

**CHARACTERIZATION OF HSV GLYCOPROTEIN-  
GLYCOSAMINOGLYCAN INTERACTIONS**

by

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## ABSTRACT

The two herpes simplex virus (HSV) serotypes, HSV-1 and HSV-2, demonstrate different tissue tropisms in the human host. There is increasing evidence that the HSV serotypes may enter host cells differently, which may account, in part, for the different behaviours of these closely related viruses. Initial adsorption of HSV to the host cell is mediated by the interaction of viral glycoproteins with cell surface glycosaminoglycans such as heparan sulfate (HS) and chondroitin sulfate (CS). To investigate the contribution of various cell surface components in the infection pathway, we isolated a mutant cell line, sog9, which is unable to synthesize glycosaminoglycans (Banfield *et al.*, 1995a). Although HSV-1 and HSV-2 infection of sog9 cells is diminished, the cells are still infected at about 0.5% efficiency, which suggests that these cells normally express at least one nonglycosaminoglycan receptor. Sog9 cells were used to test whether glycosaminoglycan analogs, such as dextran sulfate (DS), could functionally substitute for cellular glycosaminoglycans to initiate HSV infection. High-molecular-weight DS added either prior to or during inoculation stimulated HSV-1 but not HSV-2 infection by up to 35-fold. By contrast, DS added after viral adsorption had no effect on infection efficiency. Moreover, DS stimulated HSV-1 infection at 4°C, indicating that this compound impinged on an early, energy-independent step in infection. Using radiolabelled virus, it was demonstrated that HSV-1 is more efficient than HSV-2 in adsorbing to DS immobilized on microtiter wells. This raised the possibility that only HSV-1 could engage additional receptors to initiate infection in the presence of DS. To determine which viral component(s) facilitated DS stimulation, a panel of intertypic recombinants and deletion mutant viruses was investigated. These assays showed that DS stimulation of infection is mediated primarily by glycoprotein B (gB-1) and that this interaction was mediated by a domain other than the heparin-binding region in gB-1. Taken together, these results provide direct evidence that a principle role for cell surface glycosaminoglycans in HSV infection is to provide an efficient matrix for virus adsorption.

To investigate further the interactions of HSV with cell surface glycosaminoglycans, a novel cell line, sog9-EXT1, was used in this study. The

expression of EXT1, an enzyme in the heparan sulfate synthesis pathway, restores HS synthesis in sog9 cells (McCormick *et al.*, 1998). Moreover, sog9-EXT1 cells are fully susceptible to HSV-1 infection. Heparin and DS competition assays demonstrated that HSV-1 attachment to sog9-EXT1 cells is mediated by an interaction with heparan sulfate moieties on the cell surface. To determine which structural features of heparan sulfate were important for mediating attachment to sog9-EXT1 cells and control cell lines expressing HS, HSV-1 infection in the presence of chemically modified heparin compounds was examined. It was found that 2-O-, 6-O- and N-sulfate groups of heparan sulfate are involved in viral attachment. Using HSV-1 mutants deleted for the heparin-binding domains of gB and glycoprotein C (gC), it was shown that HSV-1 attachment to glycosaminoglycans on sog9-EXT1 cells is primarily mediated by gC.

The investigation of HSV-glycosaminoglycan interactions, and certainly of other aspects of the viral life cycle, is facilitated by the generation of HSV mutants. The construction of HSV recombinants has traditionally involved time-consuming purification procedures. To overcome this, attempts were made to develop i) an HSV-2 cosmid set, which contains overlapping cosmids representing the entire HSV-2 genome and ii) an HSV-2 bacterial artificial chromosome (BAC), which can be manipulated by bacterial genetics to generate the desired recombinant. This work demonstrates the potential advantages and disadvantages of using these different systems to construct HSV recombinants.



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## LIST OF ABBREVIATIONS

A	adsorbance
a.a	amino acid
ACV	acyclovir
BAC	bacterial artificial chromosome
BHV	Bovine herpesvirus
BME	beta-mercaptoethanol
BSA	bovine serum albumin
CHO	Chinese Hamster Ovary
CM	chloramphenicol
CPE	cytopathic effect
cpm	counts per minute
CS	chondroitin sulfate
dH <sub>2</sub> O	distilled water
DMEM	Dulbecco's modified eagle media
DNA	deoxyribonucleic acid
ddNTP	dideoxyribonucleoside triphosphate
dNTP	deoxyribonucleoside triphosphate
DS	dextran sulfate
DTT	dithiothreitol
EBV	Epstein Barr virus
EDTA	ethylenediaminetetraacetic acid

ER	endoplasmic reticulum
EtOH	ethanol
FBS	fetal bovine serum
Fuc	fucose
GAG	glycosaminoglycan
Gal	galactose
GalNAc	N-acetylgalactosamine
Glc	glucosamine
GlcA	glucuronic acid
GlcNAc	N-acetylglucosamine
GlcNSO <sub>3</sub>	N-sulfoglucosamine
h	hour(s)
HBD	heparin-binding domain
HCMV	human cytomegalovirus
HIV	human immunodeficiency virus
HPLC	high-pressure liquid chromatography
HS	heparan sulfate
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
Hve	herpesvirus entry mediator
IdoA	iduronic acid
IgG	immunoglobulin G
I <sub>L</sub>	inverted long segment

I <sub>ls</sub>	inverted long and short segments
I <sub>s</sub>	inverted short segment
Kbp	kilobase pairs
kDa	kilodaltons
M6P	mannose-6-phosphate
M6PR	mannose-6-phosphate receptor
Man	mannose
min	minute(s)
MOI	multiplicity of infection
MW	molecular weight
NANA	N-acetylneuraminic acid
NP-40	Nonidet P-40
P	prototype position
PBS	phosphate buffered saline
PFU	plaque forming unit(s)
PG	proteoglycan
Prr	Poliovirus receptor-related protein
PrV	pseudorabies virus
Pvr	poliovirus receptor
<i>roe</i>	rate of entry mutation
rpm	revolutions per minute
<i>syn</i>	syncytial mutation
TE	Tris-EDTA

tk	thymidine kinase
TNF	tumor necrosis factor
VHS	virion host shutoff protein
vol	volume
VZV	Varicella Zoster virus
U <sub>L</sub>	unique long segment
U <sub>S</sub>	unique short segment

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*For Mum and Dad,  
J.J. and Nan,  
with love*

## CHAPTER 1: LITERATURE REVIEW

*"Nature is to be found in her entirety nowhere more than in her smallest creatures"*

*Pliny, Roman writer*

### 1.0 INTRODUCTION

There are two herpes simplex virus (HSV) serotypes, HSV-1 and HSV-2. It is now estimated that the worldwide prevalence of HSV-1 is 70-90% (Roizman, 1993). In the U.S., the prevalence of HSV-2 is approximately 20.8% (Aurelius, 1998). HSV-1 preferentially infects the oral mucosa and is associated with facial vesicular lesions, while HSV-2 primarily infects the genital mucosa (Roizman and Sears, 1996). Why the viruses have predilections for different body sites remains unclear. Recent evidence, however, suggests that the HSV serotypes may enter host cells differently, which may account, in part, for the different behaviors of these viruses. This research examines the initial interactions of HSV-1 and HSV-2 with the host cell, providing further evidence that the association of virus with cell surface glycosaminoglycan moieties plays a pivotal role in HSV infection and is a possible determinant of HSV tissue tropism.

### 1.1 CLASSIFICATION

HSV-1 and HSV-2 belong to the family *Herpesviridae*, the sub-family *alphaherpesvirinae* and the genera *simplexvirus*. (Roizman and Sears, 1996). The alphaherpesviruses are characterized by their variable host range, short reproductive



cycles (e.g. 18 h for HSV) and rapid development of cytopathic effect (CPE) in cell culture. They are also neurotropic and can establish latency in sensory ganglia. In addition to the two HSV serotypes, varicella zoster virus (VZV), pseudorabies virus (PrV) and bovine herpesvirus (BHV) also belong to the *alphaherpesvirinae*. The two other sub-families of the *Herpesviridae* are the *betaherpesvirinae* and the *gammaherpesvirinae*. The betaherpesviruses have a relatively long reproductive cycle and infected cells often become enlarged (cytomegalia) both *in vivo* and *in vitro*. Human cytomegalovirus (HCMV) is an example of a betaherpesvirus. Finally, the *gammaherpesvirinae* are lymphotropic viruses and specifically infect B and T lymphocytes. The prototype virus of this sub-family is Epstein-Barr virus (EBV).

## 1.2 CLINICAL PATHOLOGY OF HSV-1 AND HSV-2

HSV causes two types of infections in humans, lytic and latent. The virus infects and replicates at the site of entry (lytic infection) and is then transported to the sensory neurons that innervate it, becoming latent. The virus can reactivate from latency when exposed to the proper stimulus, becoming evident once more in the form of mucocutaneous lesions (Whitley, 1996). HSV infections range from minor nuisances to life-threatening disease. In the immunocompetent host, HSV infections can be asymptomatic, but painful recurrent mucocutaneous lesions are common. Typically, HSV-1 infects the mouth and lips, causing gingivostomatitis. HSV-2 causes symptomatic lesions at genital sites and has also been recently described as causing hyperproliferative lesions (Aurelius, 1998). Severe clinical HSV problems, including keratoconjunctivitis, encephalitis and disseminated disease, are rare, but have high morbidity and mortality. Interestingly, HSV-2 has also been recently identified as a DNA tumor virus (Aurelius, 1998). It encodes a protein kinase whose expression is required for neoplastic

transformation of human cells and tumour growth in animals. It is not yet known whether the virus is associated with malignancy in the human host.

