlier sequences of the heparan sulphate binding element of gC (Feyzi *et al.,* 1997, Trybala *et al.,* 1996). The heparan sulphate recognition sequence of gC of PRV is different from that of HSV-1 (Herold *et al.,* 1996, Trybala *et al.,* 1996). Thus the specific heparan sulphate proteoglycans recognised by different viruses may vary; it is also possible that they may vary between strains.

Tal Singer *et al.* (1995) showed that much of gC binding to cells occurs via heparan sulphate. They developed an ELISA to examine the binding of purified gC to L, Vero and BHK cells, and found that gC bound to cells in a dose dependent manner eventually reaching saturation. Further experiments involving the enzymatic treatment of cells and the use of heparan

sulphate deficient cell lines demonstrated that gC binds to cell surface HS. However, they found no difference in the binding ability of a biotinylated baculovirus expressing gC to two mutant cell lines gro2C and sog9, even though sog9 cells are ten times more resistant to HSV infection that gro2 cells. They regard this as suggesting that virion components other than gC, possibly gB, are responsible for the difference in infectivity and that the residual binding of gC to the mutant cell lines may involve molecules other than glycosaminoglycans.

This group also noted that soluble gC did not inhibit plaque formation even though it is considered important for attachment. This plaque inhibition assay argues against a significant role for gC in the entry process. Another observation made by this group was that the interaction of soluble gC with cells blocks the attachment of HSV-1. Soluble gC2 also blocked HSV-1 attachment indicating that gC1 and gC2 bind to the same or similar receptors. The group argues that the blocking of attachment does indicate a significant role for the glycoprotein in attachment as it may be a weak interaction that occurs at 4°C during their assay that can be overcome by gB or other viral factors at the higher temperatures used in the plaque assays.

They made a mutant form of gC lacking residues 33 to 123 which when expressed in a baculovirus system bound significantly less well to cells than wild type gC expressed in the same system (Tal-Singer *et al.,* 1995), and competed poorly with the wild type for binding. These results suggest that residues 33-123 are important for gC attachment to cells. However, both the mutant and wild type forms of gC bound to immobilised heparin, indicating that the binding of these proteins to the cell surface involves more than a simple interaction with heparan sulphate. It is also possible that other viral components e.g. gB are capable of attachment via the non-heparan sulphate binding suggested here.

Despite the weight of historical data implicating gC of the alphahepesviruses in the adsorption of virions to the cell surface, monoclonal antibodies and polyclonal anti-gC antibodies show low or moderate inhibition of attachment (Fuller and Spear 1985) and the results of this study and the data published by Griffiths *et al.* (1998) suggest that gC of the HSV-1 strains used plays no significant role in this process. These data are similar to those obtained for the only HSV-2 strain examined so far (Gerber *et al.,* 1995) which reports no significant difference between wild type parental strain (G) and a gC deletion mutant in the number of virions binding to Hep-2 cells. They also report normal particle to p.f.u ratios for their gC-2 negative mutant.

Nor was there a delay in the penetration of adsorbed HSV-2 (G) on either Hep-2 or Vero cells. The genes for gC-1 and gC-2 have diverged to a greater extent than most of the other glycoprotein genes. Glycoprotein C-2 is significantly smaller than gC-1 (Dowbenko and Lasky, 1984; Swain *et al.,* 1985; Fink *et al.,* 1983) and diverges from its HSV-1 counterpart

at the amino terminus and also differs in the extent of glycosylation (Dall'Olio *et al.*, 1985; Serafini-Cessi *et al.*, 1984). This may indicate the lack of importance of this gene for viral binding. Alternatively this divergence in gC may indicate that differences in the interactions of HSV-1 and 2 with cell surface heparan sulphate occur, possibly influencing viral tropism. However, gC-1 and 2 use the same receptor (Tal-Singer *et al.*, 1995) and demonstrate similar dissociation constants.

At the time they were published, the Gerber results were taken to suggest that gC-2 differed widely from gC-1 since it did not play the predominant role in HSV-2 binding. However, in the light of the results presented here and the work of Griffiths *et al.*, 1998, it is suggested that gC-1 and 2 play similar roles, with gC of HSV-1 strain KOS possibly being slightly different. It is possible that the differences observed are due to differences in the role of gB and not gC with the relative contributions of the individual glycoproteins to binding being different across strains.

Neomycin and polylysine inhibit the binding of HSV-1 to cells. HSV-1 KOS was less sensitive to neomycin than strain 17syn+ indicating a strain dependent sensitivity. There is however some disagreement about how this inhibition occurs. Campadelli-Fiume *et al.* (1990b) found that wild type HSV-1 and gC negative mutants of HSV-1 and HSV-2 are blocked by neomycin and polylysine from attaching to BHK cells. They concluded from these results that the target of the polycations is the interaction of a virion component other than gC with its receptor in BHK cells.

Glycoprotein C is present in the region of HSV-1 sensitive to both neomycin and polylysine (Langeland *et al.*, 1990) although a gC negative mutant had similar drug sensitivity to that of the parental wild-type strain. These results suggest that gC is not the protein associated with the sensitivity to polylysine and neomycin, indicating that gC may not be significantly involved in binding. However, Herold and Spear (1994) report that neomycin partially inhibited the gC dependent binding of virions and that plaque formation by both gC positive and gC negative strains of HSV-1 was inhibited by neomycin. Thus they conclude that neomycin inhibits HSV-1 infection by competing with gC-1 for binding heparan sulphate glycosaminoglycans.

Since the information provided by the work shown here suggests that gC does not have a significant role in viral adsorption and gC has long been known to be dispensable *in vitro*, it is possible that the main role of gC takes place *in vivo*.

Genes that are dispensable for growth in cell culture may extend the host range of the virus in vivo. Many mutants that do not express gC grow as well as wild type HSV-1 in culture and thus gC is considered to be nonessential for virus replication *in vitro*. However, nearly all clinical isolates of HSV-1 express gC suggesting that gC may have an important role in virus

replication or survival in humans (Holland *et al.,* 1984). In the study presented here the gC negative mutant displayed the same rate of reactivation as the wild type virus indicating that the role of gC does not lie in latency/ reactivation of the virus in the mouse footpad model.

As already mentioned in section 1.4.5, gC is capable of binding complement component C3b. HSV-1 and 2, PRV, BHV-1 and EHV-1 all bind C3b, and this function has been mapped to gC or its homologues (Eisenberg *et al.,* 1987, Friedman *et al.,* 1984, Huemer *et al.,* 1993). Glycoprotein C also protects HSV-1 infected cells from lysis by antibody plus complement; as well as against lysis mediated by complement alone (Hidaka *et al.,* 1991).

Glycoprotein C negative mutant strains are unable to bind C3b and are approximately 5,000 fold more susceptible to complement neutralisation than wild type virus which indicates that gC has a vital role in protection against complement neutralisation (Friedman *et al.,* 1996). Lubinski *et al.,* 1998 demonstrated that gC is a virulence factor wihose deletion has a 10-100 fold effect on virulence.

Differences between strains are noted in complement protection as well as for the role of gC in adsorption (Friedman *et al.,* 1996, see section 1.4.3).

Friedman *et al.* (1996) also suggest a link between the complement modulation of gC and its binding to heparan sulphate. They point to the observation that heparin is an inhibitor of complement activation and thus gC binding to heparan sulphate may add to the inhibitory activity mediated by gC. Another observation that suggests a link is that of Sears (1994), showing that mammalian C3 receptors are widely distributed on cells and that activated C3 fragments on HSV may enhance infection by promoting virus attachment to complement receptors on cells.

It is possible that *in vivo* HSV gC functions are supplemented or enhanced by the function of other viral glycoproteins as appears to be the case in PRV (Mettenleiter *et al.,* 1988).

Another possible role for gC in vivo is to provide a docking mechanism for the virus that mediates rapid adhesion to cells allowing the interaction of gD with its receptors. Haywood 1994, suggests that low-affinity binding to an abundant receptor lets the virus roam over the cell surface by making and breaking weak bonds until it reaches a second receptor. This docking mechanism may be one of the roles played by HSV gC and could possibly contribute to the in vivo survival of the virus.

There is a large amount of evidence implicating gC in the adsorption of alphaherpesviruses. However, these results and those of Griffiths *et al.,* (1998) and Gerber (1995) suggest that the role of gC in adsorption may be strain dependent.

It is also possible that different HSV proteins may be involved in adsorption; the virion protein responsible may not be a glycoprotein since adsorption can occur in the absence of normal glycosylation (Langeland *et al.,* 1990).

The results presented here lead to the assumption that the heparan sulphate binding function of gB is sufficient to mediate virus attachment to heparan sulphate and to compensate for the absence of gC.

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Appendix

Figures relating to Chapter 4: Results

Figure 4.1: Structure of the HSV-1 strain 17 gC negative mutant.

A. An expansion of the HSV-1 genome showing the UL44 gene encoding gC. The positions of the 5' and 3' ends of UL44 are marked. The position of the *Xba*I site where the insertion is made is also shown. All n.p. are according to McGeoch *et al.,* 1988a.

B. An expansion of the *Xba*I site, showing where *Xba*I cuts.

C. An expansion of the *Xba*I site containing the 4bp insert (the insert is marked in the diagram) producing a stop codon towards the end of the ORF close to the transmembrane domain (indicated by solid box). The mutant produces a truncated form of the glycoprotein that is secreted and is not incorporated into the virion.

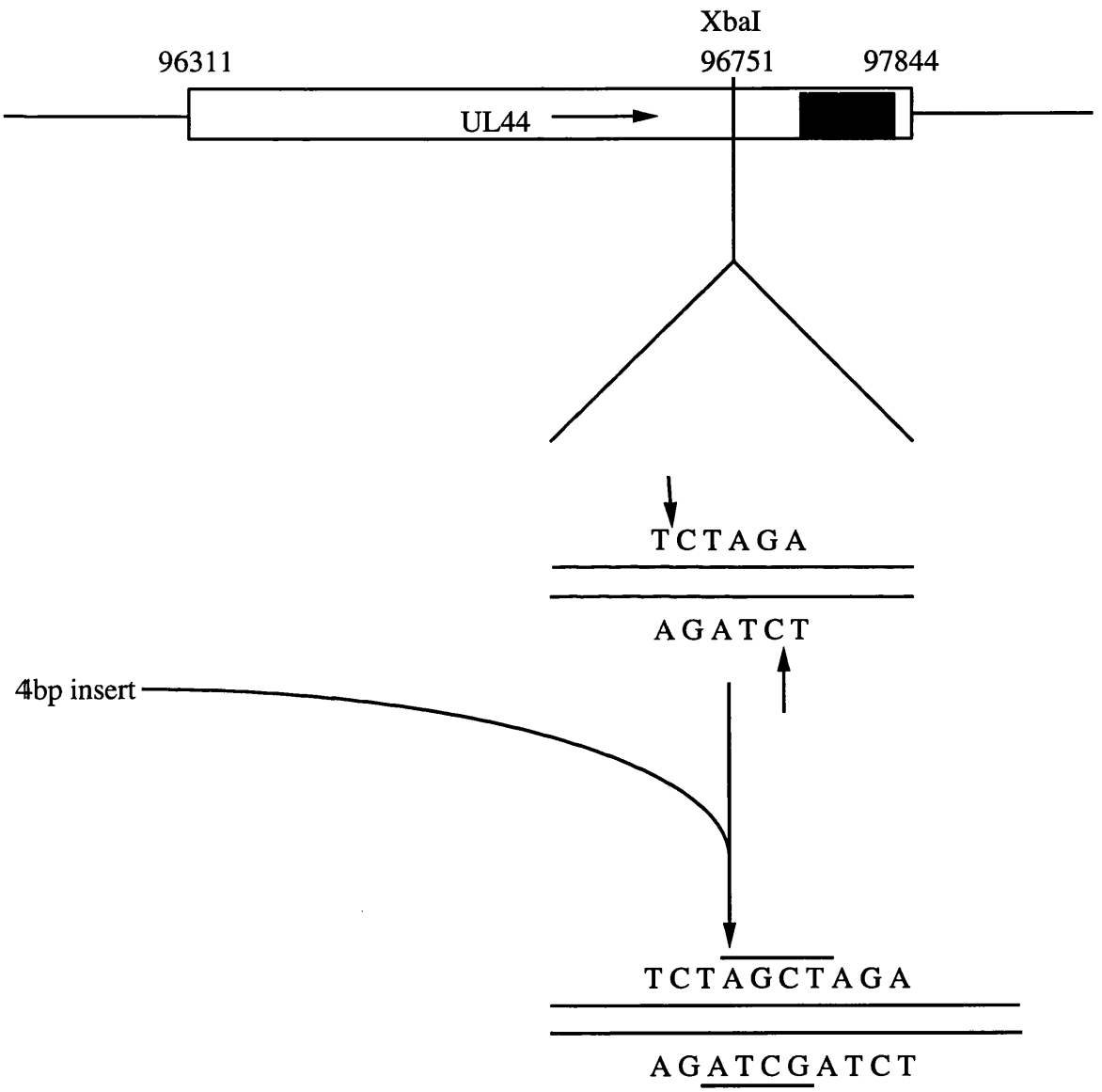
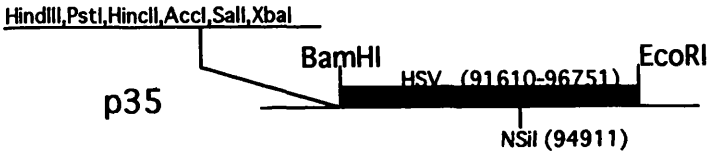

XbaI
96311
96751
97844
UL44
TCTAGA
AGATCT
4bp insert
TCTAGCTAGA
AGATCGATCT

Figure 4.2: Construction of lacZ expressing HSV-1 strain 17 viruses.



Oligo insertion at NsiI site generates p35(PacI)

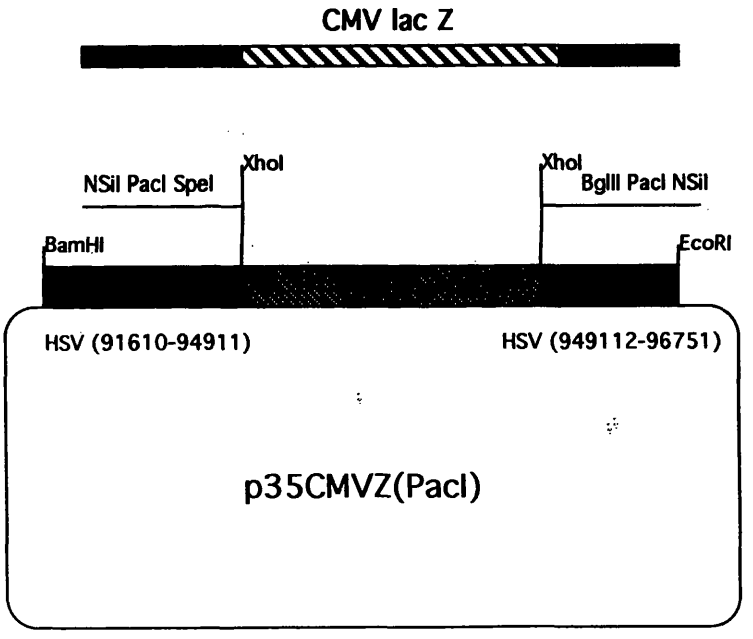
NSi I insertion oligo

NSF 5' TTTAATTAAACTAGTCTCGAGAGATCTTTAATTAAATGCA 3'

NSB 3' ACGTAAATTAATTTGATCAGAGCTCTCTAGAAATTAATTT 5'

NSiI PacI SpeI XhoI BglII PacI NSiI

CMVlacZ cassette cloned into XhoI site on p35(PacI)



Recombine with HSV-1 at UL43 locus to generate blue virus. Once isolated viral DNA digested with PacI can be used to produce HSV-1 with unique cloning site for direct insertion of lacZ neuronal promter cassettes.

Figure 4.3 Structure of R6012.
The top line of the diagram shows the HSV-1 strain F genome and below this is an expansion of the *Hpa*I-*BamH*I fragment (93730-98250). The UL44 gene was disrupted by digestion at the *Nhe*I (96276) and *Eco*RV (97649) sites, resulting in a 1,371 bp deletion after ligation of the ends (Sears *et al.*, 1991).

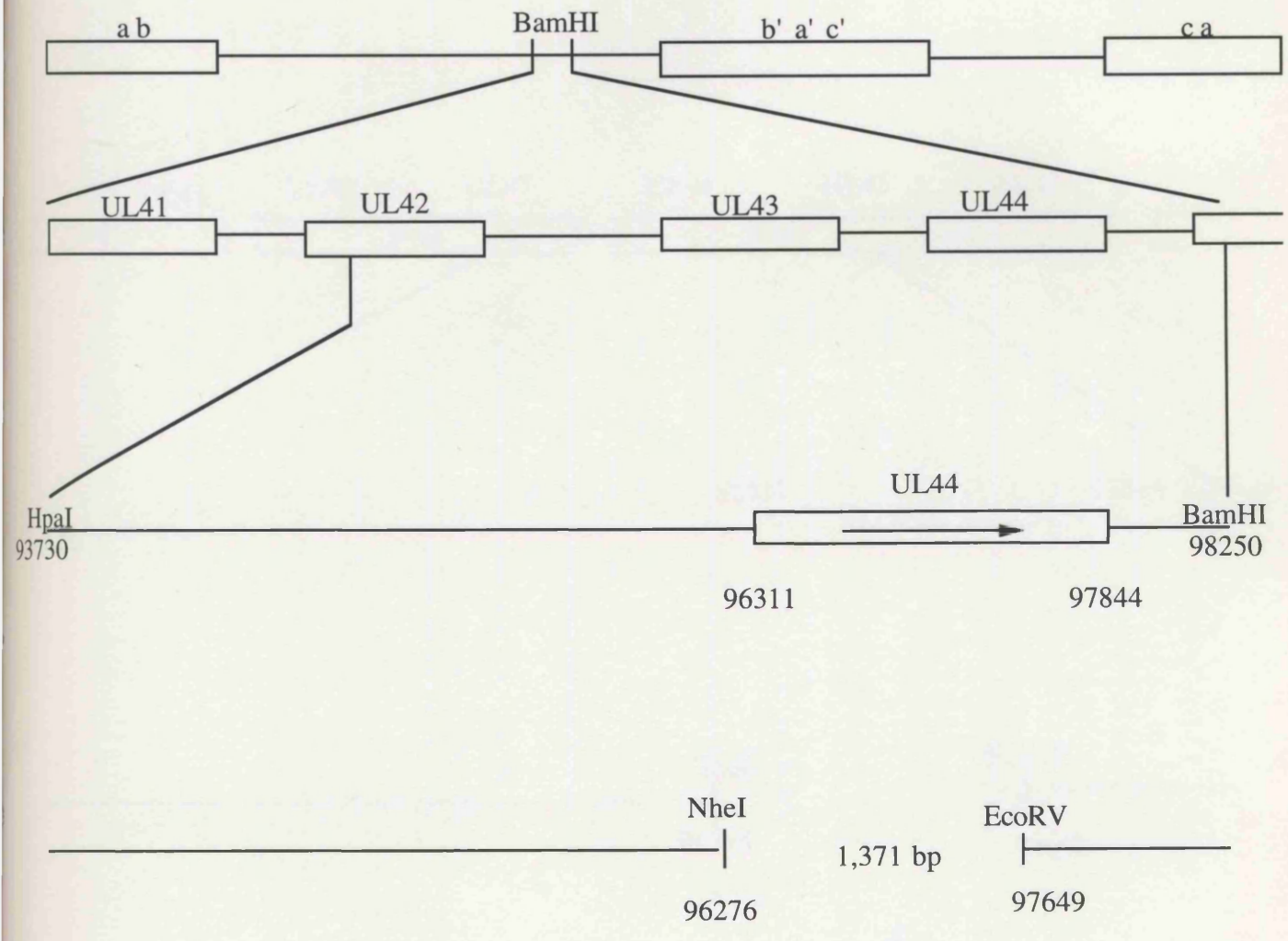

a b
BamHI
b' a' c'
c a
UL41
UL42
UL43
UL44
UL44
HpaI
93730
BamHI
98250
96311
97844
NheI
EcoRV
1,371 bp
96276
97649

Figure 4.4: Structure of SC16-ΔUL44-Z / HFEM-ΔUL44-Z.
The top line of the diagram shows the HSV-1 genome and below this is an expansion of the *Kpn*It fragment. The UL44 gene was disrupted by digestion at the *Nhe*I (96276) and *EcoR*V (98649) sites before insertion of a lacZ expression cassette (Forrester *et al.*, 1992).

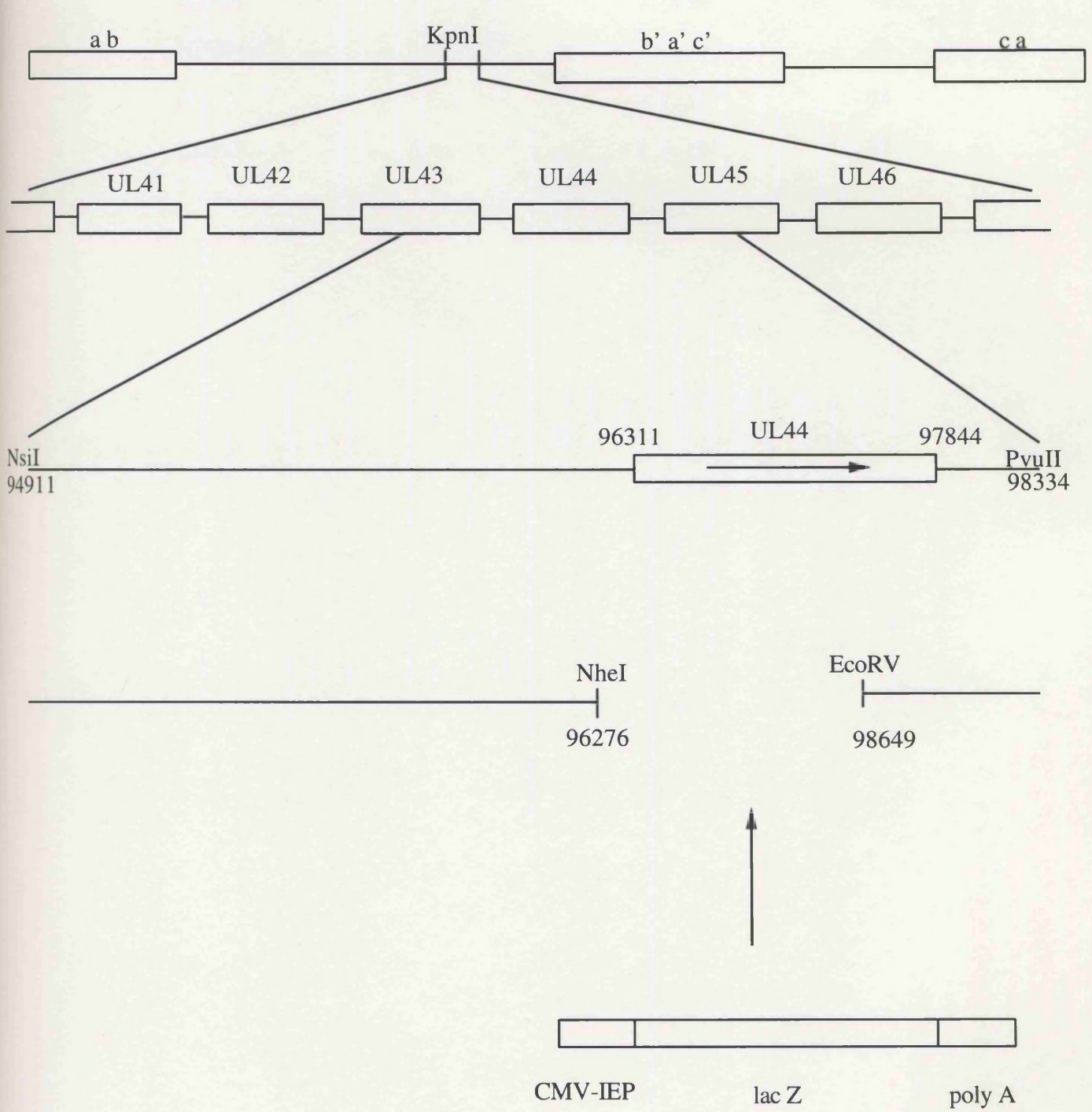

a b
KpnI
b' a' c'
c a
UL41
UL42
UL43
UL44
UL45
UL46
NsiI
94911
96311
UL44
97844
PvuII
98334
NheI
96276
EcoRV
98649
CMV-IEP
lac Z
poly A

Table 4.1: Particle to pfu ratios of the viruses used in this study.
Representation of the particle to pfu ratios of viruses used in this study.

Virus	Particle/ml	pfu	Particle:pfu
17+	$8.5x10^{11}$	$2x10^{10}$	42
gC-	$1.6x10^{11}$	$2.5x10^{9}$	62
SC16	$8.08x10^{9}$	$1.7x10^{8}$	47
SC16gC-	$2.26x10^{10}$	$2.86x10^{8}$	49
HFEM	$1.37x10^{11}$	$1.7x10^{9}$	78
HFEMgC-	$1.76x10^{11}$	$3.5x10^{9}$	49
F	$3.06x10^{11}$	$1.2x10^{10}$	24
R6012	$3.4x10^{11}$	$5.4x10^{9}$	63

Figure 4.5: Binding of radioactively labelled strain 17 wild type to C6 gliomal cells and BHK21/C13 cells.
This graph shows that the virus has a similar pattern of binding to both cells lines as judged by measuring bound counts.

Binding of labelled 17+.

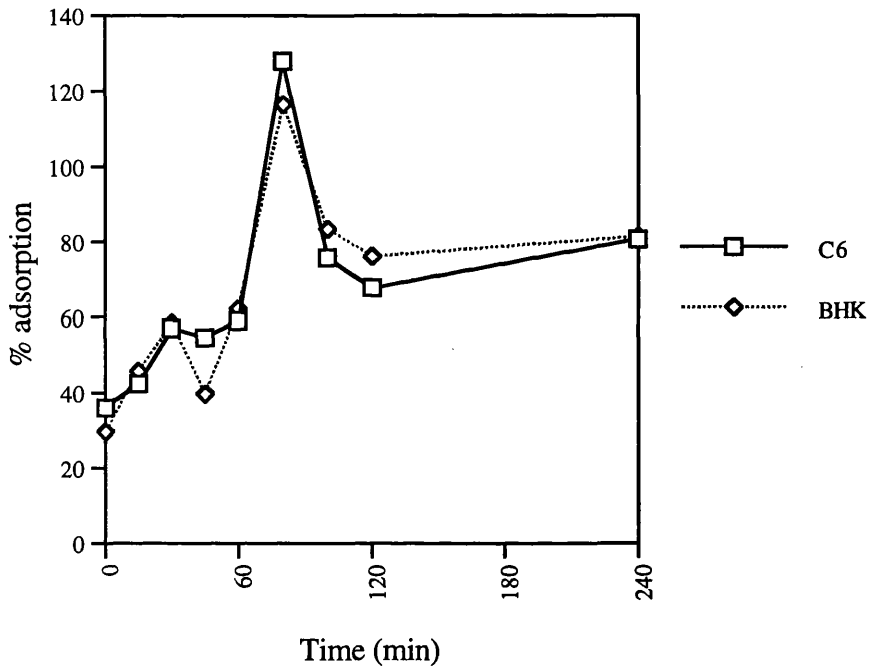


Figure 4.6: Binding of radioactively labelled strain 17 gC negative mutant to C6 gliomal cells and BHK21/C13 cells.
This graph shows that the virus has a similar pattern of binding to both cells lines as judged by measuring bound counts.

Binding of labelled strain 17 gC-.

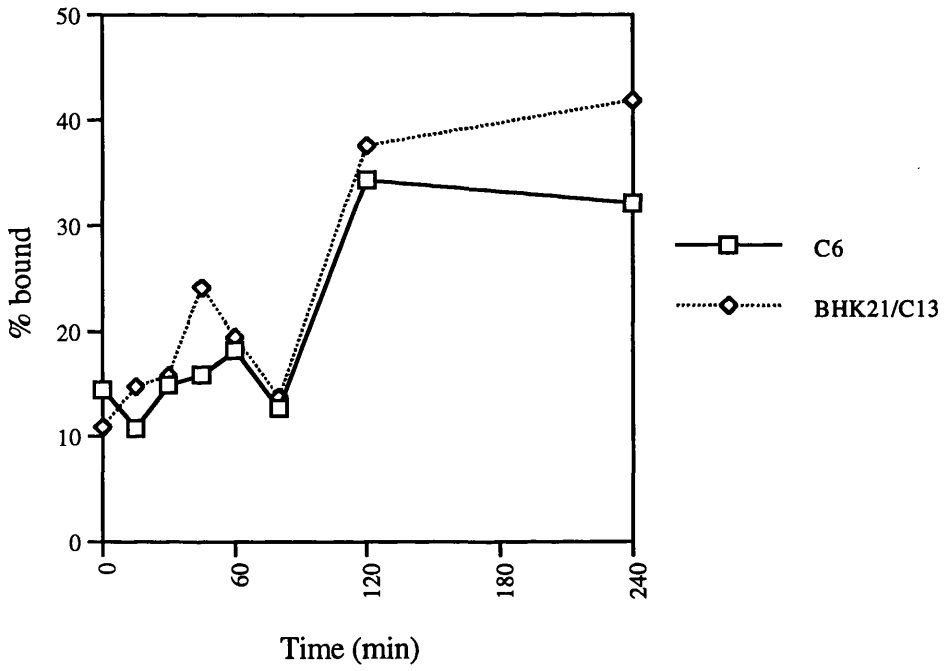


Figure 4.7: Binding of radioactively labelled strain 17 wild type and its gC negative mutant to C6 gliomal cells.
This graph shows that both viruses have similar levels of binding to C6 glioma cells as judged by measuring bound counts.

Binding of labelled 17+ and gC- to C6 gliomal cells

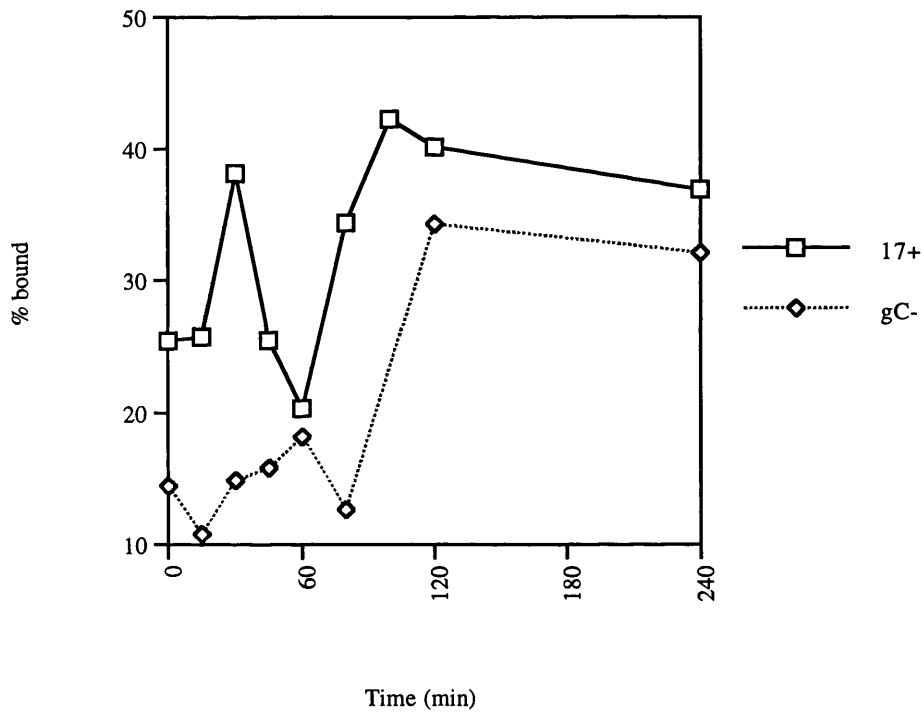
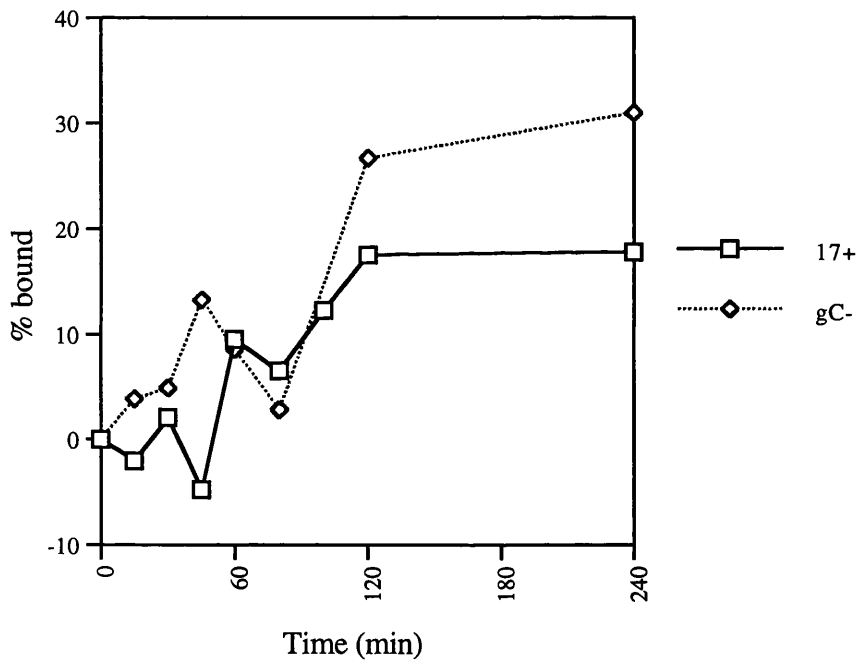


Figure 4.8: Binding of radioactively labelled strain 17 wild type and its gC negative mutant to BHK21/C13 cells.
This graph shows that both viruses have similar levels of binding to BHK21/C13 glioma cells as judged by measuring bound counts.