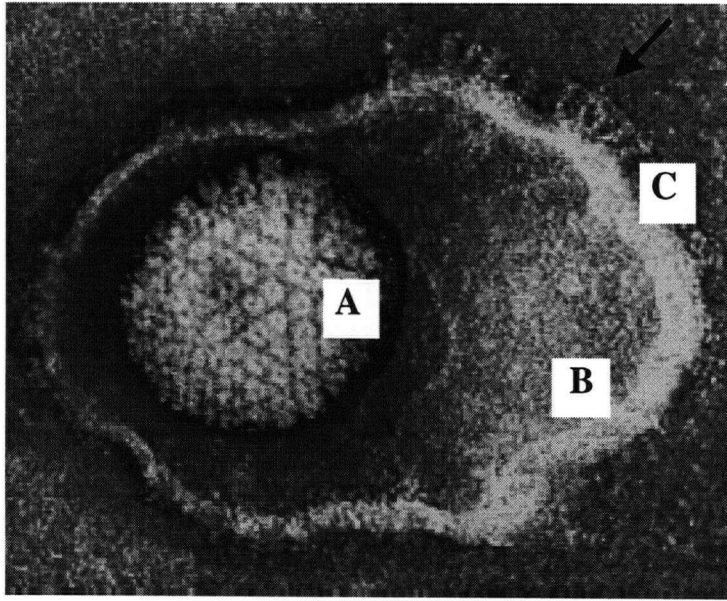
### 1.3 HSV STRUCTURE

The members of the *Herpesviridae* family are morphologically similar. They consist of four components: (a) a core containing a linear double-stranded DNA genome, (b) an icosahedral capsid surrounding the core, (c) a tegument surrounding the capsid, and (d) an outer envelope containing glycoprotein spikes (Fig. 1.1). Here, the structure of HSV will be summarized.

#### 1.3.1. The HSV Genome

Present understanding of HSV genome organization comes from studies of HSV-1. The genome exists as linear double-stranded DNA and is approximately 150kb in length (Roizman and Sears, 1996). The genome is composed of two unique regions, a 108 kb unique long sequence ( $U_L$ ) and a 13 kb unique short sequence ( $U_S$ ) (Sarisky and Weber, 1994). The  $U_L$  and  $U_S$  components are flanked by repeat sequences  $a$ ,  $b$  and  $c$ , which are 500 bp, 9 kb and 6.5 kb in length, respectively. The final structure of the genome thus appears as  $ab-U_L-b'a'c'-U_S-ca$ . The  $a$  sequences can be present in one to many copies.

An interesting feature of the HSV genome is the ability of the  $U_L$  and  $U_S$  components to invert relative to one another. Thus, studies have shown that plaque-purified HSV-1 exists as an equimolar mixture of the four isomers (Delius and Clements, 1976; Hayward *et al.*, 1975). The isomers have been designated as P (prototype),  $I_L$  (inverted  $U_L$  sequence),  $I_S$  (inverted  $U_S$  sequence) and  $I_{SL}$  (inversion of both  $U_S$  and  $U_L$ ) (Fig. 7.3, Appendix) (Roizman and Sears, 1996). The precise mechanism by which the HSV genome isomerizes remains unclear, although studies have indicated that homologous



**Figure 1.1.** Electron micrograph of HSV particle (X100,000). The HSV virion consists of a double stranded linear DNA genome contained within an icosahedral capsid (A). Surrounding the capsid is the amorphous tegument (B). The virion envelope (C) contains glycoprotein spikes which are indicated by the arrow.

recombination through any of the repeats of the HSV-1 genome (the *a*, *b* and *c* sequences) will promote inversion of the  $U_L$  and  $U_S$  components (Sarisky and Weber, 1994; Martin and Weber, 1996). The function of the inversions in the viral life cycle is also enigmatic, since genomes restricted to one orientation, as a result of deletion of the internal repeats, can still yield infectious virus (Poffenberger *et al.*, 1983).

The HSV-1 genome contains three origins of DNA replication:  $ori_L$ , which is situated in the  $U_L$  segment of the genome, and two copies of  $ori_S$  in the *c* sequences that flank the  $U_S$  segment. HSV-1 is known to express at least 84 different proteins, 38 of which are essential for growth in cell culture (Aurelius, 1998). By contrast, the ORFs that are essential for HSV-2 replication have not been identified. Although the HSV-2 genome is organized similar to that of HSV-1, the overall DNA sequence similarity between the two viruses is only 50% (Aurelius, 1998).

### **1.3.2. The HSV Capsid**

By electron microscopy, the capsid of HSV-1 appears as an icosahedron containing 162 capsomers (Davison *et al.*, 1992). There are 150 hexameric capsomers and 12 pentameric capsomers. The capsid is approximately 100 nm in diameter and is assembled in the nuclei of virus-infected cells (Roizman, 1993).

### **1.3.3. The HSV Tegument**

The space between the capsid and the envelope is termed the tegument. The thickness of the tegument is variable and frequently distributed asymmetrically (Roizman, 1993). The tegument contains several important regulatory proteins, including the transactivator VP16 and the virion host shut-off (VHS) protein, which together create an environment that promotes viral DNA replication (Ward and Roizman, 1994).

#### 1.3.4. The HSV Envelope

The viral envelope is acquired as the DNA capsid buds from the inner nuclear membrane into the cytoplasm of the host cell (Roizman and Sears, 1996). The viral envelope is primarily composed of lipids and contains numerous glycoproteins, which appear as 8-24 nm long spikes when viewed by electron microscopy (Asher *et al.*, 1969; Epstein *et al.*, 1962; Stannard *et al.*, 1987). In HSV-1, ten envelope glycoproteins have been identified to date: gB, gC, gD, gE, gG, gH, gI, gJ, gL and gM. Several of these glycoproteins are important for mediating viral entry and egress, although only gB, gD, gH, gL are essential for virus replication (Little *et al.*, 1981; Cai *et al.*, 1988; Desai *et al.*, 1988; Ligas and Johnson, 1988; Herold *et al.*, 1991; Fuller and Lee, 1992; Baines *et al.*, 1993; Roop *et al.*, 1993; Laquerre *et al.*, 1998b). HSV synthesizes an additional glycoprotein, gK, although this glycoprotein does not appear to be incorporated into the envelope of infectious virus (Jayachandra *et al.*, 1997). Synthesis of the HSV envelope glycoproteins is described later in Section 1.5.1.

In addition to the glycoproteins, the HSV-1 envelope also contains several non-glycosylated membrane proteins, including U<sub>L</sub>20 (Ward *et al.*, 1994), U<sub>L</sub>45 (Cockrell and Muggeridge, 1998) and U<sub>L</sub>43 (MacLean *et al.*, 1991). These proteins are not essential for viral growth in tissue culture, although UL20 and UL45 have been shown to function in viral egress and cell-cell fusion, respectively (Baines *et al.*, 1991; Visalli and Brandt, 1993; Haanes *et al.*, 1994).

### 1.4 THE HSV LIFE CYCLE

#### 1.4.1 The HSV Lytic Cycle

The HSV replication cycle is relatively short, lasting approximately 18 hours, and results in the production of infectious viral progeny and the destruction of the host cell (Ward and Roizman, 1994). In cell culture, virally infected cells become round in appearance and lose their adhesive properties. Other cytopathic effects include

degradation of cell nucleoli and chromatin. The lytic cycle of HSV can be divided into three stages: (a) viral entry, (b) viral replication, and (c) viral egress (Fig. 1.2). As it is the subject of this thesis, viral entry will be described later in detail.

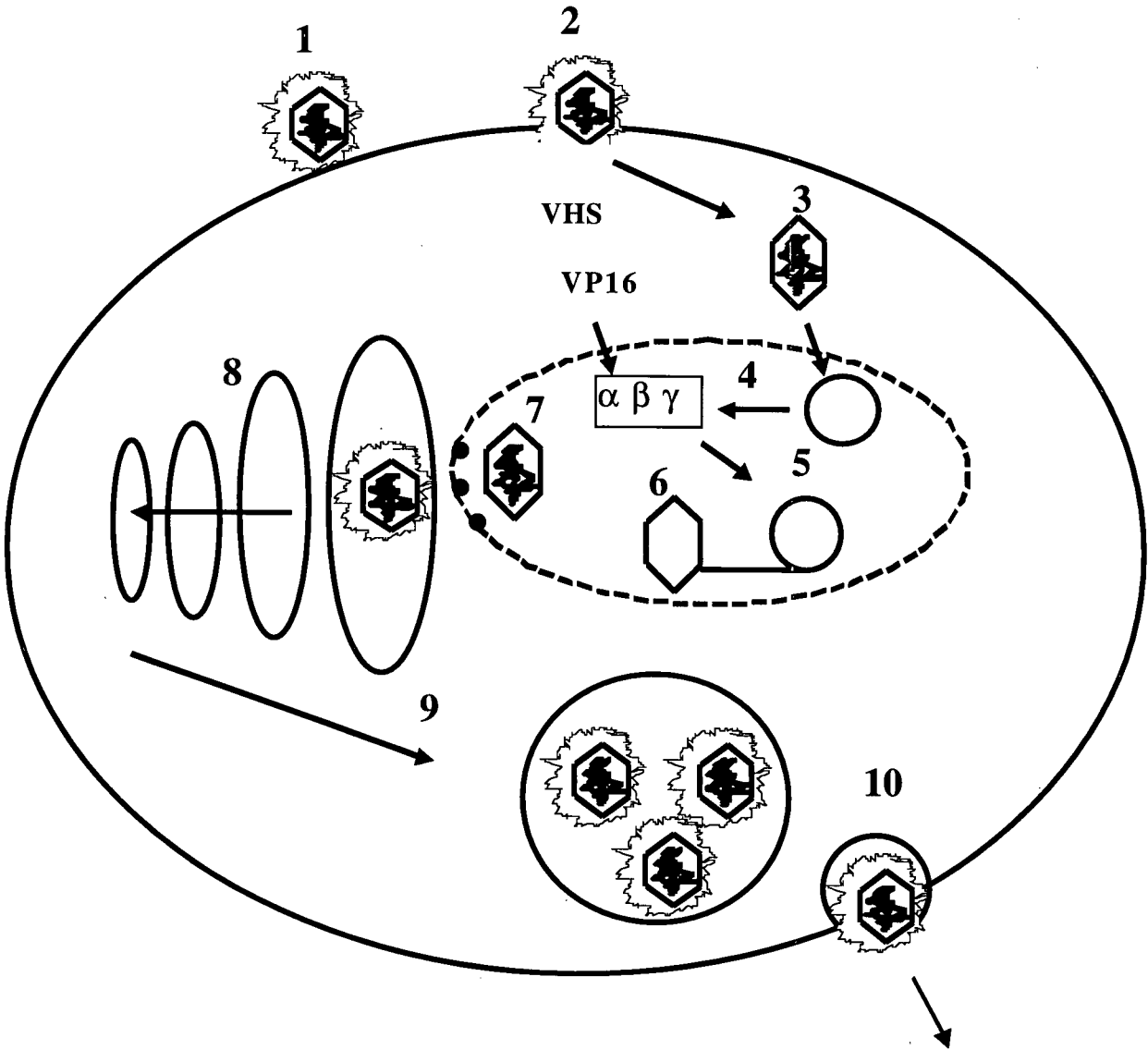
#### 1.4.1.1. Viral Replication

Upon virus entry (see Section 1.5), the viral capsid is released into the cell cytoplasm and transported to the nuclear pores. The cellular cytoskeleton probably mediates the transport of HSV-1 capsids to nuclear pores, since drugs such as nocodazole, which disrupts microtubules, have been shown to inhibit neuritic transport of HSV-1 (Dales and Chardonnet, 1977; Kristensson *et al.*, 1986). Once the virus reaches the nuclear pores, capsid uncoating occurs. The linear DNA is released into the cell nucleus and immediately circularizes (Roizman and Sears, 1996). The viral DNA is now ready to undergo replication.