Binding of labelled strain 17+ and gC- to BHK21/C13 cells.



Adsorption experiments.

The graphs presented here in figures 4.9 - 4.11 and 4.17 to 4.43 show the level of adsorption at each timepoint. In each experiment the adsorption at the final timepoint for each virus (t240) is considered to be 100%. Where error bars are present they represent the standard deviation between experiments. Values given for each timepoint are the average of triplicate repeats within an experiment. The t50s for each graph are tabulated in Table 4.2.
Cmparisons between viruses were always made within the same assay.

Figure 4.9: Adsorption kinetics of HSV-1 strain 17 and its gC negative mutant on BHK21/C13 cells.

BHK, 17+ and gC-.

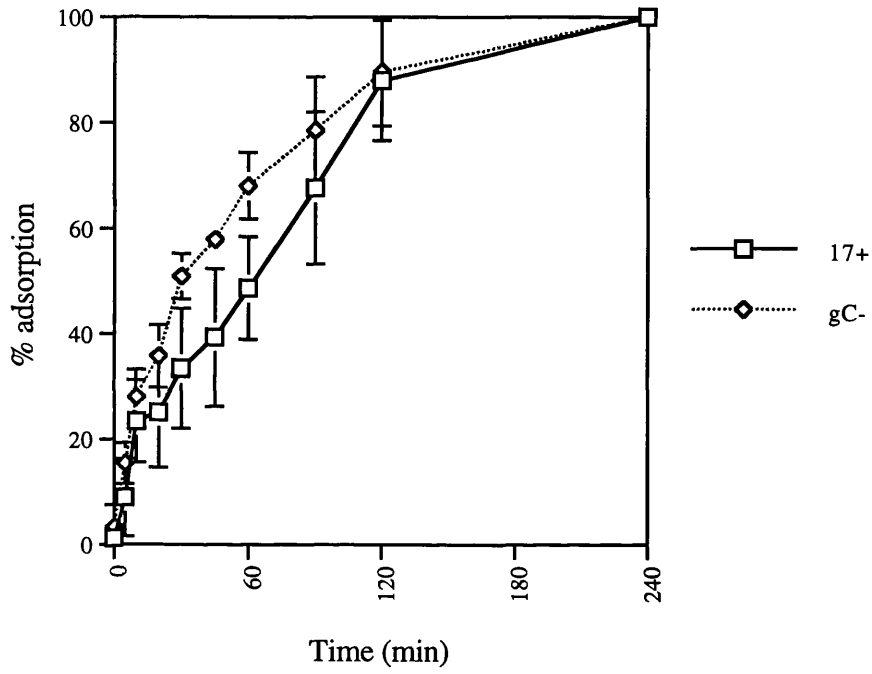


Figure 4.10: Adsorption kinetics of HSV-1 strain 17 lacZ and its gC negative lacZ expressing mutant on BHK21/C13 cells.
No standard deviation is shown due to the fact that there were insufficient repeats of the assay for statistical analysis.

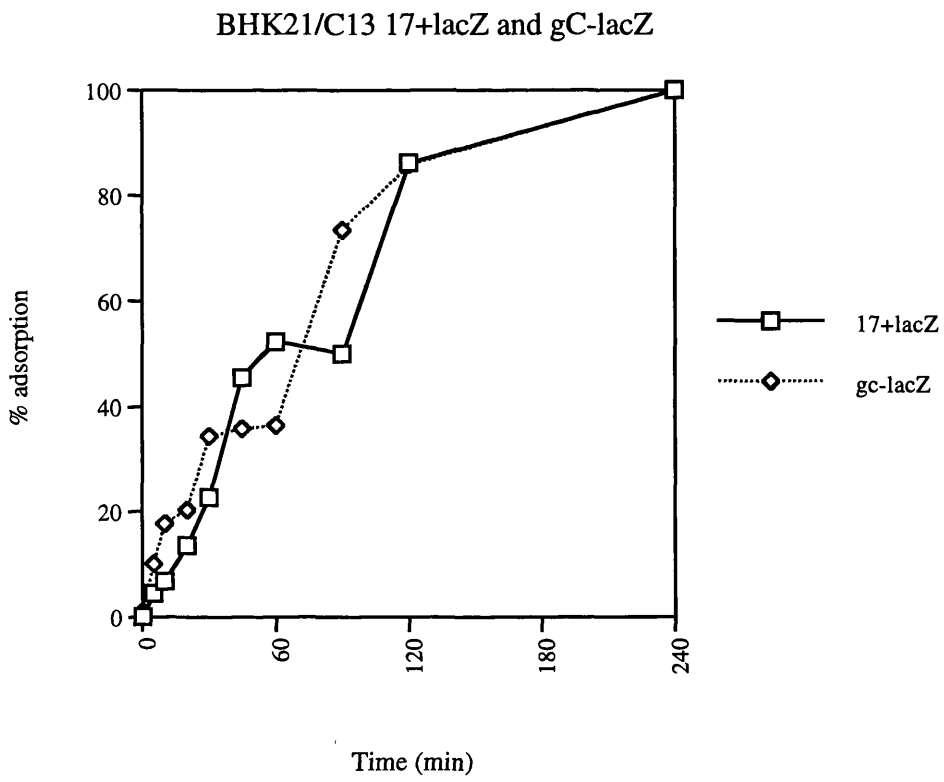

BHK21/C13 17+lacZ and gC-lacZ
% adsorption
100
80
60
40
20
0
0
60
120
180
240
Time (min)
17+lacZ
gc-lacZ

Table 4.2: Adsorption t50 values.
This table shows the t50 value (the time in minutes taken to reach 50% adsorption) for each pair of viruses being compared and the cell line used.
The table shows the values for all cell lines used in this project (BHK21/C13, Vero, HeLa and 3T6).
Values were obtained from a series of different experiments.

Cell line	Viruses	t50
BHK21/C13	17+	42
	gC-	61
BHK21/C13	17+ lacZ	60
	gC- lacZ	62
BHK21/C13	gC-	43
	gC- lacZ	36
BHK21/C13	SC16	63
	SC16gC-	48
BHK21/C13	HFEM	58
	HFEMgC-	47
BHK21/C13	F	44
	R6012	48
BHK21/C13	17+	42
	17+ lacZ	48
BHK21/C13	17+	42
	gC-lacZ	36
BHK21/C13	17+	38
	SC16	63
BHK21/C13	17+	40
	SC16gC-	44
BHK21/C13	17+	37
	HFEM	42
BHK21/C13	17+	36
	HFEMgC-	58
BHK21/C13	17+	56
	F	47
BHK21/C13	17+	56
	R6012	62

Cell line	Viruses	t50
Vero	17+	52
	gC-	57
Vero	17+ lacZ	53
	gC- lacZ	61
Vero	gC-	53
	gC- lacZ	61
Vero	SC16	56
	SC16gC-	73
Vero	HFEM	33
	HFEMgC-	29
Vero	F	52
	R6012	53
Vero	17+	53
	17+ lacZ	59
Vero	17+	53
	gC-lacZ	61
Vero	17+	62
	SC16	56
Vero	17+	52
	SC16gC-	69
Vero	17+	33
	HFEM	33
Vero	17+	33
	HFEMgC-	29
Vero	17+	53
	F	52
Vero	17+	53
	R6012	53

Cell line	Viruses	t50
HeLa	17+ lacZ	69
	gC-lacZ	73
HeLa	17+ lacZ	70
	SC16gC-	66
HeLa	17+ lacZ	76
	HFEMgC-	77
3T6	17+ lacZ	58
	gC-lacZ	55
3T6	17+ lacZ	58
	SC16gC-	48
3T6	17+ lacZ	56
	HFEMgC-	59

Figure 4.11: Adsorption kinetics of all HSV-1 strain 17 viruses used in this study on BHK21/C13 cells.

BHK21/C13, HSV-1 strain 17 viruses

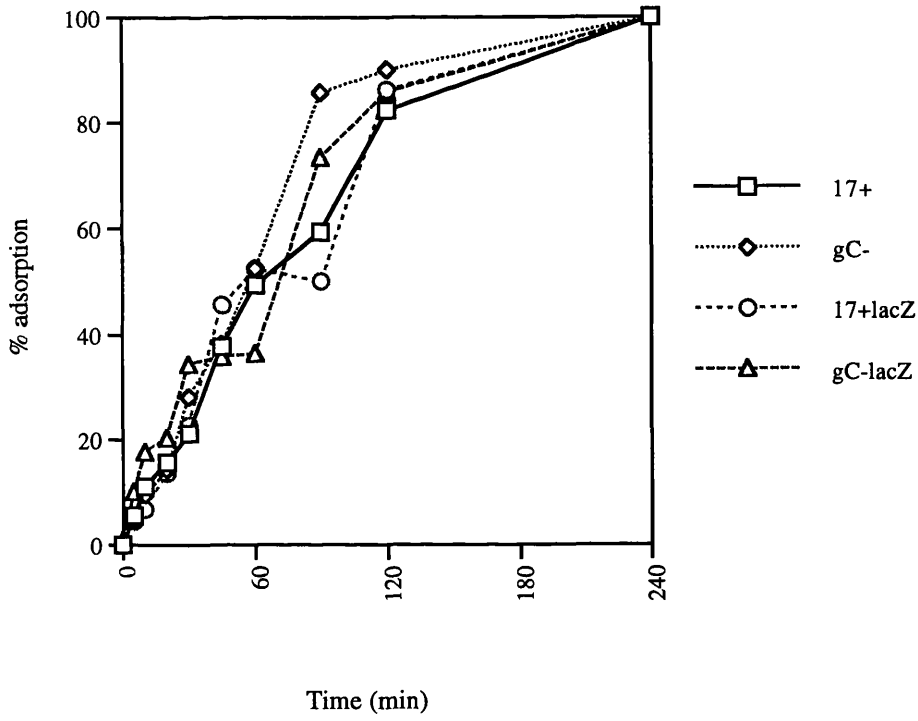


Figure 4.12: Rate of penetration into BHK21/C13 cells by HSV-1 strain 17 and its gC negative mutant.

This figure shows the rate of viral penetration as compared to time. Both HSV-1 strain 17 and its gC negative mutant display the same rate of penetration.

Penetration of BHK21/C13 cells by 17+ and gC-.

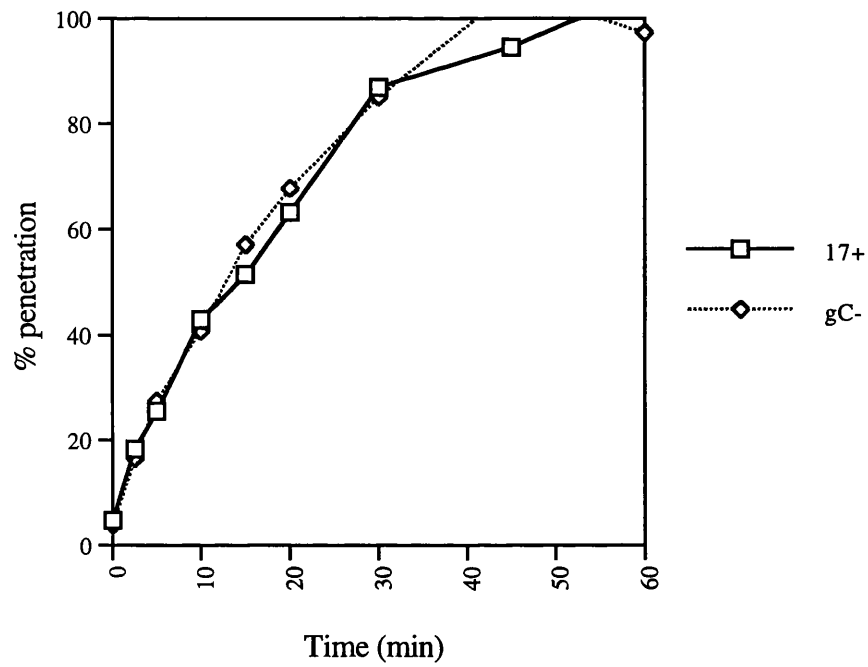


Table 4.3: Pathogenicity studies of HSV-1 strain 17 wild type and gC negative mutant following peripheral inoculation.
This table summarises the results obtained following footpad injection of the strain 17 viruses. They have similar LD_{50}s with less than 10 fold difference.

Dose / Virus	10^4	10^5	10^6	10^6	10^7	LD50
Strain 17	2/6*	3/8	8/8	8/8	ND	10^5
gC negative	0/6	3/8	4/8	4/4	4/4	10^6

* number of deaths/number injected

ND = not done

Table 4.4: Intracranial pathogenicity studies of HSV-1 strain 17 wild type and gC negative mutant.

This table summarises the results obtained following intrcranial inoculation of the strain 17 viruses. They have similar LD_{50}s with less than 10 fold difference.

Virus \ Dose	10^1	10^2	10^3	10^4	LD50
17+	7/7*	6/6	6/6	6/6	$<10^1$
gC-	1/6	5/6	5/7	7/7	$5x10^1$

*= no. deaths/no. injected

Figure 4.13: Percentage reactivation of HSV-1 strain 17 and its gC negative mutant from latently infected ganglia.
This graph shows that both viruses followed a simmilar pattern of reactivation.

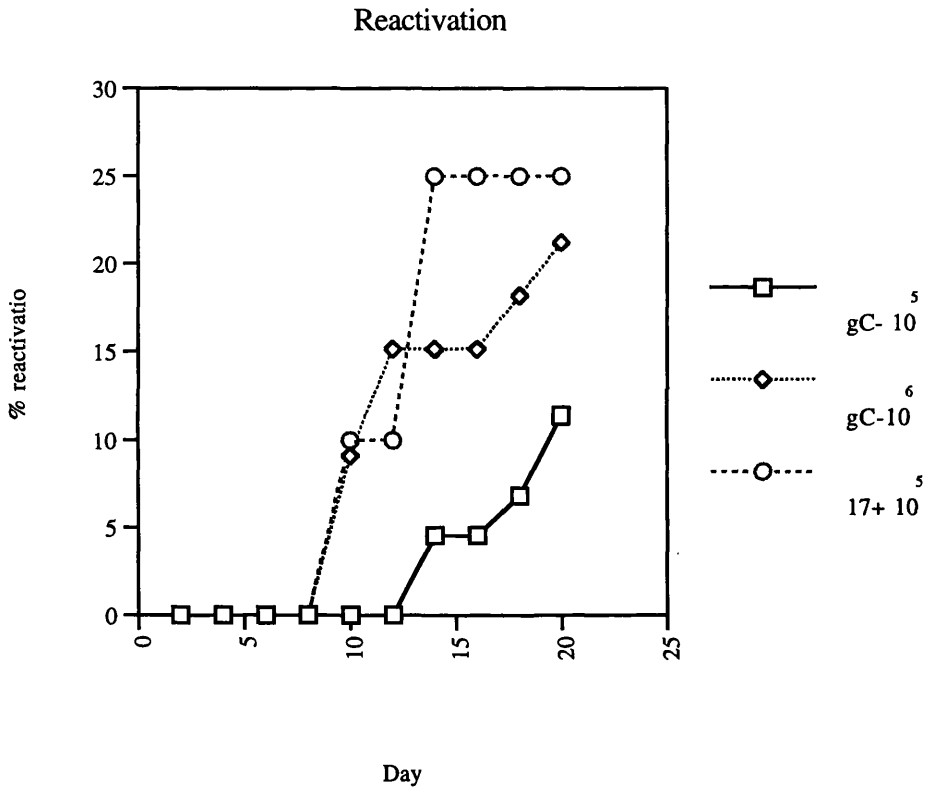

Reactivation
% reactivatio
30
25
20
15
10
5
0
0
5
10
15
20
25
Day
gC- 10 5
gC-10 6
17+ 10 5

Figure 3.14 Structure of HSV-1 gC negative mutants used in this study.