Two viral tegument proteins, VHS and VP16, are released into the cell cytoplasm concomitant with viral entry. These proteins create an environment that is favourable for viral gene expression (Ward and Roizman, 1994). VHS mediates the degradation of host cell mRNA, and in this manner downregulates host protein synthesis (Kwong and Frenkel, 1989; Read and Frenkel, 1993). VP16 acts *in trans* to initiate the transcription of viral genes (Batterson and Roizman, 1983; Campbell *et al.*, 1984). HSV gene expression is coordinately regulated and occurs sequentially in a cascade fashion (Roizman and Sears, 1996). The three sets of genes expressed are the  $\alpha$ ,  $\beta$  and  $\gamma$  genes.

The  $\alpha$  genes are the first to be expressed, and their expression is maximal 2 to 4 h post-infection. The  $\alpha$  genes primarily encode regulatory proteins, which in turn are required for the induction of the  $\beta$  genes. The  $\beta$  group of polypeptides, whose rate of synthesis peaks at approximately 5 to 7 h post-infection, are involved in viral DNA synthesis. The  $\beta$  proteins also include the viral thymidine kinase (tk) and the viral DNA polymerase. The viral tk and DNA polymerase are targets of HSV antiviral agents, which

**Figure 1.2.** Schematic representation of the HSV reproductive cycle. (1) HSV attachment to cell surface receptors is followed by (2) fusion of the virion envelope with the cell membrane (penetration). (3) The nucleocapsid and two tegument proteins, VHS and VP16, are released into the cell cytoplasm. (4) The nucleocapsid is transported to nuclear pores where the viral genome is released into the nucleus and circularizes. VP16 initiates transcription of the  $\alpha$  genes. Subsequently, the  $\beta$  genes are transcribed, whose protein products are necessary for (5) DNA replication. Concomitant with DNA replication, is transcription of the  $\gamma$  genes, which encode the capsid proteins and glycoproteins. (6) Concatamer length DNA is packaged into preformed capsids. (7) Nucleocapsids acquire an envelope by budding from glycoprotein-modified patches on the inner nuclear membrane. (8) In the model of egress shown here, enveloped viruses travel through the ER and Golgi apparatus, at which time the glycoproteins are modified. (9) Mature enveloped virions are transported to the cell membrane in vesicular structures and are then released (10) into the extracellular space. See text for details.





include the guanosine analog acyclovir (ACV). Unlike its cellular counterpart, HSV tk recognizes nucleoside analogs, such as ACV, as substrates and phosphorylates them. HSV DNA polymerase is less selective than cellular DNA polymerases and can incorporate the triphosphate form of ACV into viral DNA, at which point DNA synthesis is terminated.

DNA synthesis begins following the production of the  $\beta$  proteins. It is detectable as early as 3 h and continues for up to 15 h post-infection. DNA synthesis is a requirement for the expression of some, but not all, of the  $\gamma$  genes. Collectively, the  $\gamma$  genes encode for structural components of the virion, including capsid proteins and envelope glycoproteins. Although some of the  $\gamma$  genes (e.g. gC) are expressed late in infection, it should be noted that others, such as the genes specifying gB and gD, are expressed early in infection. Once synthesized in the rough endoplasmic reticulum (ER), the viral glycoproteins are transported to the inner nuclear membrane, Golgi membranes and the cell plasma membrane.

Viral DNA is synthesized by a rolling-circle mechanism in globular structures in the nucleus called replication compartments. This yields long head-to-tail concatamers (Jacob *et al.*, 1979; Liptak *et al.*, 1996; Boehmer and Lehman, 1997). Genome length DNA is then cleaved and packaged into preformed capsids. Associated with DNA replication, cleavage and packaging, is the isomerization of the DNA (Dutch *et al.*, 1992). Mature capsids containing DNA appear in the nucleus within 6 h post-infection.

#### 1.4.1.2. Viral Egress

The egress of HSV from an infected host cell is believed to initiate when mature capsids attach to patches in the inner nuclear membrane that contain immature viral envelope glycoproteins. Capsids bud through these regions, acquiring an envelope (Darlington and Moss, 1968; Roizman and Sears, 1996). Subsequent routes taken by the particles as they travel to the extracellular space, and by which the envelope

glycoproteins are processed, remain unclear. One view is that the enveloped virions are transported in transport vesicles through the endoplasmic reticulum (ER) and Golgi complex, finally reaching the plasma membrane where they are released. The glycoproteins are believed to mature as the virus is transported along the secretory pathway (Campadelli-Fiume *et al.*, 1991). Evidence for this model comes from a study by Johnson and Spear (1982) who demonstrated that monensin, an ionophore that disrupts the budding of vesicles from the Golgi, inhibits the transport of HSV particles to the cell surface. Treatment of HSV infected cells with monensin led to the accumulation of enveloped virus particles in what appeared to be Golgi-derived vacuoles. This supports the hypothesis that the viral glycoproteins are modified as the enveloped virus moves through the Golgi complex.

An alternative model for egress involves fusion of enveloped virus particles in the perinuclear space with the outer nuclear membrane, thereby releasing naked nucleocapsids into the cell cytoplasm (Jones and Grose, 1988; Whealy *et al.*, 1991). The nucleocapsids are then re-enveloped in the *trans* Golgi. This would yield a virion envelope with fully matured glycoproteins. Evidence for this model comes from a study by Whealy *et al.* (1991), who demonstrated that treatment of PrV-infected cells with brefeldin A, an inhibitor that destroys the Golgi apparatus, resulted in the accumulation of envelope-free capsids in the cytoplasm. In controls, the capsids were associated with Golgi vesicles and by electron microscopy, appeared to acquire a membrane envelope from the *trans* Golgi. There is evidence that HSV-1 may use a similar egress pathway. Brefeldin A treatment of HSV-1 infected cells results in the accumulation of naked nucleocapsids in the cell cytoplasm (Cheung *et al.*, 1991). Moreover, Browne and colleagues demonstrated that restriction of the expression of glycoprotein H to the ER-inner nuclear membrane by means of an ER-retention motif, resulted in the release of virus containing no detectable amounts of the glycoprotein (1996). These data suggest that the HSV-1 virion acquires an envelope from a subcellular component other than the ER-inner nuclear membrane. Support for this model also comes from the general observation that naked nucleocapsids

are prevalent in HSV-infected cells and are found adjacent to membrane-bound vesicular structures (possibly Golgi-derived vesicles) (Roizman and Sears, 1996).

Virus egress is dependent on Golgi-dependent maturation of viral glycoproteins. As discussed later (Section 1.5.1), defects in glycoprotein processing block the transport of virus to the extracellular space. The process of virion translocation across the cytoplasm to the extracellular space is also regulated by various viral components, including the protein product encoded by the UL20 gene. In several cell lines, the Golgi apparatus fragments as a result of infection, and in these cells, virions devoid of UL20 get trapped in the perinuclear space (Baines *et al.*, 1991; Campadelli-Fiume *et al.*, 1993). UL20 thus appears to facilitate viral exocytosis when the Golgi apparatus is not intact. In addition to UL20, gH (Browne *et al.*, 1996; Desai *et al.*, 1988), gD (Campadelli-Fiume *et al.*, 1991), and gK (Hutchinson and Johnson, 1995; Jayachandra *et al.*, 1997) are involved in the transport of enveloped virus: virions accumulate within the cytoplasm when any one of these proteins is absent.

#### 1.4.2 HSV Latency

Much of the clinical disease arising from HSV infection is due to the ability of the virus to interact with neurons of the peripheral nervous system. This allows the virus to survive throughout the life of the infected individual in a latent state and evade the host immune response. Following primary infection and replication at peripheral sites, virus attaches to and enters sensory nerve terminals (Vahlne *et al.*, 1978). HSV particles travel along axons to neuronal cell bodies within the sensory ganglia (McLennan and Darby, 1980). These are preferentially the trigeminal and dorsal root ganglia for HSV-1 and HSV-2, respectively (Efstathiou *et al.*, 1986; Galloway *et al.*, 1982). Latent virus is reactivated by hormonal imbalance, emotional stress or injury to tissues that are innervated by neurons which are latently infected with the virus. Figure 1.3 illustrates herpes simplex virus latent infection. HSV latency has recently been reviewed by Whitely *et al.* (1998) and Efstathiou *et al.* (1999). Despite extensive research, however,

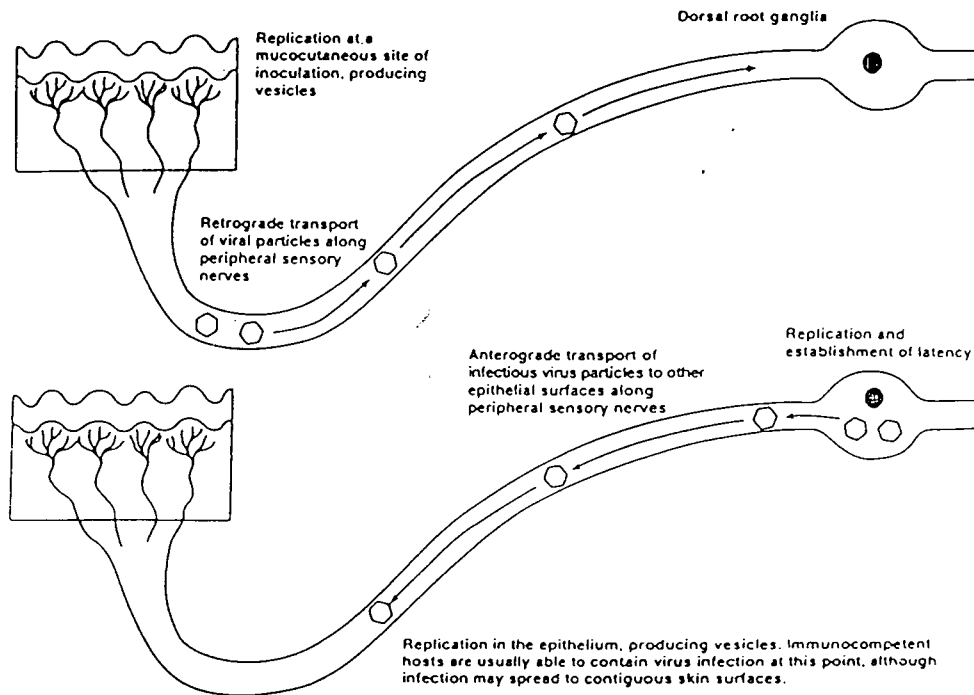


Figure 1.3. Illustration of HSV latency. From Whitley *et al.*, 1998.

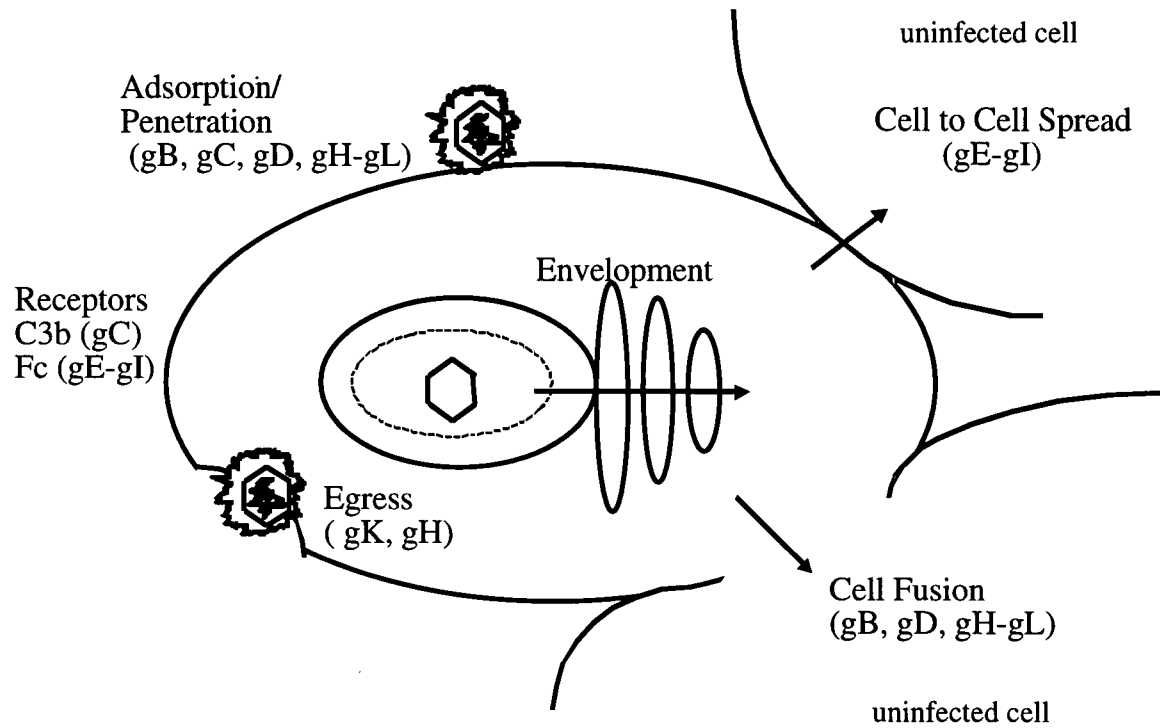
the mechanisms by which the virus establishes and maintains a latent state or is reactivated, remain poorly understood.

## 1.5 THE HSV ENTRY PATHWAY

In general, entry of enveloped viruses into cells requires the binding of virus to receptors on the cell surface, followed by either direct fusion of the virion envelope with the cell plasma membrane or endocytosis of the virion particle into the cell. Viral entry by endocytosis requires a low pH to initiate membrane fusion, as weak bases that increase pH inhibit virus entry. Data from electron microscopy studies suggests that HSV can penetrate cells by either fusion at the plasma membrane and/or by endocytosis (Morgan *et al.*, 1968; Dales and Silverberg, 1969). There is compelling evidence, however, that HSV entry occurs by a pH-independent fusion mechanism, rather than by endocytosis. Agents that inhibit endocytosis (e.g. chloroquine) do not block HSV entry (Fuller *et al.*, 1989). Moreover, it appears that entry of HSV by endocytosis results in degradation of the virus and thus a non-productive infection (Campadelli-Fiume *et al.*, 1988). Numerous lines of evidence indicate that HSV-1 entry is comprised of a cascade of virus-cell interactions, involving several viral glycoproteins and at least two cell surface components. The process of HSV-1 entry into cells can be divided into three distinct phases: binding, stable attachment of the virion particle to the cell surface, and penetration. Before discussing details of the entry pathway, the synthesis of viral glycoproteins that mediate HSV entry into host cells will be described in detail.

### 1.5.1 HSV Glycoproteins: Genetic Loci, Synthesis and Processing

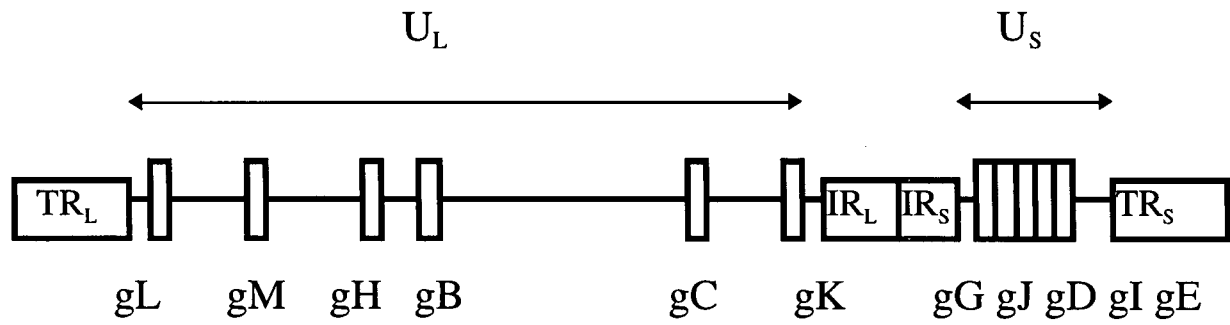
The HSV envelope glycoproteins are important determinants of HSV pathogenicity. The glycoproteins play critical roles in viral entry, egress, cell fusion and immune evasion. They also influence tissue tropism and host range. The functions of the glycoproteins in HSV-1 infection are illustrated in Figure 1.4. The organization of



**Figure 1.4** Functions of HSV glycoproteins.

glycoprotein genes in the genome of HSV-1 is shown in Figure 1.5. Precise map locations of the glycoproteins have come from nucleotide sequence analyses. It is significant that several of the glycoproteins are conserved among members of the *Alphaherpesvirinae* (Table 1). Five families of glycoproteins, gB, gC, gD, gH and gL, participate in the entry into cells of HSV-1, HSV-2, PrV and BHV-1 (Spear, 1993). gB and gH appear to be the most strongly conserved of the glycoprotein families with respect to amino acid sequence (Manservigi and Cassai, 1989). In fact all herpesviruses (alpha-, beta- and gamma-) encode members of gB and gH families. This likely reflects the important functions these glycoproteins have in viral infection. It is also interesting that glycoproteins not essential for viral replication are clustered in the U<sub>S</sub> region. Of all the HSV U<sub>S</sub>-encoded glycoproteins, only glycoprotein D is required for viral infection. However, in both PrV and BHV, gD has been shown to be dispensable for growth in cell culture, likewise to the other U<sub>S</sub>-encoded glycoproteins (Schmidt *et al.*, 1997; Schroder *et al.*, 1997). These observations support the hypothesis that this cluster of dispensable genes might have been acquired during the evolution of herpesviruses, perhaps having evolved from one dispensable precursor gene (McGeoch 1990; Schroder *et al.*, 1997).

The HSV glycoproteins have the characteristics of integral membrane proteins. Nucleotide sequence analyses of gB-1, gC-1 and gD-1, for example, have shown that (i) the N-terminal portions of these proteins are hydrophobic and act as signal sequences which are cleaved while (ii) the C-terminal regions possess a hydrophobic transmembrane anchoring region and a highly basic carboxy tail (Watson *et al.*, 1982; Frink *et al.*, 1983; Bzick *et al.*, 1984). In general, the biosynthesis of HSV glycoproteins is similar to that of eukaryotic cell glycosylated proteins (Campadelli-Fiume and Sefarini-Cessi, 1985). Non-glycosylated precursors of the viral glycoproteins are synthesized on polyribosomes bound to the rough endoplasmic reticulum. Concomitantly, the proteins are then transported into the lumen of the rough endoplasmic reticulum by the signal sequence. The unglycosylated protein precursors then undergo glycosylation using host machinery. Studies have shown that glycoproteins specified by HSV-1 contain both O-linked



**Figure 1.5.** Organization of glycoprotein genes in the HSV-1 genome. (TR) terminal repeat; (IR) internal repeat.



**Table 1.** Homology between glycoproteins encoded by alphaherpesviruses

HSV	PRV	BHV-1	Essential for Viral Replication?	Function
gB	gB	gI	Yes	binding; penetration; cell fusion; cell-cell spread; neuroinvasive determinant (HSV)
gC	gC	gIII	No	binding; C3b receptor (HSV-1); egress (PrV)
gD	gD	gIV	Yes for HSV No for BHV and PrV	stable attachment; penetration; cell fusion and cell-cell spread; neuroinvasive determinant (HSV)
gH	gH	gII	Yes	penetration; cell fusion ; cell-cell spread; egress (HSV)
gL	gL	gL	Yes	penetration; cell fusion; cell-cell spread
gK	gK	?	No	egress (HSV and PrV); cell fusion (HSV)
gE	gE	gE	No	cell-cell spread; Fc receptor (HSV-1)
gI	gI	gI	No	cell-cell spread; Fc receptor (HSV-1)
gG	gG	gG	No	unknown
gJ	not present	gJ	No	unknown
gM	gM	gM	No	possibly cell-cell spread (HSV and PrV)
?	gN	?	No	gN forms complex with gM in PrV

(Manservigi and Cassai, 1991; Maclean *et al.*, 1993; Spear, 1993; Ward and Roizman, 1994; van Drunen Littel-van den Hurke *et al.*, 1996; Schmidt *et al.*, 1997; Schroder *et al.*, 1997; Yoshitake *et al.*, 1997; Jones *et al.*, 1998; Klupp *et al.*, 1998; Schroder and Keil, 1999).