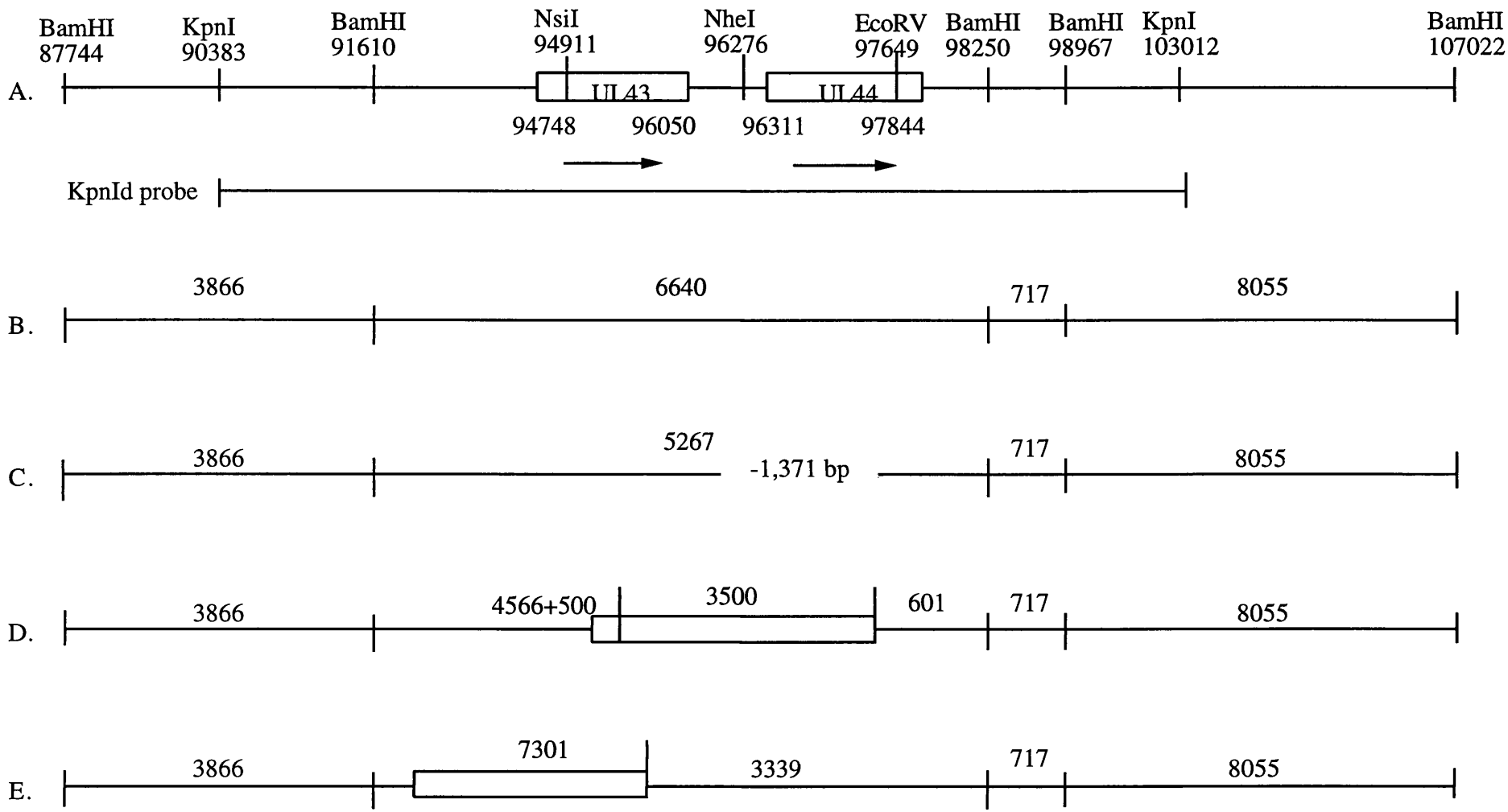
A. An expansion of the HSV-1 genome from np 87744-107022. The position of the *BamHI*, *Kpn*I, *Nsi*I, *Nhe*I and *EcoR*V sites are shown as are the locations of UL43 and UL44 which are indicated by solid boxes and run in the 5' to 3' direction.

B. The sizes of the fragments obtained from this region by digestion of HSV-1 DNA with *BamHI*.

C. The sizes obtained by *BamHI* digestion of the HSV-1 strain F gC negative virus, R6012, showing the position of the end points of the deletion. The size of the deletion is shown in the marked box.

D. The sizes obtained by *BamHI* digestion of the HSV-1 mutant viruses, SC16-ΔUL44-Z and HFEM-ΔUL44-Z. Here the deletion in gC (as for R6012, line C) is replaced by a 3.1Kb insert indicated by a solid box.

E. The sizes obtained bu *BamHI* digestion of the HSV-1 strain 17 virus with a lacZ insert in the *Nsi*I site in UL43


BamHI 87744
KpnI 90383
BamHI 91610
NsiI 94911
NheI 96276
EcoRV 97649
BamHI 98250
BamHI 98967
KpnI 103012
BamHI 107022
A.
UL43
UL44
94748
96050
96311
97844
KpnId probe
B.
3866
6640
717
8055
C.
3866
5267
-1,371 bp
717
8055
D.
3866
4566+500
3500
601
717
8055
E.
3866
7301
3339
717
8055

Not to scale.

Figure 4.15: Profile of HSV-1 gC negative viruses.
An autoradiograph of HSV-1 DNA digested with *BamHI*, Southern bloted and probed with 32P labelled *KpnId* (see figure 4.14 for example sizes of bands).
Lane 1: a Kb ladder was run to provide molecular weight markers. The sizes of the bands are marked on the left hand side.

Lane 2: HFEM-ΔUL44-Z the 6.64kb band is replaced by bands of 5.1kb (indicated by *) and 600bp. The 600bp band is not visible on this gel.
Lane 3: HFEM wild type virus with the predicted bands of 8.05kb, 6.64 kb, 3.86 kb and 717bp.

Lane 4: gC negative mutant, SC16-ΔUL44-Z which produces the same pattern of bands as HFEM-ΔUL44-Z (Lane 2).
Lane 5: SC16 wild type virus, with the predicted bands of 8.05kb, 6.64 kb, 3.86 kb and 717bp.
Lane 6: gC negative lacZ expressing form of HSV-1 strain 17, where the wild type band of 6.64kb is replaced by bands of 3.3kb (indicated by **) and 7.3kb
Lane 7: wild type strain 17 lacZ virus, where the wild type band of 6.64kb is replaced by bands of 3.3kb (indicated by **) and 7.3kb
Lane 8: the strain 17 gC- mutant, with bands of 8.05kb, 6.64 kb, 3.86 kb and 717bp.
Lane 10: R6012, the gC negative mutant for strain F. In this case the 6.64kb band is replaced by a 5.26kb band due to the deletion in UL44.
Lane 11:wild type strain F with bands of 8.05kb, 6.64 kb, 3.86 kb and 717bp.

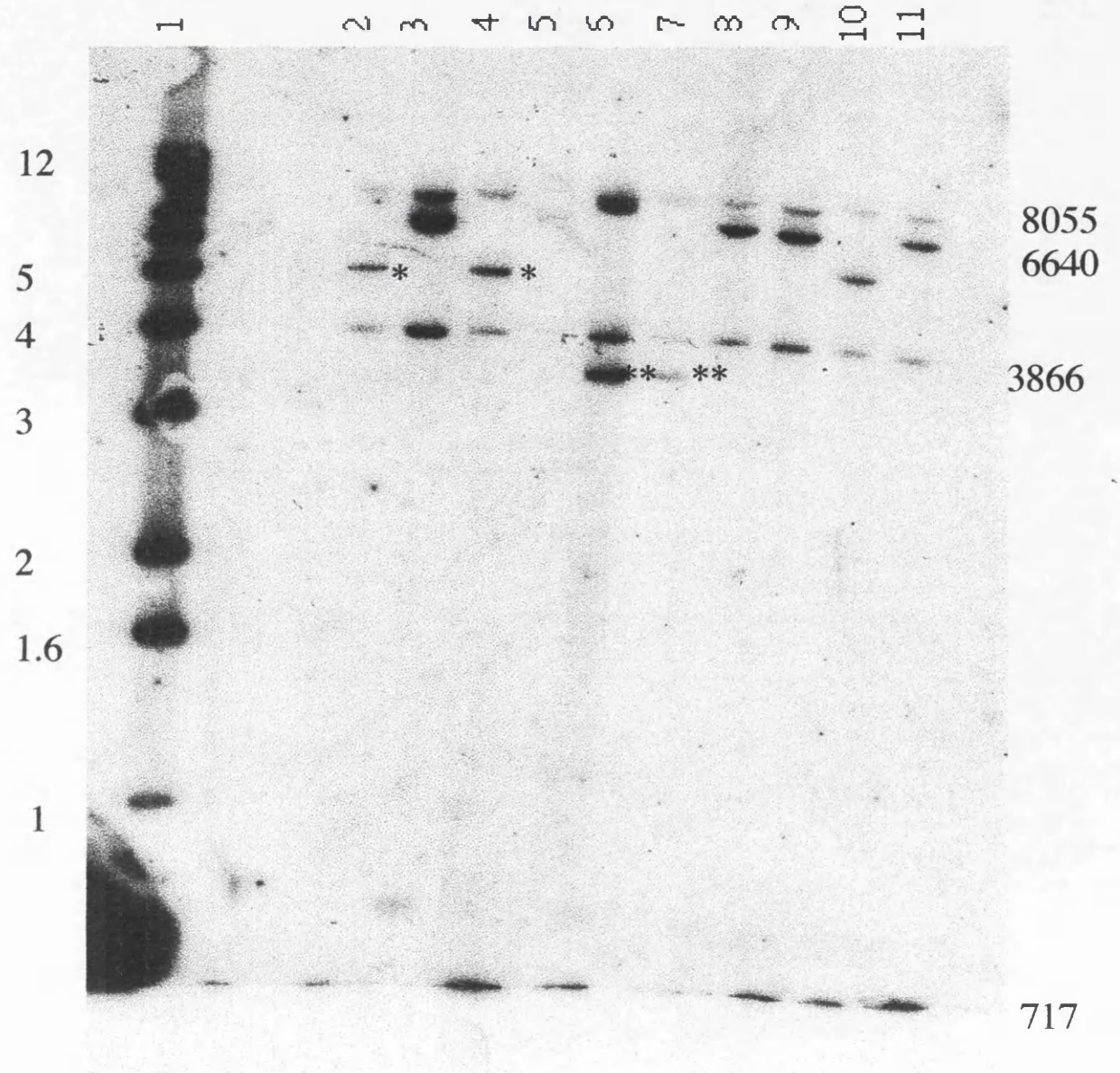

1
2
3
4
5
6
7
8
9
10
11
12
5
4
3
2
1.6
1
8055
6640
3866
717
*
*
**
**

Figure 4.16: Western Blot of gC produced by HSV-1 wild type and gC negative strains

Western blot of extracts prepared on BHK21/C13 cells infected with various HSV-1 viruses, harvested 20 hrs post infection. The incubation temperature was 37°. Samples were run on a 7.5% SDS PAGE, and probed with antibodies against gC. Bands of 60K, 55K and 52K are detected in all wild type samples.

The marker lane shows bands at 66K and 46K.
The wild type viruses all have a band at the site of gC (60K). They also have a second smaller band that is precursor gC (55K, 52K). In the case of strain F there are two precursor gC bands and there is more precursor gC than gC. This is due to differences in rate of progression of infection.
The gC negative mutant lanes do not show bands of gC or Precursor gC, they do however show a non specific band at apporoximately 40K. This band is also seen in the mock infected lane.

60K
55K
52K
Marker
Mock
HFEMgC-
HFEM
SC16gC-
SC16
R6012
F
F
17+ lacZ
gC- lacZ
17 gC-
17+
17+
gC
pre-gC

Figure 4.17: Adsorption kinetics of HSV-1 strain SC16 and its gC negative mutant SC16- UL44-Z on BHK21/C13 cells.

BHK, SC16 and SC16gC-.

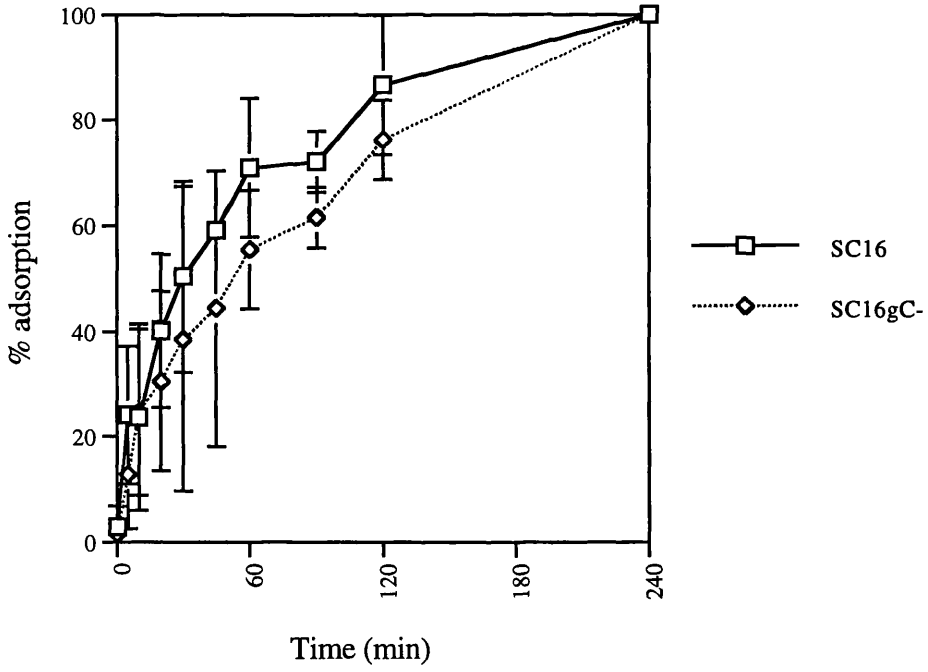


Figure 4.18: Adsorption kinetics of HSV-1 strain HFEM and its gC negative mutant HFEM - UL44-Z on BHK21/C13 cells.

BHK21/C13, HFEM and HFEMgC-.

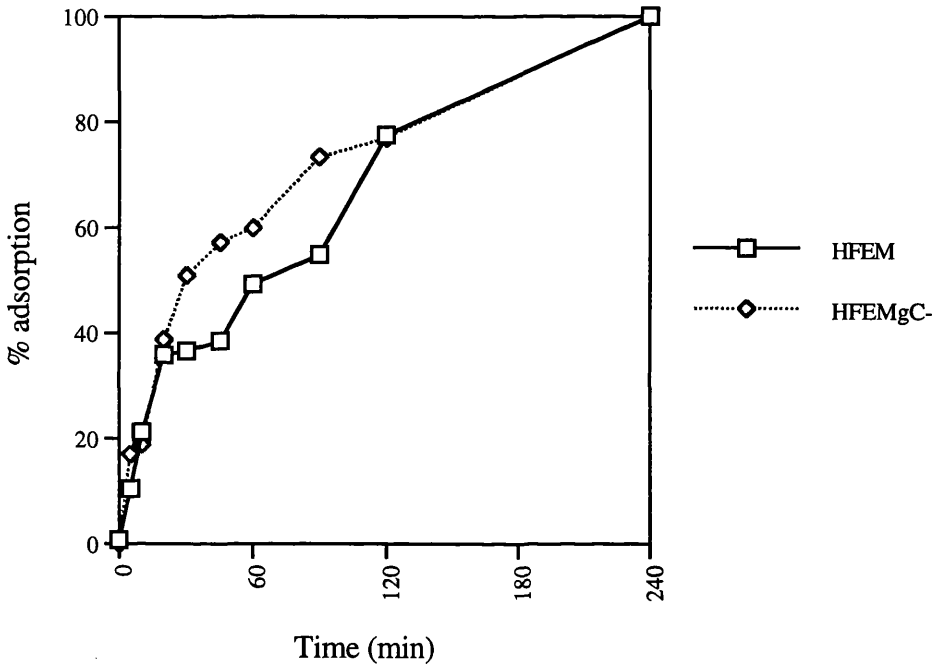


Figure 4.19: Adsorption kinetics of HSV-1 strain F and its gC negative mutant R6012 on BHK21/C13 cells.

BHK21/C13, F and FgC-.

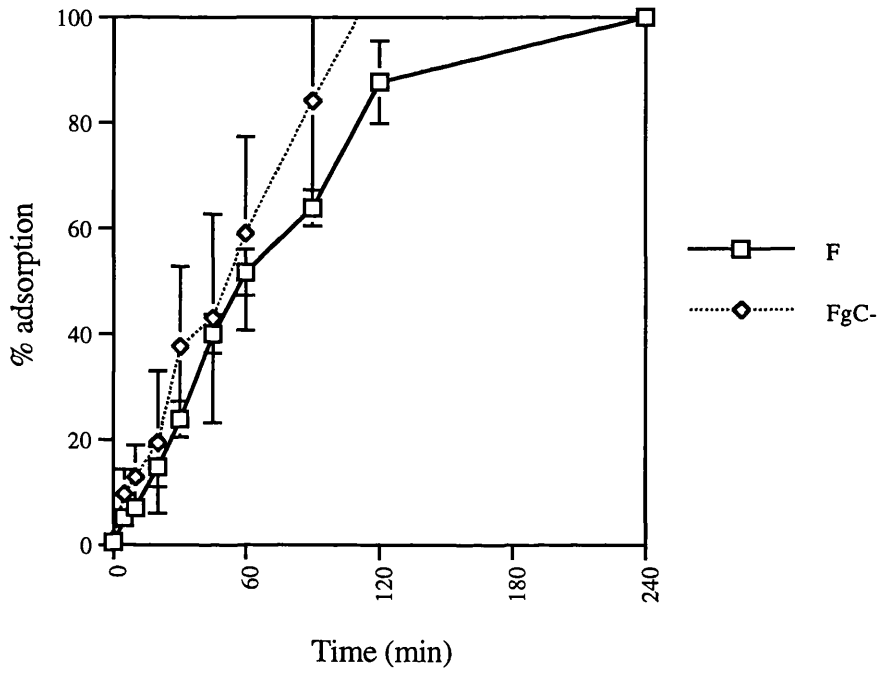


Figure 4.20: Adsorption kinetics of HSV-1 strains 17 and SC16 on BHK21/C13 cells.