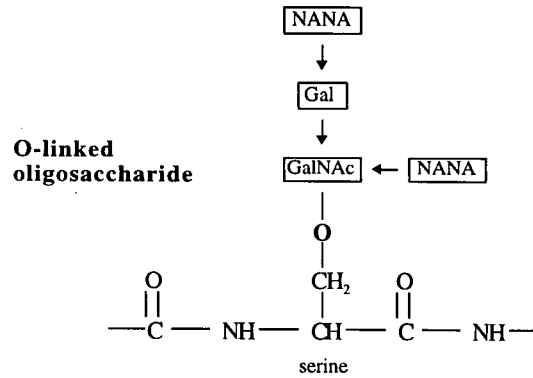
(Oloffson *et al.*, 1981; Johnson and Spear, 1983) as well as N-linked oligosaccharides (Sefarini-Cessi and Campadelli-Fiume, 1981; Wenske *et al.*, 1982).

N-linked glycosylation is initiated in the ER, when a preformed branched oligosaccharide [(glucose)<sub>3</sub>-(mannose)<sub>9</sub>-(N-acetylglucosamine)<sub>2</sub>] linked to a dolichol phosphate lipid carrier is transferred *en bloc* to asparagine residues on the nascent polypeptide. These asparagine residues are found within the tripeptide recognition sequences Asn-X-Ser or Asn-X-Thr (where X is any amino acid except proline) (Marshall, 1972). The asparagine-linked (N-linked) oligosaccharides are further modified during transit through the Golgi apparatus. This processing will produce an oligosaccharide chain referred to as the high mannose oligosaccharide, which contains only mannose and N-acetylglucosamine (Fig. 1.6B). In some instances, N-linked glycans are not processed beyond this stage, such as the adenovirus E3 glycoprotein (Kornfeld and Wold, 1981). Complex N-linked glycans are produced when high mannose forms undergo further trimming in the Golgi. These oligosaccharides have the core structure [(mannose)<sub>3</sub>-(N-acetylglucosamine)<sub>2</sub>] and several side chains containing N-acetylglucosamine, galactose and N-acetylneuraminic acid residues (Fig. 1.6C). Fucose can also be added to the side chains. The mature forms of HSV glycoproteins possess complex glycans (Campadelli-Fiume and Sefarini-Cessi, 1985). HSV-1 gB is an exception to this, in that not all of its high mannose glycans undergo conversion. Thus, it carries both types of N-linked glycans (Wenske *et al.*, 1982; Johnson and Spear, 1983).

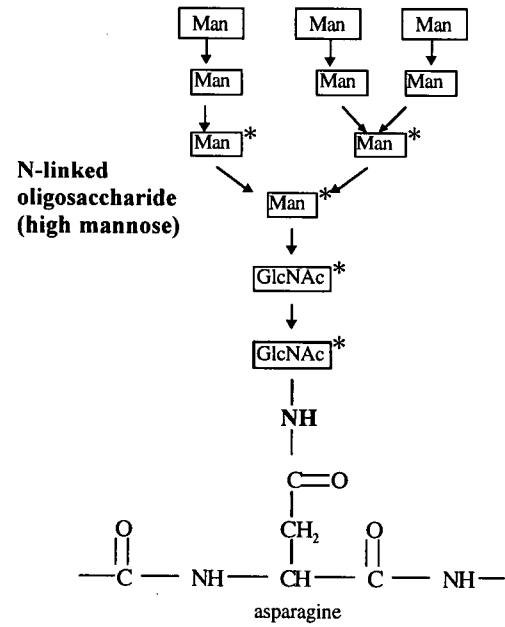
By contrast to N-glycosylation, O-glycosylation of HSV glycoproteins is a late post-translational event (Johnson and Spear, 1993). O-glycosylation initiates when N-acetylgalactosamine is linked to a serine or threonine residue in the polypeptide chain (Fig. 1.6A). For HSV glycoproteins, this appears to occur in the Golgi apparatus. The assembly of sugars added later (galactosamine, N-acetylgalactosamine, fucose and sialic acid) involves the sequential action of specific glycosyltransferases located in the Golgi complex. Significantly, O-glycosylation of viral proteins occurs less frequently than N-

**Figure 1.6.** Structure of typical N-linked and O-linked oligosaccharides. (A) Structure of an O-linked oligosaccharide, here shown linked to a serine hydroxyl group. (B) Structure of a high-mannose N-linked oligosaccharide, linked to asparagine. (C) Structure of a complex N-linked oligosaccharide. Complex N-linked oligosaccharides are formed by modifications to the high mannose form in the Golgi complex. Asterisks indicate the five residues always found in N-linked oligosaccharides. (NANA), N-acetylneuraminic acid (sialic acid); (Gal), galactose; (GlcNAc), N-acetylglucosamine; (GalNAc), N-acetylgalactosamine; (Man), mannose; (Fuc), fucose.

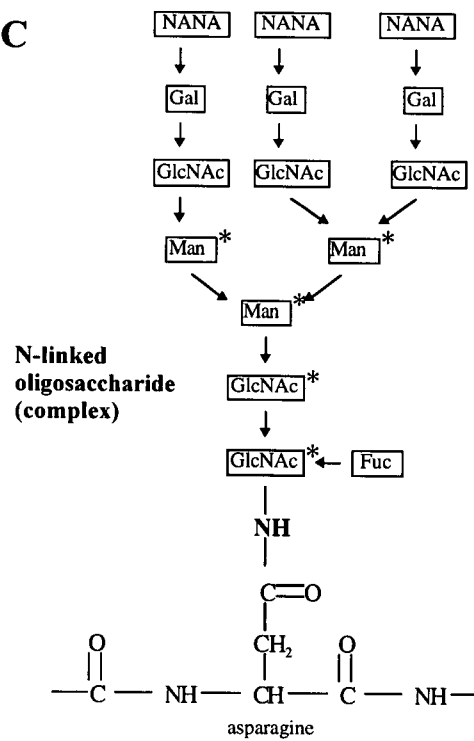
**A**



**B**



**C**



glycosylation (Olofsson *et al.*, 1981; Johnson and Spear, 1983). It has been postulated that the conversion of high mannose forms to complex N-linked glycans, may interfere with accessibility of serine/threonine sites to the transferase (Sefarini-Cessi *et al.*, 1983a).

Structures of N and O-linked oligosaccharides vary immensely, and the glycan side chains of an individual glycoprotein can thus be quite heterogeneous. Johnson and Spear (1993) noted that O-linked oligosaccharides from gC and gD ranged in size from 215 daltons to as large as 2750 daltons. For both N- and O- glycosylation, the conformation of the protein may, in part, determine the extent of glycosylation and degree of heterogeneity (Roizman and Sears, 1996).

The role of N-glycosylation with regard to HSV infectivity has been well characterized. Studies using the antibiotic tunicamycin, which inhibits the dolichol-dependent transfer of the mannose-rich backbone of the oligosaccharide chain to the protein, have demonstrated that N-glycosylation of viral glycoproteins is necessary for the production of infectious progeny (Pizer *et al.*, 1980). A subsequent study demonstrated that in tunicamycin-treated cells, virion envelopment is defective (Peake *et al.*, 1982). To test directly whether the conversion of high-mannose oligosaccharides to complex glycans is required for infectivity, Sefarini-Cessi and colleagues (1983) examined HSV-1 infection in cell lines defective in Golgi enzymes responsible for the addition of terminal sugars to N-linked glycans. In this cell line, therefore, the HSV glycoproteins were not fully processed. It was interesting then that this had no effect on the production of infectious progeny. The authors concluded that the presence of complex glycans is not a requirement for HSV infectivity and that high mannose forms are sufficient in this regard. The conversion of high mannose forms to complex N-linked glycans does, however, appear to facilitate virion egress and virus-induced cell-fusion (Sefarini-Cessi *et al.*, 1983). Several other groups have also shown that viral egress is hindered when there are defects in the glycosylation pathway (Johnson and Spear, 1982; Peake *et al.*, 1982).

The role of O-glycosylation in HSV infectivity is not as well understood. An interesting observation, however, was made by Tal-Singer and colleagues (1995) who

noted that in HSV-1 gC, most of the O-linked saccharides were clustered in the N-terminus. In mucins, which are large mucous glycoproteins, clustering of O-linked glycans is believed to force the protein into an extended fibre-like structure (Jentoft, 1990). Indeed, gC-1 has a rod-like appearance when examined by electron microscopy, and it may be that this conformation facilitates the protein's interaction with cell surface glycosaminoglycans and thereby mediate the initial attachment of virus to cell surface glycosaminoglycans (Tal-Singer *et al.*, 1995).

### 1.5.2 Viral Binding

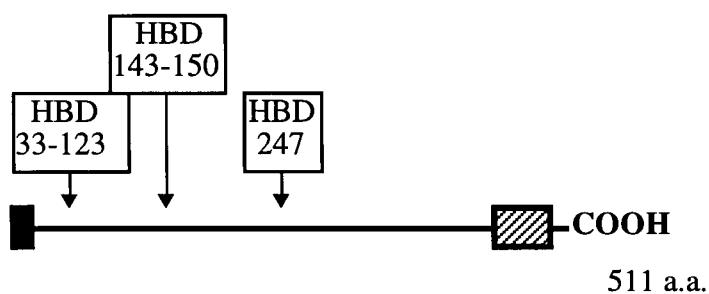
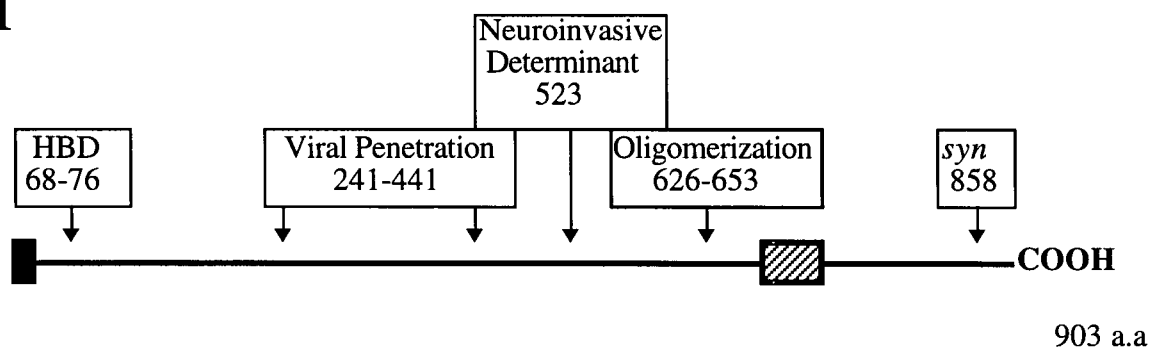
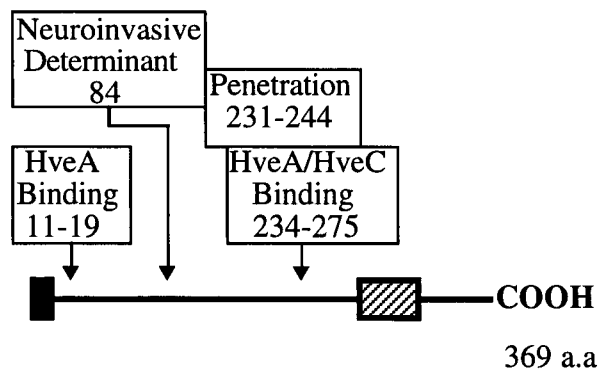
The current model for HSV entry predicts that there is an initial interaction of HSV particles with cell surface glycosaminoglycans (GAGs) which are abundant on most cell surfaces. Evidence for this interaction stems from observations that soluble heparan sulfate (HS) and heparin, which is structurally similar to HS, can reduce HSV infection by over 90% when present during inoculation (Gruenheid *et al.*, 1993). Because soluble glycosaminoglycans do not appear to reduce the infectivity of the virion itself, it is likely that soluble GAGs act as competitive inhibitors to viral attachment. Removal of cell surface GAGs, by incubation of the host cell with heparin- and chondroitin-lyases, also reduces HSV infection, which suggests that HS and chondroitin sulfate (CS) have roles in HSV infection (WuDunn and Spear, 1989). Furthermore, animal cell mutants with defects in GAG synthesis show either complete (Shieh *et al.*, 1992) or partial resistance to HSV infection (Gruenheid *et al.*, 1993; Banfield *et al.*, 1995a).

HSV was the first virus shown to bind HS (WuDunn and Spear, 1989). Other herpesviruses have since been found to use HS as an initial receptor, including pseudorabies virus (Mettenleiter *et al.*, 1990), cytomegalovirus (Neyts *et al.*, 1992), bovine herpesvirus 1 (Okazaki *et al.*, 1991) and human herpesvirus 7 (Secchiero *et al.*, 1997). Recent work has also demonstrated that foot-and-mouth-disease virus (Jackson *et al.*, 1996), respiratory syncytial virus (Krusat and Streckert, 1997), dengue virus (Chen *et*

*al.*, 1997), adeno-associated virus type 2 (Summerford and Samulski, 1988) and human immunodeficiency virus type 1 (Patel *et al.*, 1993), all use cell surface HS GAGs to attach to cells. Like HSV, many of the aforementioned viruses use additional cellular receptors besides HS. The functional role of HS in HSV infection appears to be to concentrate viral particles at the cell surface, thereby increasing the frequency of a subsequent interaction with its secondary receptor (Johnson and Ligas, 1988).

Initial attachment of HSV virions to cell surface heparan sulfate and chondroitin sulfate GAGs is mediated by gC and/or gB. The gene encoding HSV-1 gC (gC-1) has been extensively studied. Sequence data predict a primary protein product of 511 amino acids with a predicted molecular weight of 55,000 (Swain *et al.*, 1985). By contrast, HSV-2 gC-2 is 480 amino acids in length and has a predicted molecular weight of 51,600. Relative to the other HSV glycoproteins, gC-1 and gC-2 show the least degree of conservation, with limited areas of DNA and amino acid similarity. The functional arrangement, however, seems to be similar. Both glycoproteins have a hydrophobic signal sequence, a hydrophobic membrane-anchoring region, and a short acidic carboxy terminus. Both N- and O-linked oligosaccharides have been described for the two proteins (Wenske *et al.*, 1982; Olofsson *et al.*, 1983; Swain *et al.*, 1985; Johnson and Spear, 1983). The functional domains of gC-1 are depicted in Figure 1.7.

The involvement of HSV-1 gC in virion attachment to host cells is supported by several lines of evidence. First, virions devoid of gC are impaired in their ability to bind to cells (Herold *et al.*, 1991; Herold *et al.*, 1994; Laquerre *et al.*, 1998b). Second, neutralizing antibodies against gC-1 can block the binding of particles to host cells (Fuller and Spear, 1985). That gC-1 interacts with HS GAGs is demonstrated by the ability of soluble gC-1 to adsorb to heparin affinity columns (Herold *et al.*, 1991). Moreover, heparitinase treatment of cells, which removes any cell surface HS GAGs, reduces the attachment of soluble gC-1 by 50% (Tal-Singer *et al.*, 1995). Finally, removal of the heparin-binding domains of gC-1 significantly reduces the attachment efficiency of virus to cells (Trybala *et al.*, 1994; Tal-Singer *et al.*, 1995). As discussed later (section 1.7.2),

**gC-1****gB-1****gD-1**

**Figure 1.7** Functional domains of the HSV-1 glycoproteins gC-1, gB-1 and gD-1. The filled boxes represent signal sequences. Hatched boxes indicate transmembrane regions. The amino acids comprising the different glycoprotein domains are indicated. (HBD) Heparin-binding domain; (*syn*) syncytial; (Hve) Herpes virus entry mediator.



these heparin-binding regions have high positive charge densities, thereby promoting binding to negatively charged heparan sulfate moieties. It should be stressed, however, that the polycationic nature of heparan sulfate-binding domains (HBDs) is but one parameter that governs the interaction of proteins with GAGs.

The involvement of gC-1 in the initial adsorption of HSV-1 to host cells is made complicated by the observation that gC is not essential for viral infection (Herold *et al.*, 1991; Laquerre *et al.*, 1998b). Virions devoid of gC-1 are still infectious, although less efficient in attachment. This prompted Herold and co-workers (1994) to investigate whether there was gC-independent mode of binding. The ability of HSV-1 gC-negative virus to bind to cells devoid of HS GAGs was found to be significantly reduced compared to wild-type virus, indicating that gC-independent binding of HSV-1 requires cell surface HS. Moreover, HSV-1 particles devoid of both gB-1 and gC-1 have a drastically reduced ability to bind to cells compared to gC1-deficient and wild-type virions. These data, along with the finding that gB has an affinity for heparin under physiological conditions, led to the conclusion that in the absence of gC-1, gB-1 mediates adsorption to HS GAGs (Herold *et al.*, 1991).

gC-1 homologs in PrV and BHV-1 also function in viral attachment to HS GAGs (Karger and Mettenleiter, 1993). It was originally thought that HSV-2 gC would function to mediate HSV-2 attachment to cellular HS moieties since soluble gC-2, like gC-1, binds to heparin affinity columns (Gerber *et al.*, 1995). However, by contrast to HSV-1 gC, HSV-2 gC does not appear to play a predominant role in viral adsorption, because gC2-negative virions can bind to the cell surface as efficiently as wild-type HSV-2 (Gerber *et al.*, 1995). This raises the interesting possibility that gB-2, which is also a heparin-binding protein (Williams and Straus, 1997), may be the principle glycoprotein involved in mediating HSV-2 viral attachment to GAGs.

Glycoprotein B is required for productive infection of both HSV-1 and HSV-2. gB-1 and gB-2 are highly conserved, having an overall nucleotide and amino acid sequence similarity of 86% (Stuve *et al.*, 1987). For both proteins, the length of the entire coding

sequence is 904 amino acids, although the mature gB-2 is 7 amino acids longer than gB-1 due to its shorter signal peptide (the length of the signal peptides for gB-1 and gB-2 are 29 and 22 amino acids, respectively). These glycoproteins have 3 domains. For gB-1 and gB-2 respectively, they include an amino terminal hydrophilic extracellular domain of 696 and 700 amino acids, a transmembrane anchor region of 69 and 54 amino acids, and a charged carboxy terminus 109 and 106 amino acids, the latter two domains being highly conserved between the proteins (Pellet *et al.*, 1985; Stuve *et al.*, 1987). Marked divergence, however, is observed within the amino-terminal 85 amino acids of the protein (Stuve *et al.*, 1987). HSV-1 gB has the consensus sequence for six N-glycosylation sites and contains 10 cysteine residues. These are conserved in gB-2. Figure 1.7 shows the functional domains of gB-1.

The biologically active form of gB is a multimer (Claesson-Welsh *et al.*, 1986; Cai *et al.*, 1988; Highlander *et al.*, 1991). It has been demonstrated that a region in the gB-1 ectodomain, comprising residues 626 to 653, is sufficient for oligomerization (Laquerre *et al.*, 1996). gB monomers self-associate through a hydrophobic interaction. The formation of disulfide bridges involving cysteines on the monomer units is not required for dimerization, although this contributes to oligomer stability. Other viral glycoproteins, including those of human immunodeficiency virus (Earl *et al.*, 1990) and Semliki Forest Virus (Kielian *et al.*, 1990) form oligomers. Oligomerization of viral glycoproteins can impart new functions to the protein (Doms *et al.*, 1993). Thus oligomeric forms can possess functions, such as receptor binding, that the monomer units would not.