BHK21/C13, 17+ and SC16.

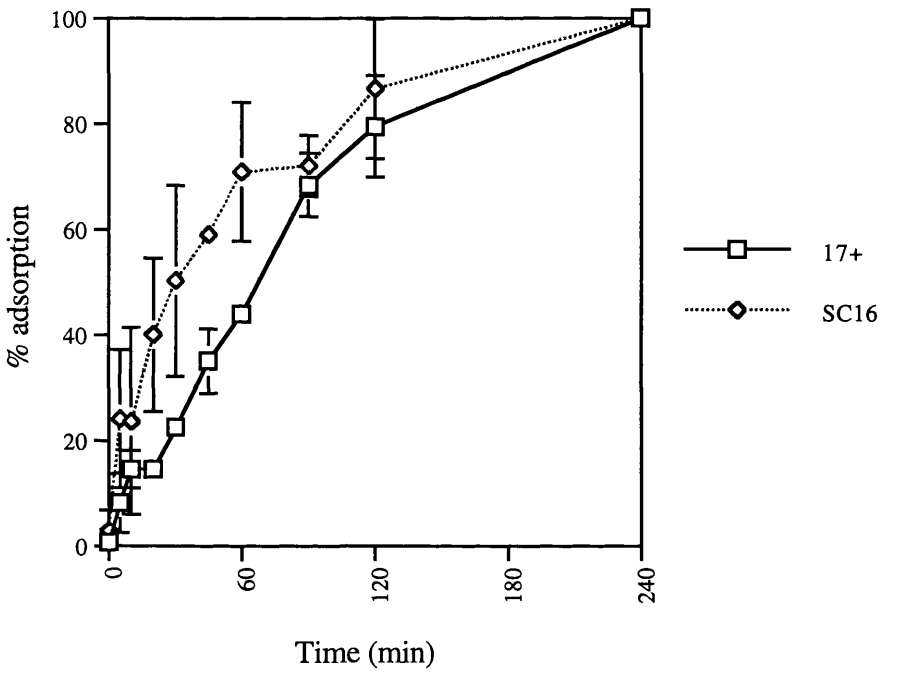


Figure 4.21: Adsorption kinetics of HSV-1 strains 17 and SC16- UL44-z on BHK21/C13 cells.

BHK, 17+ and SC16gC-.

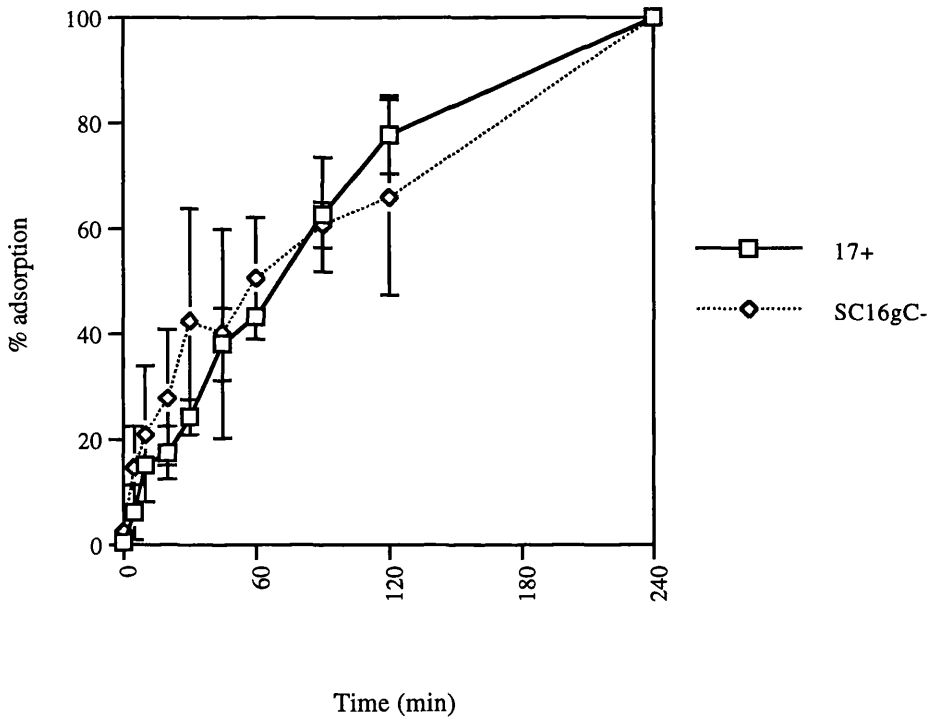


Figure 4.22: Adsorption kinetics of HSV-1 strains 17 and HFEM on BHK21/C13 cells.

BHK21/C13, 17+ and HFEM.

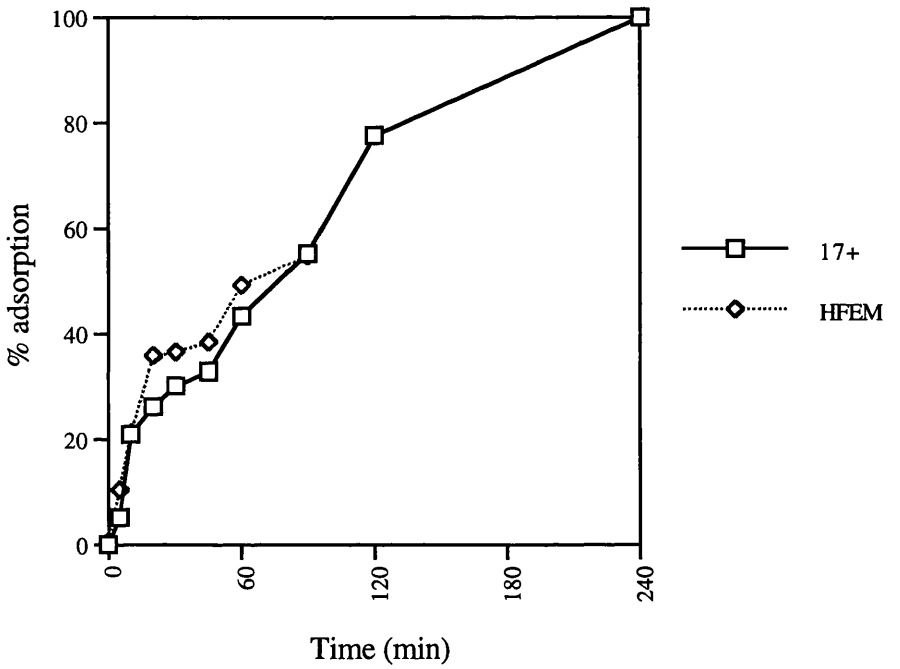


Figure 4.23: Adsorption kinetics of HSV-1 strains 17 and HFEM-ΔUL44-Z on BHK21/C13 cells.

BHK21/C13, 17+ and HFEMgC-.

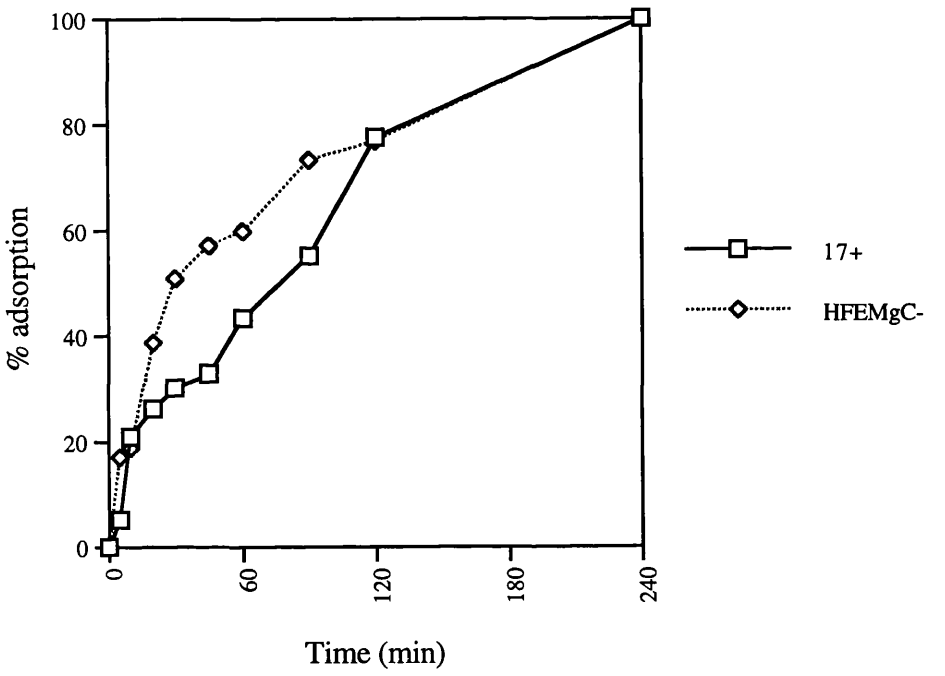


Figure 4.24: Adsorption kinetics of HSV-1 strains 17 and F on BHK21/C13 cells.

BHK21/C13, 17+ and F.

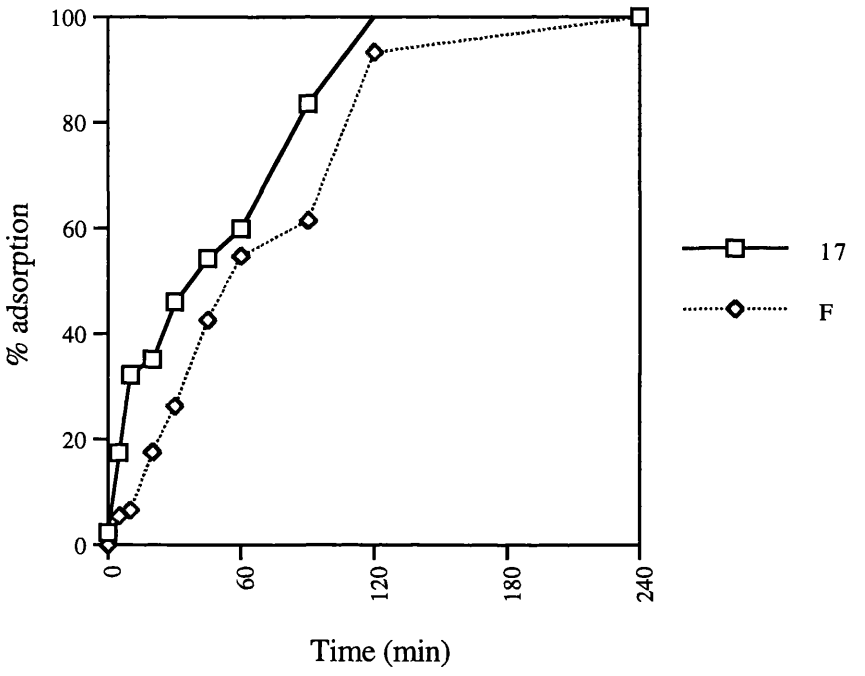
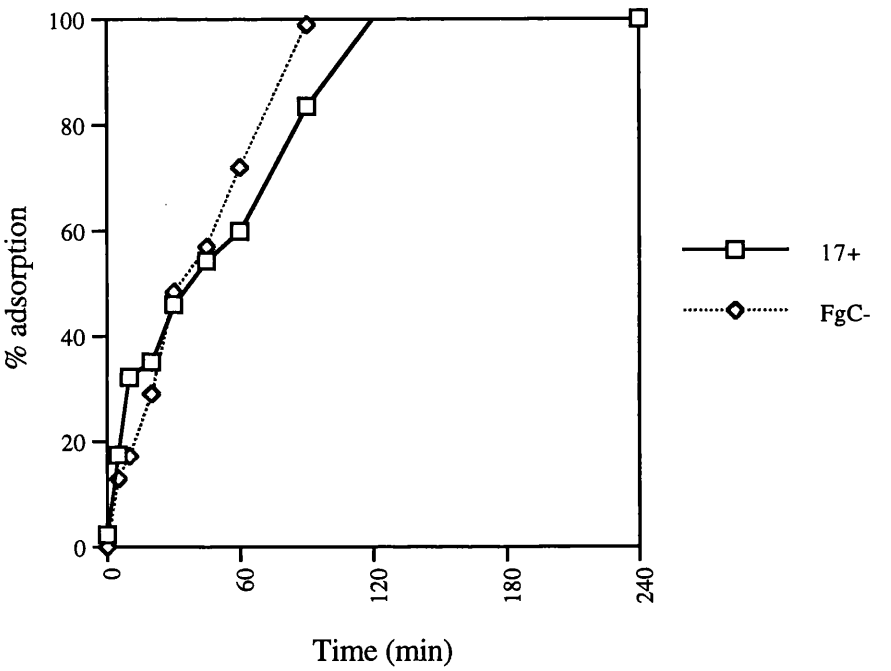


Figure 3.25: Adsorption kinetics of HSV-1 strain 17 and the strain F gC negative mutant, R6012 on BHK21/C13 cells.

BHK21/C13, 17+ and FgC-.



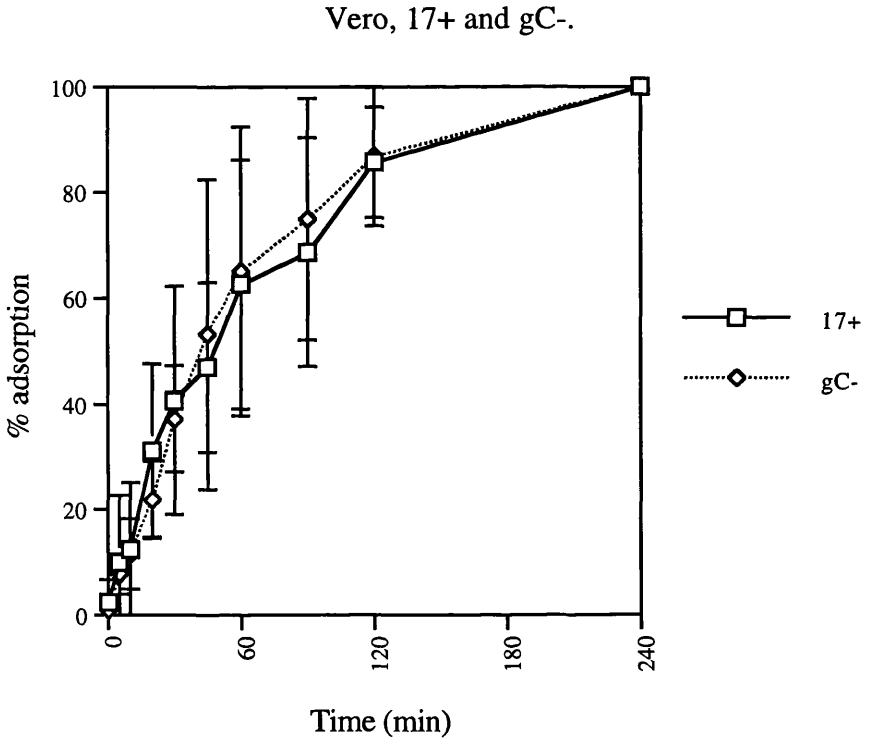

Vero, 17+ and gC-.
100
80
60
40
20
0
% adsorption
0
60
120
180
240
Time (min)
17+
gC-

Figure 4.27: Adsorption kinetics of all HSV-1 strain 17 viruses used in this study on Vero cells.

Vero, parental and lacZ expressing HSV-1 strain 17 viruses.

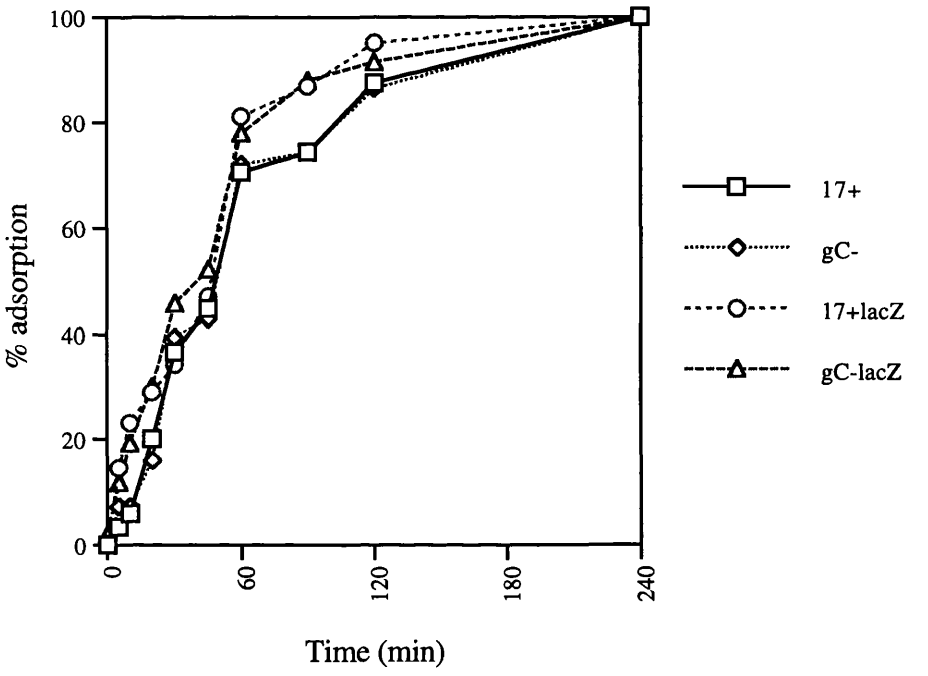


Figure 4.28: Adsorption kinetics of HSV-1 strain SC16 and its gC negative mutant SC16- UL44-Z on Vero cells.

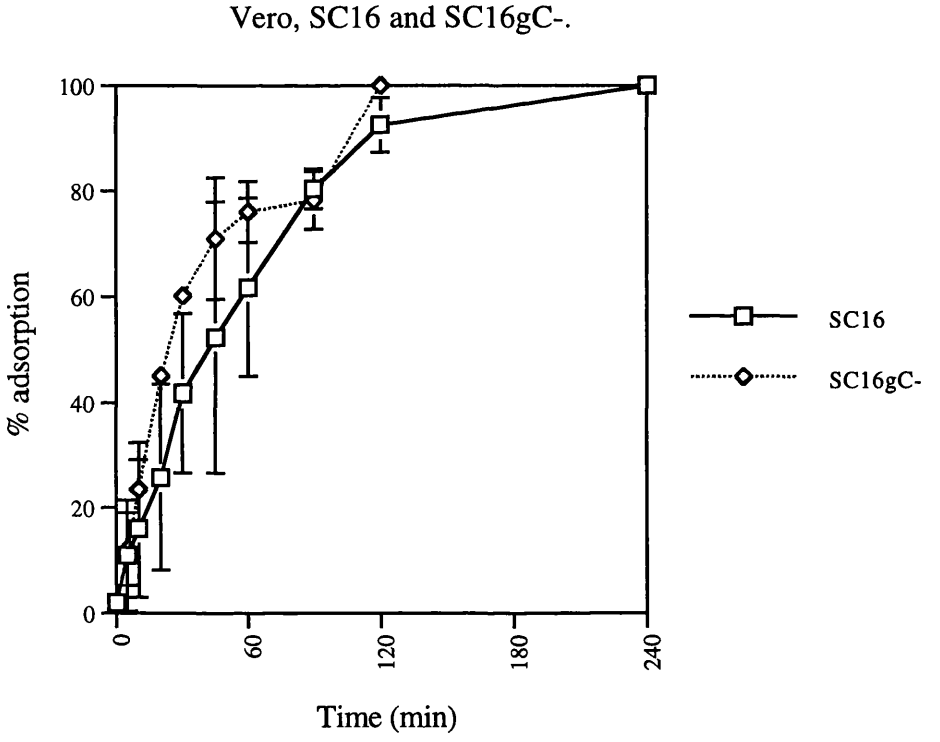

Vero, SC16 and SC16gC-.
100
80
60
40
20
0
% adsorption
0
60
120
180
240
Time (min)
SC16
SC16gC-

Figure 4.29: Adsorption kinetics of HSV-1 strain HFEM and its gC negative mutant HFEM- UL44-Z on Vero cells.

Vero, 17+ and HFEMgC-.

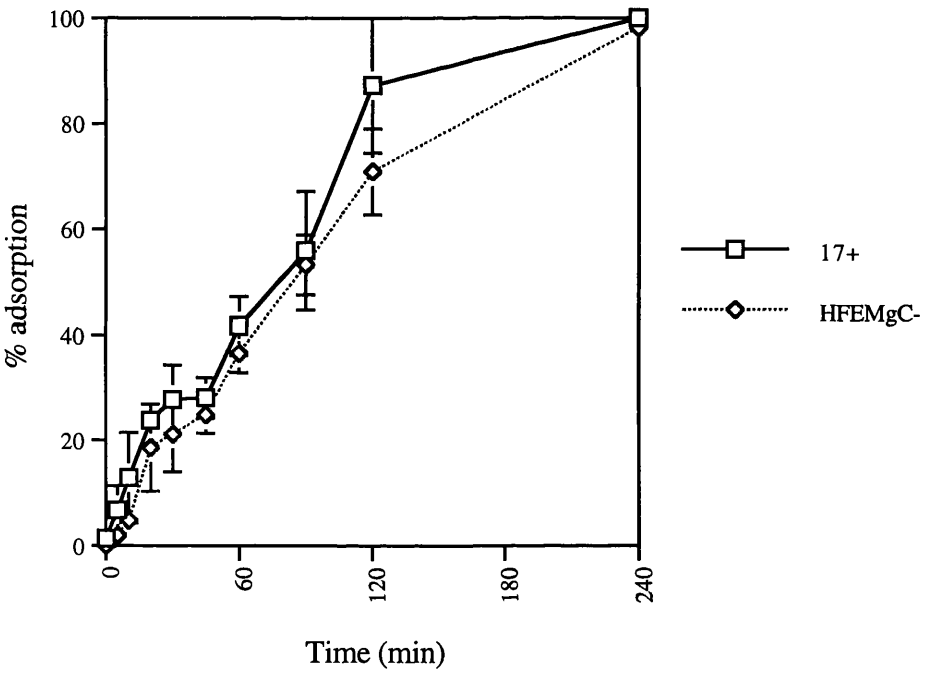


Figure 4.30: Adsorption kinetics of HSV-1 strain F and its gC negative mutant R6012 on Vero cells.

Vero, 17+ and SC16.

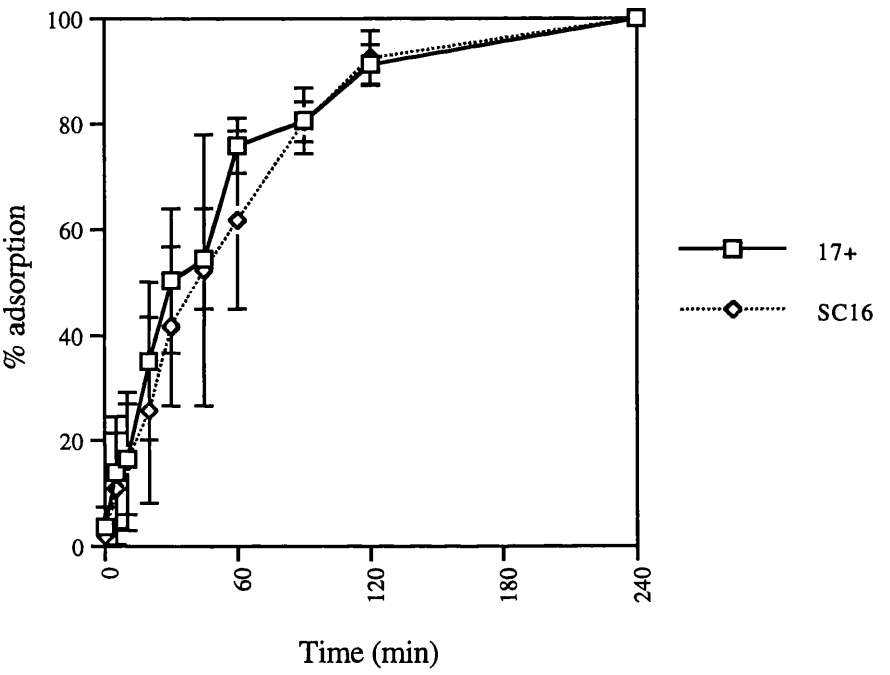


Figure 4.31: Adsorption kinetics of HSV-1 strains 17 and SC16 on Vero cells.

Vero, 17+ and SC16.

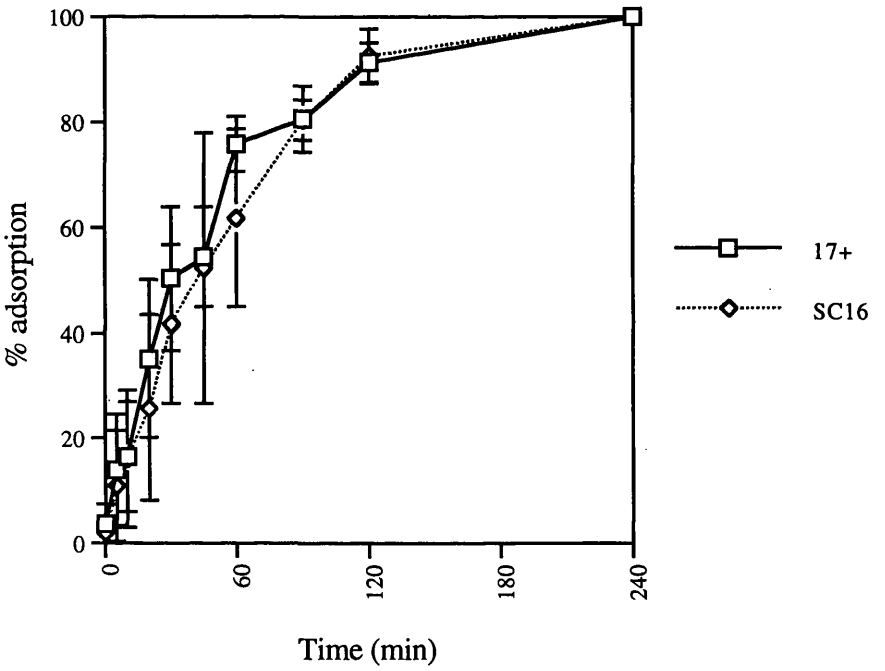


Figure 4.32: Adsorption kinetics of HSV-1 strain 17 and the SC16 gC negative mutant SC16- UL44-Z on Vero cells.

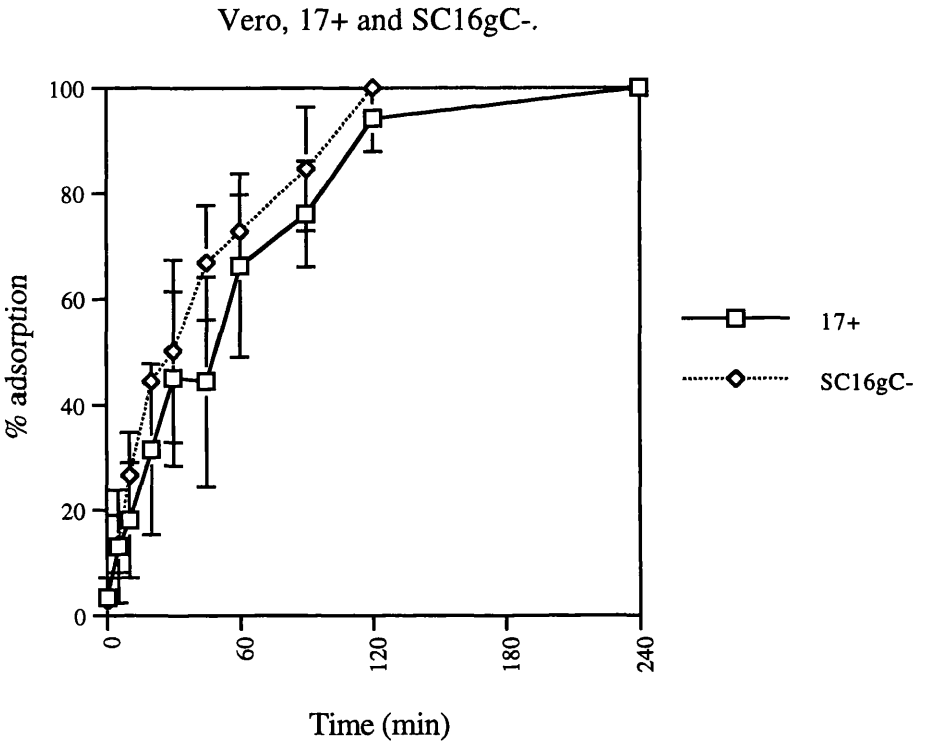

Vero, 17+ and SC16gC-.
100
80
60
40
20
0
% adsorption
0
60
120
180
240
Time (min)
17+
SC16gC-

Figure 4.33: Adsorption kinetics of HSV-1 strains 17 and HFEM on Vero cells.

Vero, 17+ and HFEM.

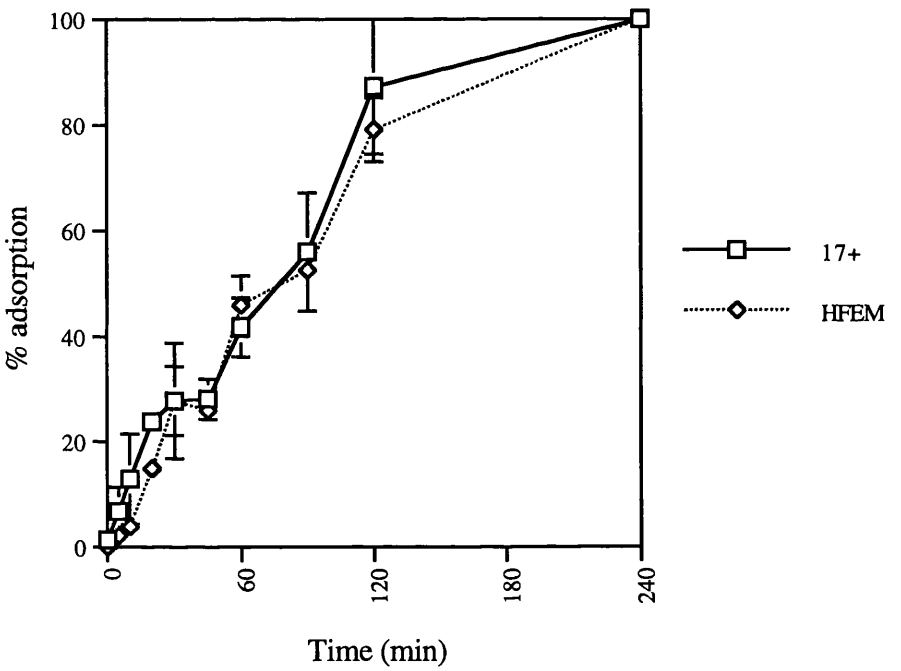


Figure 4.34: Adsorption kinetics of HSV-1 strain 17 and the HFEM gC negative mutant HFEM- UL44-Z on Vero cells.

Vero, 17+ and HFEMgC-.

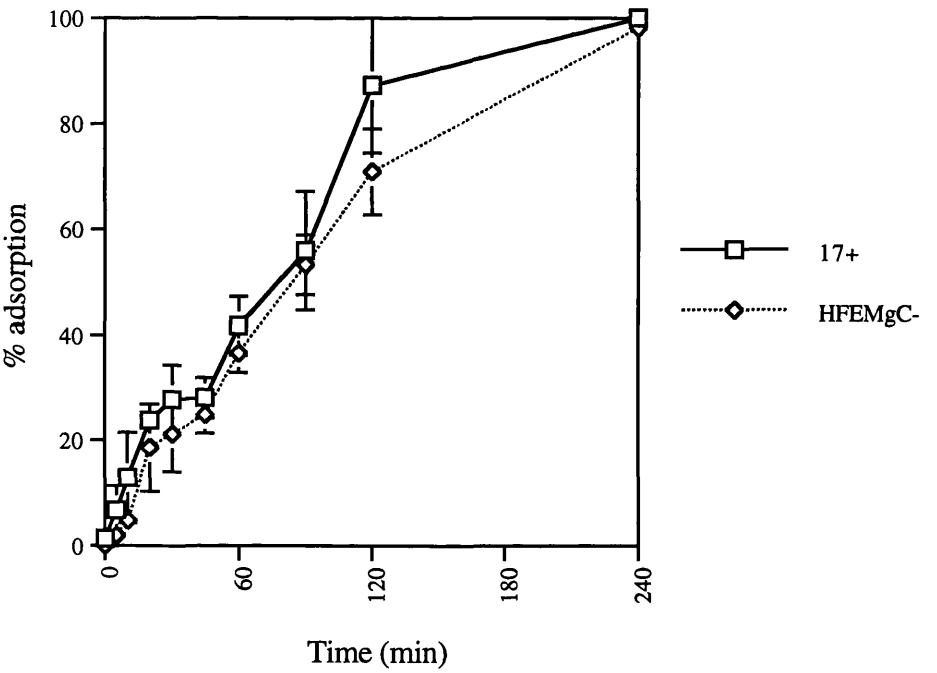


Figure 4.35: Adsorption kinetics of HSV-1 strains 17 and F gC on Vero cells.