Recently, Laquerre and colleagues (1998b) identified the heparin-binding domain of gB-1 (see Section 1.7.2). While this domain is not required for HSV-1 infectivity, it is involved in viral adsorption. A mutant HSV-1 deficient in the heparin-binding domain of gB-1 was shown to bind to cells less efficiently than wild-type virus (20% reduction in binding). Significantly, this reduction in binding was not as great as that observed for a gC-deficient HSV-1 virus (65% reduced-binding). Having both mutations in a single virus (i.e. deletions in the HBD of gB-1 as well as the gC-coding sequence) was additive, and

reduced binding to cells by 80%. Thus, the relative contribution of gC-1 to HS-binding is greater than that of gB-1. It is also worthy to note that the HBD of gB-1 is located within the region most divergent between gB-1 and gB-2. Future studies will need to determine whether this domain functions similarly in the two glycoproteins with respect to affinity and specificity for GAGs.

### 1.5.3 Stable Attachment of Virus to the Host Cell Surface

Several lines of evidence support a model whereby HSV entry involves a second non-GAG receptor. For example, sog9 cells, despite being GAG-deficient, are still susceptible to HSV infection (Banfield *et al.*, 1995a). Subramanian and colleagues (1994) isolated swine testis cells which were defective in HSV-1 entry, despite expressing a full complement of functional cell surface HS moieties. This indicated that these cells lacked a functional non-HS receptor required for efficient viral entry. Finally, Sears *et al.* (1991), have provided conclusive evidence that HSV-1 is able to bind at least two cell surface receptors. MDCK cells, a polarized cell line, express HS GAGs primarily on the basal cell surface. It was demonstrated that gC-deficient virions are only able to infect via the basal surface of MDCK cells whereas wild-type HSV-1 was able to infect at both the basal and apical surfaces. These data suggest that one receptor interacts with gC and is found on the apical cell surface, while a second receptor is found only at the basal cell surface.

The accumulative work of several laboratories has shown that gD interacts with its own cellular receptor. Glycoprotein D is found in the envelope of HSV-1 and HSV-2, and for both viruses, this protein is required for entry into mammalian cells (Highlander *et al.*, 1987; Johnson and Ligas, 1988; Ligas and Johnson, 1988). The two proteins are structurally similar (Eisenberg *et al.*, 1980; Lasky and Downbenko, 1984; Chiang *et al.*, 1994) and appear to be functionally interchangeable, since gD-2 can be recombined into HSV-1 with no effect on virus infection (Muggeridge *et al.*, 1992). The gene for HSV-1 gD encodes for a protein 394 amino acids in length which like gB and gC, has an N-

terminal ectodomain and a hydrophobic transmembrane anchor sequence near the carboxy terminus (Fig. 1.7) (Matthews *et al.*, 1983).

Evidence that gD interacts with its own receptor came first from the observation that gD-1 interferes with infection by HSV-1 (Spear, 1993). For example, cells which constitutively express gD-1 are resistant to infection by HSV-1 (Campadelli-Fiume *et al.*, 1988; Johnson and Spear, 1989). Presumably the gD produced by the cell sequesters the cellular receptors and prevents them from binding to gD in the virus. This resistance to infection is due to the failure of the virus to penetrate into gD-expressing cells, not the inability of the virus to bind to HS GAGs. gD-mediated interference is also observed with HSV-2, PrV and BHV-1 (Spear, 1993). Moreover, it appears that in certain cell types, some of these viruses use a common gD receptor for entry, since cells expressing a member of the gD family can be resistant to infection by a heterologous virus (Spear, 1993). Johnson and colleagues (1990) have demonstrated that soluble forms of HSV-1 and HSV-2 gD bind to a limited number of sites on the cell surface, approximately  $4 \times 10^5$  to  $5 \times 10^5$  per cell (Johnson *et al.*, 1990). Binding of soluble gD-1 can also be reduced by treating cells with proteases but is unaffected when cell surface HS is removed. Thus, the gD receptor is not an HS moiety.

Brunetti *et al.* (1994) later proposed that HSV-1 gD interacts with host cell mannose-6-phosphate receptors (MPRs). MPRs are found on the surfaces of cells, primarily in clathrin coated pits (Dahms *et al.*, 1989). Soluble gD-1 bound to the 275-kDa MPR and the 46-kDa MPR. Moreover, the authors observed that gD-1 was modified by mannose-6-phosphate moieties. This group subsequently demonstrated that soluble forms of the MPRs could inhibit HSV-1 entry into monkey cells (Brunetti *et al.*, 1995). However, mouse cells lacking the receptor are still susceptible to infection, suggesting that MPRs are not essential for entry in all cell types.

### 1.5.3.1. Hve A

Collectively, the above data provided strong evidence for at least one other receptor involved in HSV entry. To determine the identity of this receptor, Montgomery and colleagues (1996) transfected an expression library into CHO cells, which express GAGs but are resistant to entry of HSV-1, PrV and BHV-1 (Shieh *et al.*, 1992; Warner *et al.*, 1998). CHO cells are partially susceptible to HSV-2 infection. One clone, which encoded a member of the TNF receptor family, restored full susceptibility to HSV-1 and HSV-2 infection in the CHO cells and was shown to have herpesvirus entry activity. This cell surface protein has now been designated as herpesvirus entry protein A (HveA). The interaction of HSV-1 and HSV-2 with HveA was later shown to be mediated by gD (Whitbeck *et al.*, 1997; Nicola *et al.*, 1998).

Characteristic of the TNF receptor family, Hve A, also known as TR2 (Kwon *et al.*, 1997) and ATAR (*another TRAF-associated receptor*) (Hsu *et al.*, 1997), is a type I membrane glycoprotein with cysteine-rich repeats in the ectodomain (Naismith and Sprang, 1998). Lymphotoxin  $\alpha$  and LIGHT (homologous to *lymphotoxins*, exhibits inducible expression, and competes with gD for HveA, a receptor expressed by *T* lymphocytes) are members of the TNF family and are ligands for HveA (Mauri *et al.*, 1998). The TNF-related cytokine-receptor systems feed into signalling pathways involved in either cell death or cell survival. This raises the possibility that HSV gD-1 may be able to modify HveA-signalling activities during viral entry which may, in turn, aid in virus survival and persistence. Although HveA is expressed in many tissue types, including lung and liver, the receptor is expressed most abundantly in lymphoid organs and cells (Marsters *et al.*, 1997). This corroborates the findings of Montgomery and colleagues (1996), who observed that anti-Hve A antibody prevented HSV-1 infection of human T lymphocytes but not of other human cell types. This demonstrated that (i) HveA is the principle co-receptor into human lymphoid cells and (ii) other receptors must exist for HSV entry, since HSV can also infect cells of neuronal and epithelial origin.

### 1.5.3.2. HveB/Prr2

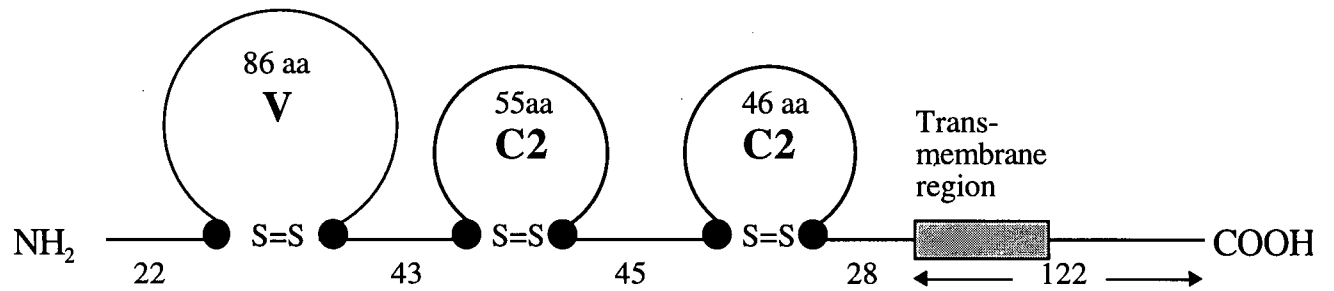
A subsequent study by Warner and colleagues (1998) demonstrated that poliovirus receptor-related protein 2 (Prr2) (Eberle *et al.*, 1995) mediated entry of wild-type HSV-2 strains, particular viable HSV-1 gD mutants and PrV into CHO cells. Prr2, designated as herpesvirus entry protein B (HveB), failed to support entry of wild-type HSV-1 strains or BHV-1. The structure of Prr2/HveB is illustrated in Figure 1.8. Prr2/HveB is expressed in keratinocytes and neuronal cells. Prr2 is a member of the poliovirus receptor subfamily of the immunoglobulin (Ig) superfamily. The poliovirus receptor family also includes the poliovirus receptor (Pvr) and poliovirus receptor-related protein 1 (Prr1) (Fig. 1.8). By contrast to Pvr, Prr1 and Prr2 do not function in poliovirus entry and are only related to Pvr by amino acid sequence (Eberle *et al.*, 1995). Recently, Prr1 and Prr2 were renamed as nectin-1 and nectin-2 $\alpha$ , respectively (Takahashi *et al.*, 1999). The name nectin comes from the Latin “necto” meaning “to connect”. Both nectin-1 and nectin-2 $\alpha$  have been shown to function as cell-cell adhesion molecules (Aoki *et al.* 1997; Takahashi *et al.*, 1999).