Vero, 17+ and F.

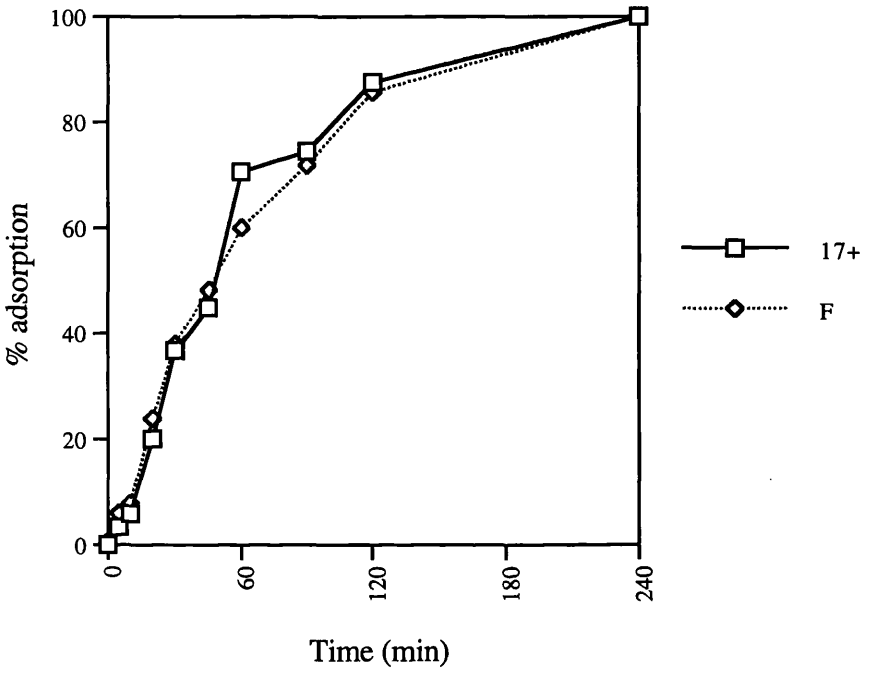


Figure 4.36: Adsorption kinetics of HSV-1 strain 17 and the HSV strain F gC negative mutant R6012 on Vero cells.

Vero 17+ and FgC-.

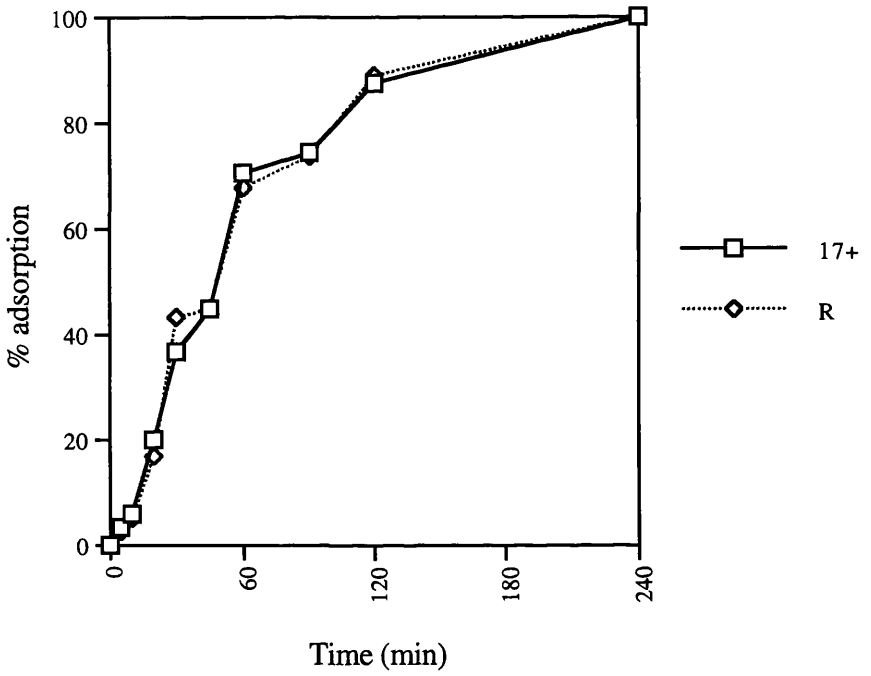


Figure 4.37: Comparison of adsorption as measured by Giemsa and β-galactosidase staining.

These two methods of staining were compared using HSV-1 strain 17 wild type and the lac Z expressing form of this virus in order to ensure that they gave similar plaque numbers and therefore values for adsorption.

Giemsa stain v's lacZ.

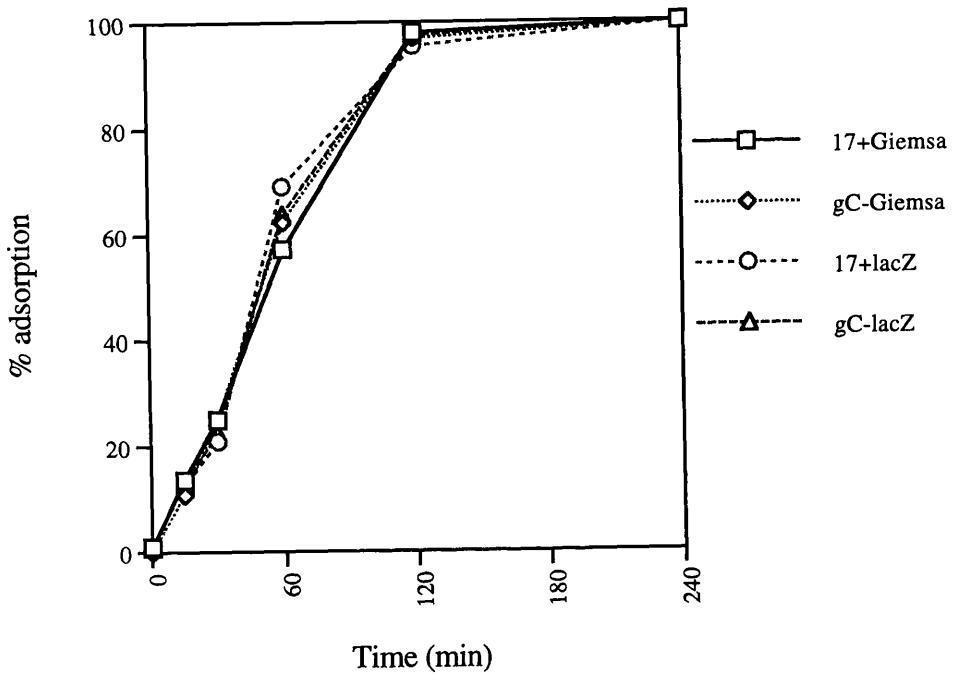


Figure 4.38: Adsorption kinetics of HSV-1 strain 17 lacZ and its gC negative lacZ expressing mutant on HeLa cells.

HeLa 17+ lacZ and gC- lacZ.

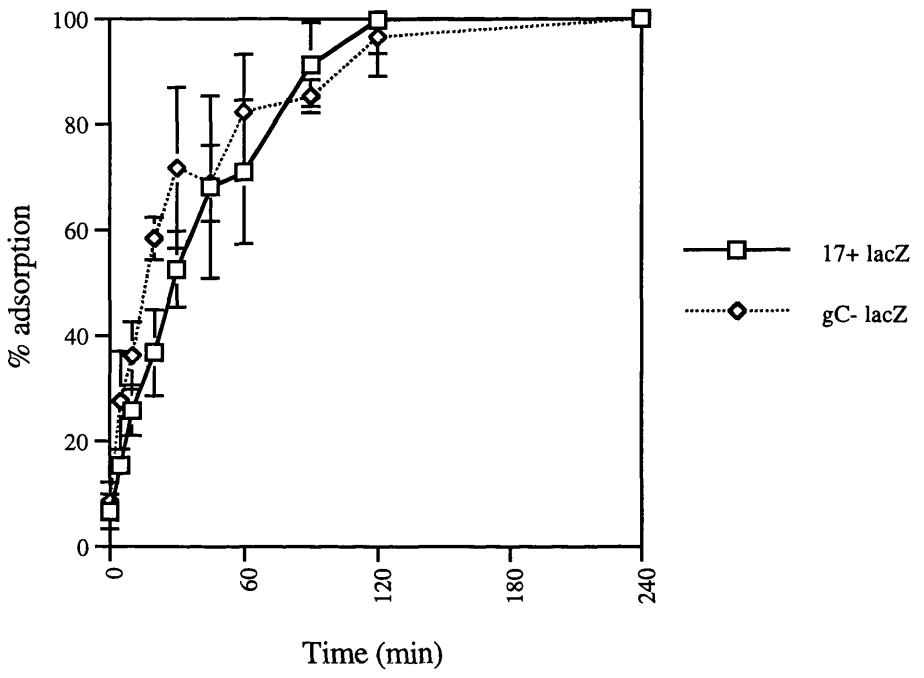


Figure 4.39: Adsorption kinetics of HSV-1 strains 17 lacZ and SC16-ΔUL44-Z on HeLa cells.

HeLa, 17+ lacZ and SC16 gC-.

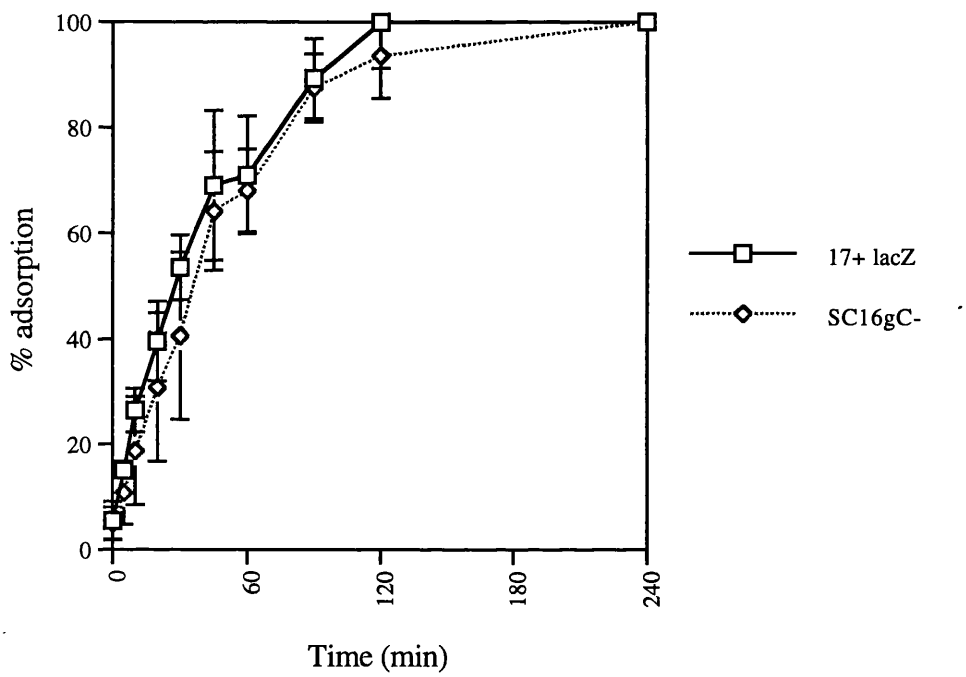


Figure 4.40: Adsorption kinetics of HSV-1 strains 17 lacZ and HFEM-ΔUL44-Z on HeLa cells.

HeLa, 17+ lacZ and HFEMgC-

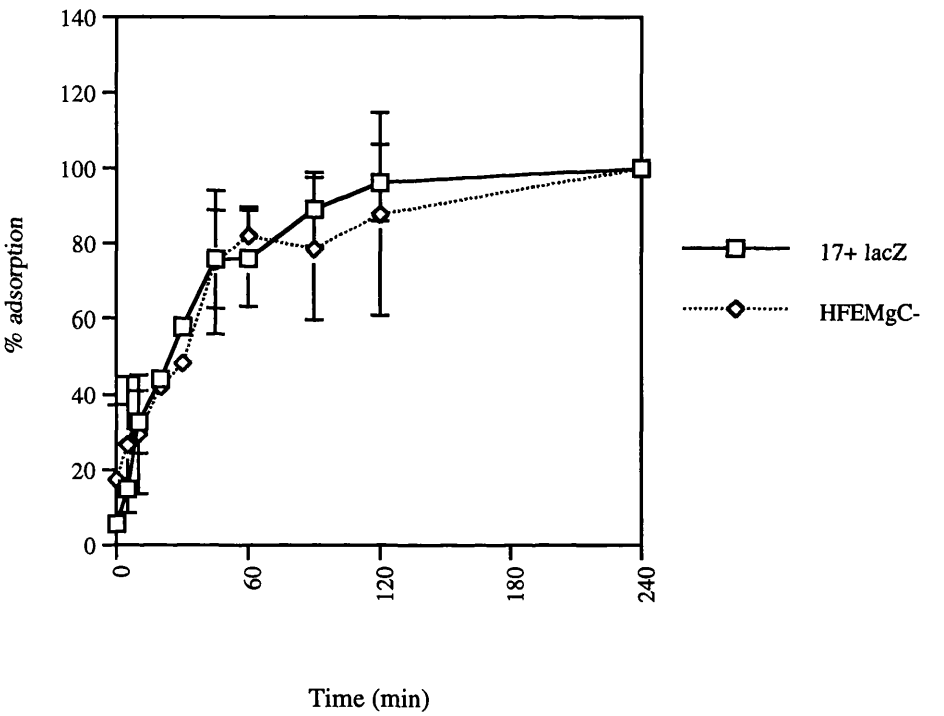


Figure 4.41: Adsorption kinetics of HSV-1 strain 17 lacZ and its gC negative lacZ expressing mutant on 3T6 cells.

3T6, 17+lacZ and gC- lacZ.

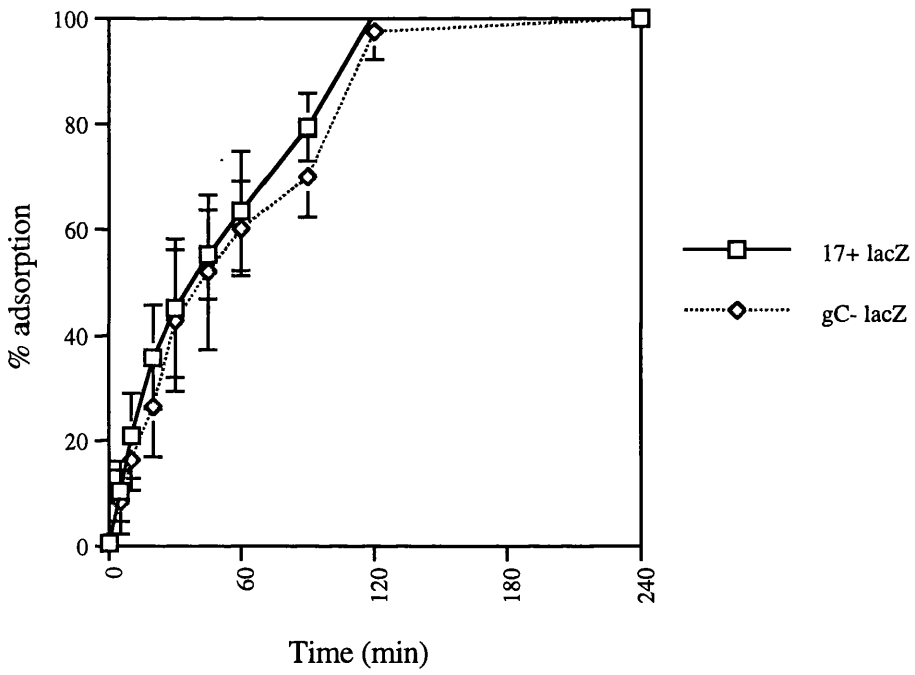


Figure 4.42: Adsorption kinetics of HSV-1 strains 17 lacZ and SC16-ΔUL44-Z on 3T6 cells.

3T6, 17+lacZ and SC16gC-.

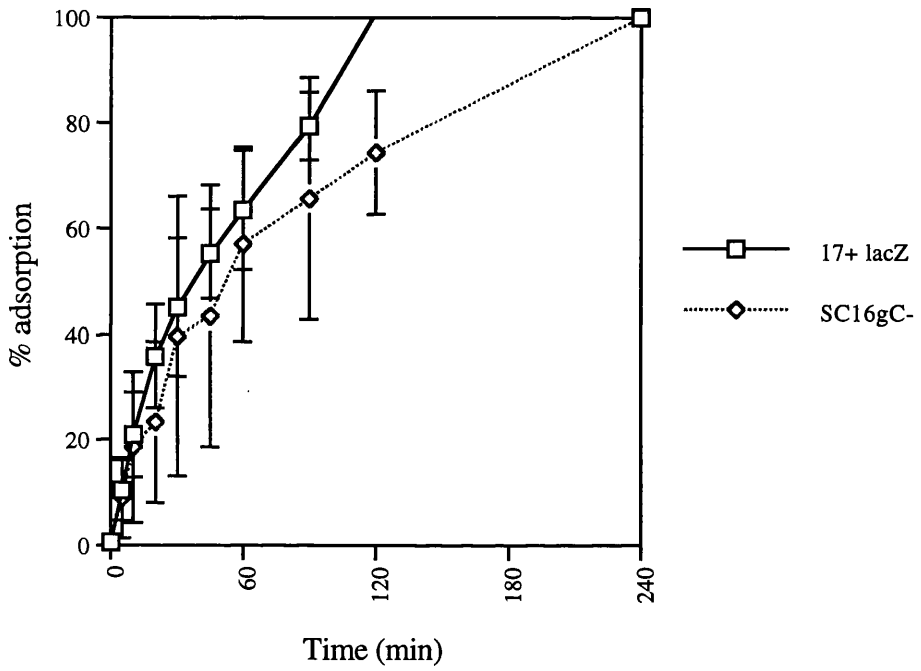
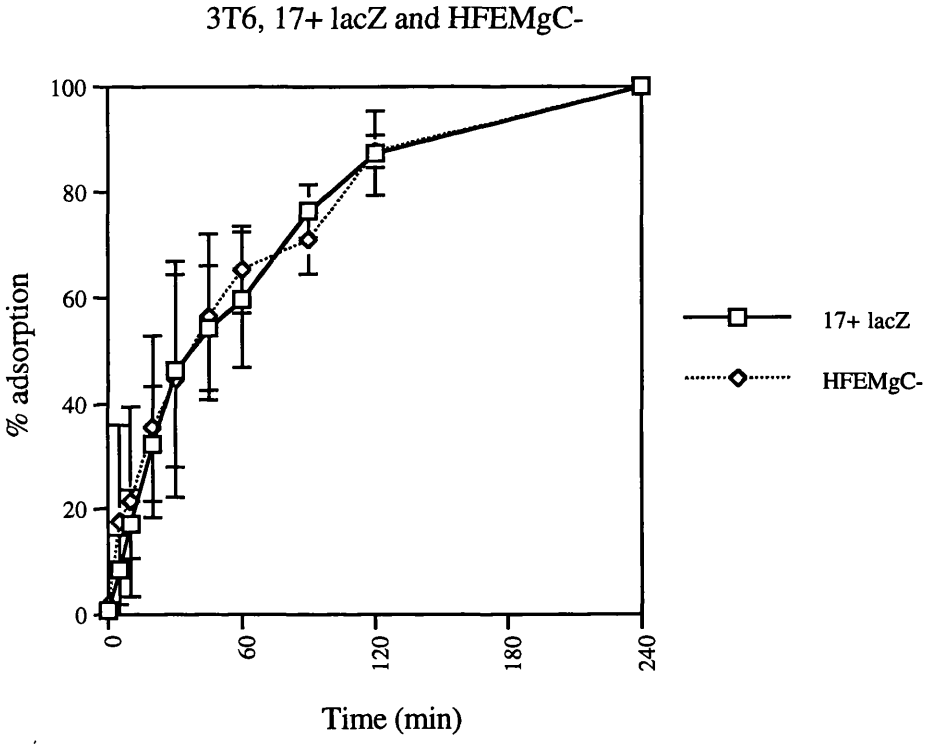


Figure 4.43: Adsorption kinetics of HSV-1 strains 17 lacZ and HFEM-ΔUL44-Z on 3T6 cells.


3T6, 17+ lacZ and HFEMgC-
100
80
60
40
20
0
% adsorption
0
60
120
180
240
Time (min)
17+ lacZ
HFEMgC-

Heparin inhibition experiments.

The graphs presented here in figures 4.44 to 4.49 show that addition of exogenous heparin results in inhibition of viral binding. Binding in the absence of exogenous heparin is considered as 100% binding and all other values are expressed as a percentage of this value. The gC negative viruses are generally less sensitive to the presence of exogenous heparin than their parental viruses.

Figure 4.44: Inhibition of adsorption of HSV-1 strain 17 and its gC negative mutant by addition of exogenous heparin.

Heparin inhibition, 17+ and gC-.

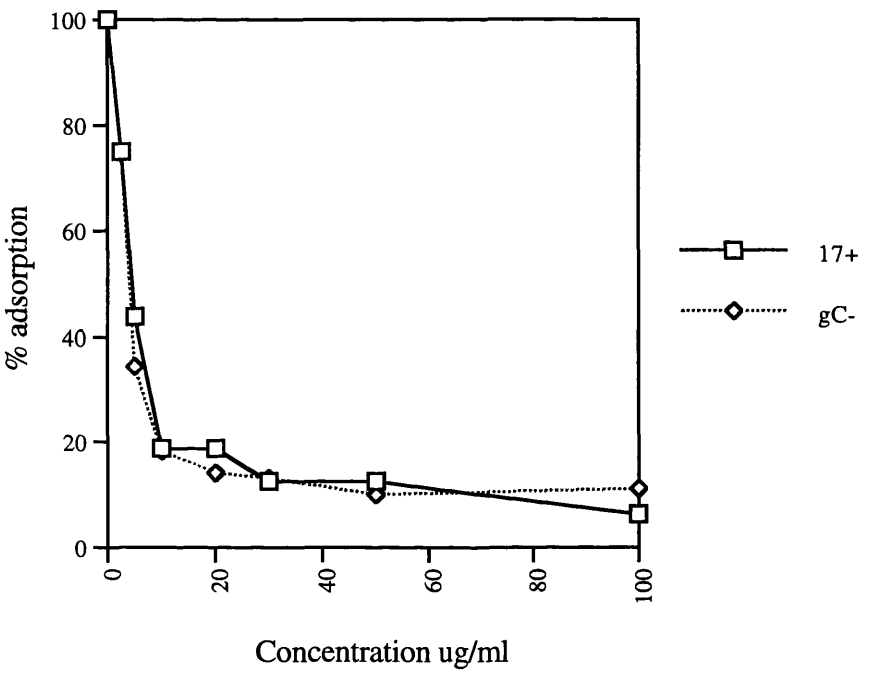


Figure 4.45: Inhibition of adsorption of all HSV-1 strain 17 viruses used in this study by addition of exogenous heparin.

Heparin inhibition, strain 17.

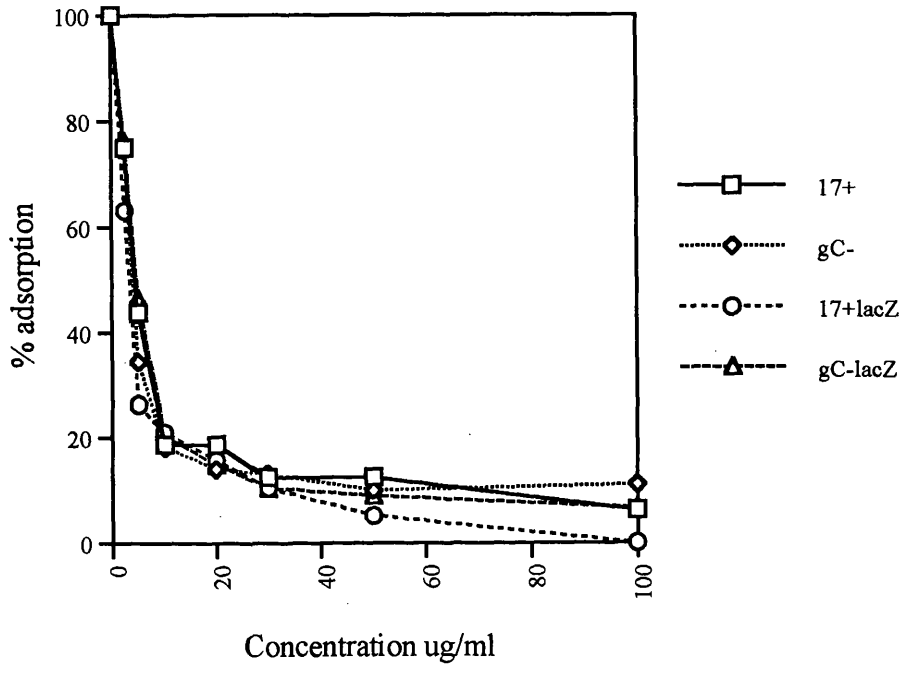


Figure 4.46: Inhibition of adsorption of all HSV-1 viruses used in this study by addition of exogenous heparin.

Heparin inhibition, all viruses.

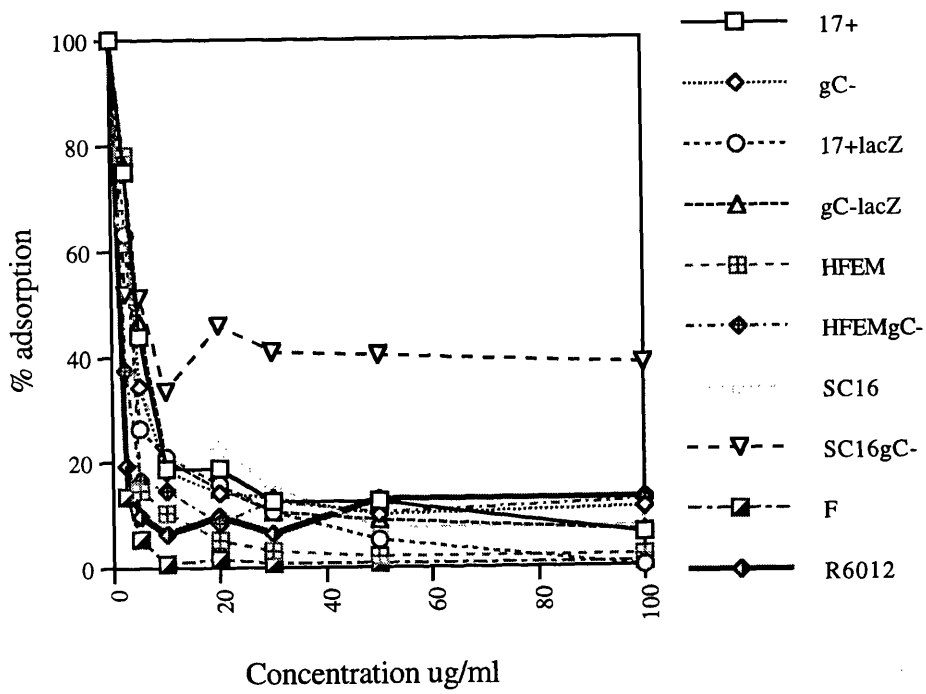


Figure 4.47: Inhibition of adsorption of HSV-1 strain SC16 and its gC negative mutant SC16-ΔUL44-Z by addition of exogenous heparin.

Heparin inhibition, SC16 and SC16gC-.

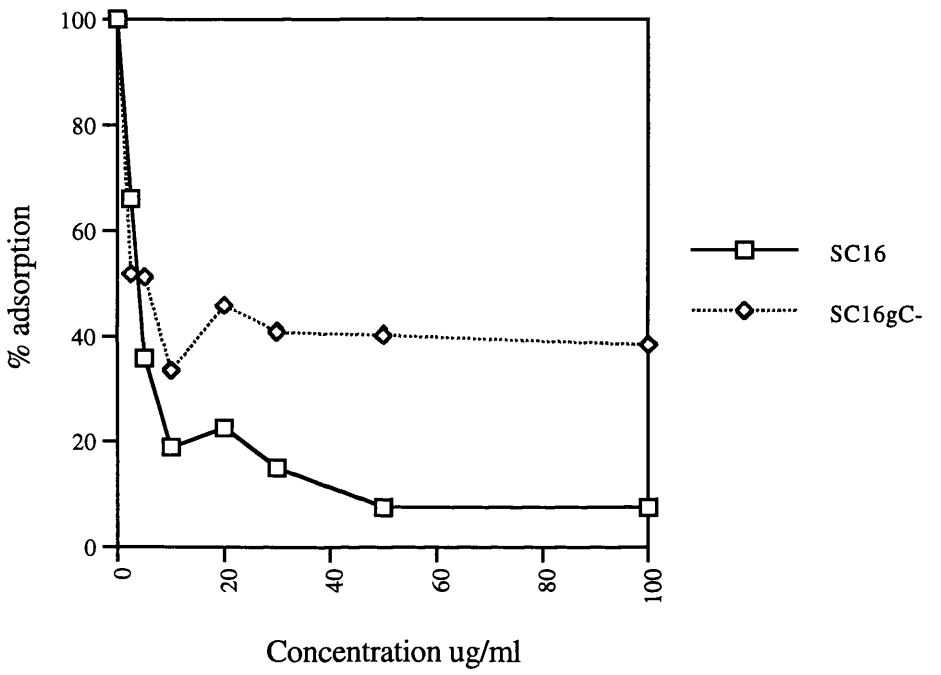


Figure 4.48: Inhibition of adsorption of HSV-1 strain HFEM and its gC negative mutant HFEM-ΔUL44-Z by addition of exogenous heparin.

Heparin inhibition, HFEM and HFEMgC-.

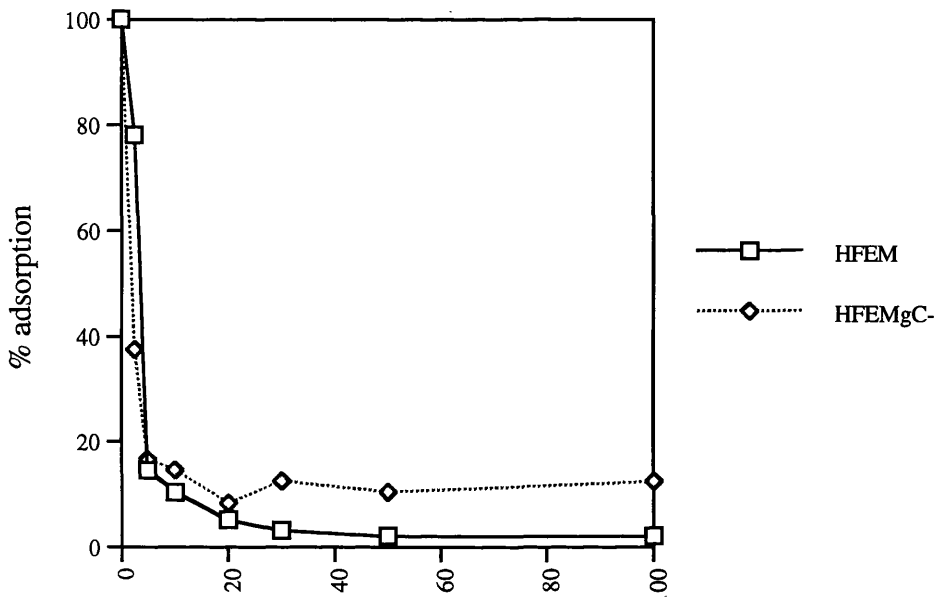


Figure 4.49: Inhibition of adsorption of HSV-1 strain F and its gC negative mutant R6012 by addition of exogenous heparin.

Heparin inhibition, F and FgC-.

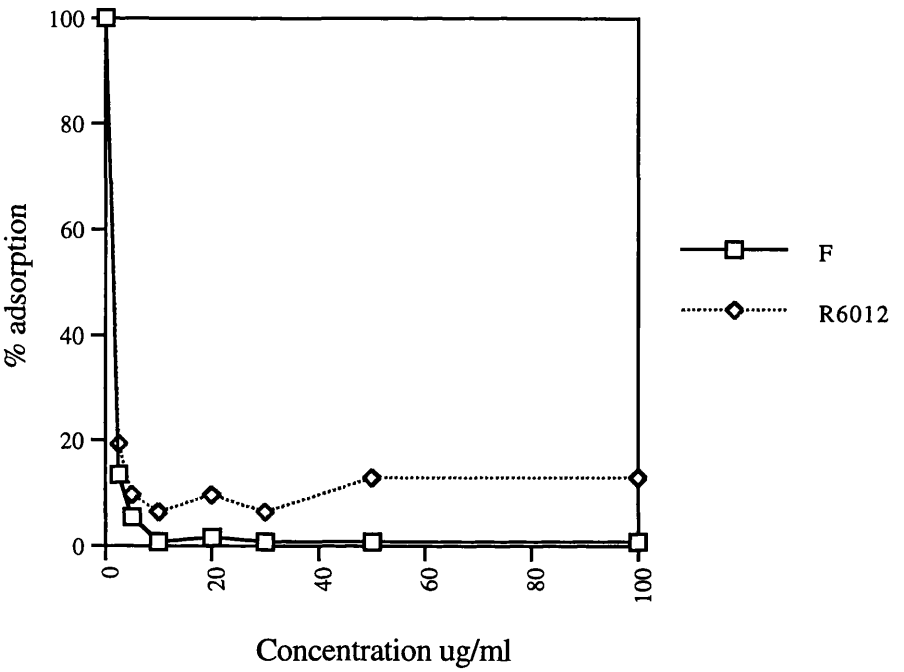


Figure 4.50: Inhibition of adsorption of all HSV-1 gC negative mutants used in this study by exogenous heparin.

Heparin inhibition, gC negative viruses.