### 1.5.3.3. HveC/Prr1

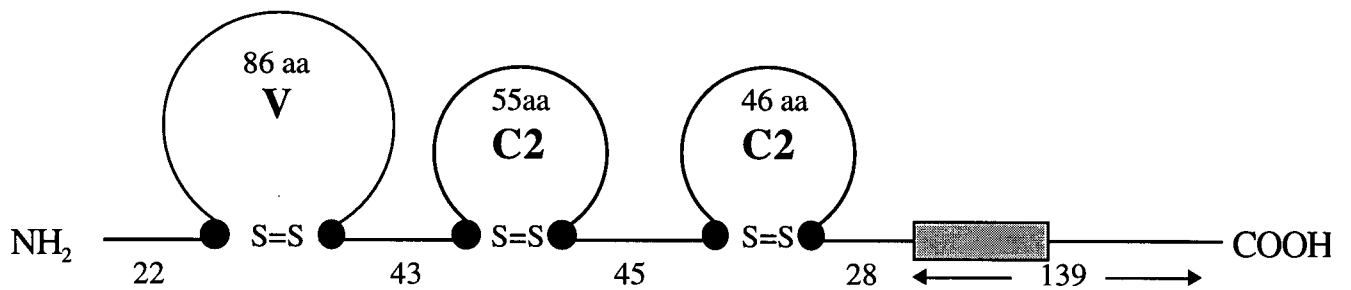
The ability of wild-type HSV-2, but not HSV-1, to use HveB as a receptor could account, in part, for serotype differences in tissue tropism and pathogenicity. Neither HveA nor HveB, however, function as a co-receptor of both serotypes into epithelial cells, the mucosal epithelia being the site of initial replication. Moreover, neither Hve A nor Hve B can serve as a co-receptor for the other alphaherpesviruses, PrV and BHV-1. For these reasons, Geraghty and colleagues (1998) set out to isolate a common coreceptor for HSV-1, HSV-2, PrV and BHV-1. In their study, they identified poliovirus receptor-related protein 1 (Prr1) protein (Lopez *et al.*, 1995), designated as herpesvirus entry protein C (HveC), as being able to mediate entry of all these viruses into resistant CHO cells (Fig.1.8). Additionally, they demonstrated that Pvr itself mediates BHV-1 and PrV infection, but not HSV infection. Cocchi *et al.* (1998b) later identified an isoform of

**Figure 1.8 .** Schematic representation of the molecular structures of HveB/Prr2, HveC/Prr1, HIgR, and PVR. These receptors are members of the Ig superfamily. Shown here are the V and C domains formed by cysteine bonds and the number of amino acid residues in each region. From Warner *et al.*(1996) and Cocchi *et al.* (1998).

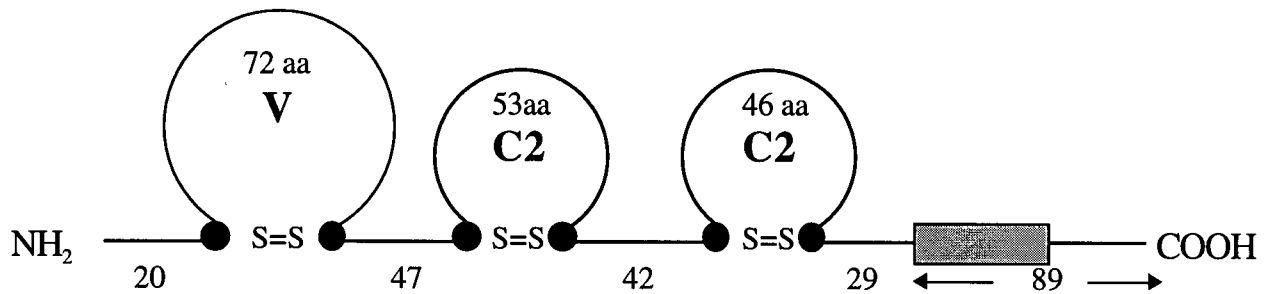
## HveB/Prr2



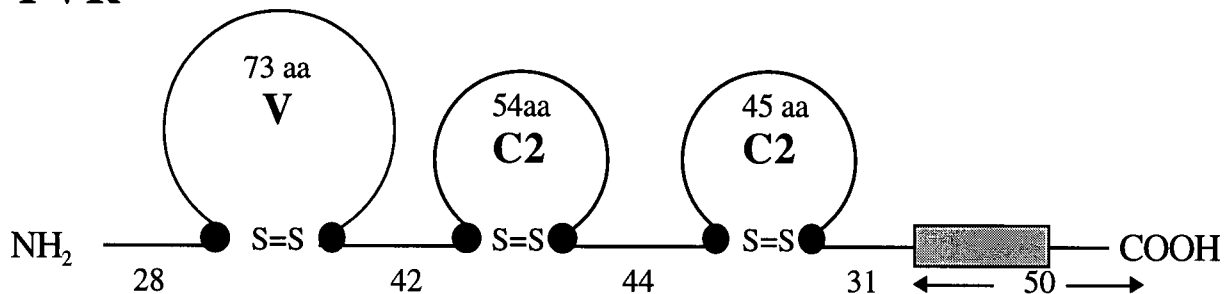
## HveC/Prr1



## HIgR



## PVR





HveC, designated the herpes Ig-like receptor (HIgR), as being able to mediate HSV-1, HSV-2 and BHV-1 infection (Fig. 1.8). HIgR appears to be a splice variant of HveC; the two share an identical ectodomain, comprising one variable (V) and two constant (C2) domains. The proteins differ in their cytoplasmic and transmembrane domains. Significantly, HveC and HIgR are expressed in human epithelial and neuronal cells and as such are strongly favoured as the receptors that allow both HSV-1 and HSV-2 to infect epithelial mucosa and spread to the nervous system (Geraghty *et al.*, 1998; Cocchi *et al.* 1998b).

Likewise to HveA, HveC and HIgR interact with HSV-1 gD. Moreover, Cocchi and colleagues (1998a) demonstrated that the V domain of HIgR interacts with gD. The gD-HveA and gD-Hve C interactions have been studied in detail (Krummenacher *et al.*, 1998). Soluble truncated forms of Hve A and Hve C directly bind to HSV gD both in solution and at the surface of virions. Competition assays with neutralizing anti-gD monoclonal antibodies showed that the region between amino acids 234 and 275 in gD-1 may contain a common domain which interacts with both HveA and HveC. However, the gD-HveA and gD-HveC interactions also had a number of significant differences. Disruption of the N-terminal domain of gD by linker insertion at amino acid 34 significantly reduced binding of gD to Hve A but not Hve C. Moreover, competition assays with anti-gD monoclonal antibodies directed against N-terminal amino acids 11 to 19 blocked the interaction between HveA and HSV-1 (KOS) but did not block the interaction of Hve C with virus. Collectively, these data indicate that the N-terminal region of gD is important for interactions with Hve A but not HveC (Krummenacher *et al.*, 1998).

The Ig superfamily members Pvr, Prr-1, Prr-2 and HIgR share a similar molecular structure defined by the six conserved cysteine residues (Fig. 1.8). Alphaherpesviruses may have evolved to recognize these highly conserved domains in the Ig superfamily. Clearly, however, different HSV-1 and HSV-2 strains vary in their ability to utilize HveA, HveB, Hve C and HIgR for infection. This, and the fact that these receptors are

expressed in distinct cell types, will, in part, determine the pattern of viral spread in an infected individual.

#### 1.5.4. Viral Penetration

The HSV glycoproteins gB, gD, gH and gL are required for viral penetration and are essential for productive HSV infection (Cai *et al.*, 1988; Ligas and Johnson, 1988; Forrester *et al.*, 1992; Hutchinson *et al.*, 1992). Homologs of gB, gD, gH and gL in PrV and BHV-1 have also been shown to be involved in penetration (Spear, 1993; Karger and Mettenleiter, 1993; van Drunen Littel-van den Hurk *et al.*, 1996; Klupp *et al.*, 1988). Unlike HSV gD, however, BHV and PrV gD homologs are not essential for this process (Schmidt *et al.*, 1997; Schroder *et al.*, 1997). HSV mutants deleted for either gB, gD and/or gH-gL are able to attach to cells but unable to penetrate. Polyethylene glycol, a membrane fusogen, can enable adsorbed mutant viruses to penetrate and initiate infection. Furthermore, anti-gB, gD and gH neutralizing antibodies permit attachment to cells but prevent penetration (Fuller and Spear, 1987; Fuller *et al.*, 1989; Navarro *et al.*, 1992). The activity of gH is dependent on gL (Hutchinson *et al.*, 1992). gH and gL form a hetero-oligomer which is incorporated into virions. Co-expression of gL and gH is required for normal post-translational modifications, folding and intracellular transport of both glycoproteins (Gompels and Minson, 1989; Roop *et al.*, 1993). Thus, Roop *et al.* (1993) observed that an HSV-1 mutant deleted in gL was unable to incorporate gH into the virion envelop and enter into cells, although it could adsorb efficiently to the cell surface.

Domains critical for viral penetration have been mapped for HSV-1 gB. Anti-gB monoclonal antibodies that inhibit viral penetration map to amino acid residues 241 to 441 in the gB molecule, a region that is centrally located within the ectodomain (Fig. 1.7) (Highlander *et al.*, 1988). With respect to gD-1, earlier studies identified a functional region comprising residues 231 to 244, which when altered significantly disrupted gD function (Feenstra *et al.*, 1990; Muggeridge *et al.*, 1990). It is interesting that this region overlaps with the region identified as interacting with HveA and HveC. It will be

important to establish how the interaction of gD with its cellular receptor facilitates the fusion of the virion envelope with the cell plasma membrane.

Despite these findings, the molecular mechanisms underlying HSV penetration remain unclear. It is evident, however, that gB-1, gD-1, gH-1, gL-1 and also gC-1 can associate with one another in the virion envelope (Handler *et al.*, 1996a). Chemical cross-linking studies have shown that these glycoproteins can form homodimers and hetero-oligomers of gD linked to gB, gC linked to gB and gD, and gH-gL linked to gD and gC. The gH-gL complex does not appear to associate with gB. These findings were expanded by another study which examined cross-linking patterns of these glycoproteins during entry (Handler *et al.*, 1996b). Changes in cross-linking profiles were not detected during viral attachment. However, complexes of gB and gD which were present during attachment disappeared as penetration proceeded. It is possible that during penetration, the glycoproteins undergo a conformational change or that the virion envelope undergoes a physical change (such as during the formation of a fusion pore). Together, these findings support the concept that the HSV viral glycoproteins form a complex during entry. This is not necessarily surprising, since all known virus fusion proteins are oligomers and many cellular fusion events are dependent on the formation of multi-subunit complexes (White, 1990).

#### **1.5.5. Cell-to-Cell Spread and Cell-to-Cell Fusion**

Two processes related to, but distinct from viral entry, are cell-to-cell spread of virus and the fusion of cells infected with virus. During wild-type HSV infection in tissue culture, it is typical for cells to become rounded as single cells, with very little fusion of infected cells (Hutchinson *et al.*, 1992b). Plaque formation results from virus spreading from an infected cell directly into adjacent uninfected cells. Cell-to-cell spread requires the glycoproteins gB, gD and gH-gL. If viruses deficient in these glycoproteins are grown on complementing cell lines (to provide the missing glycoprotein) the mutant viruses can

enter cells but cannot spread to adjacent cells and form plaques (Cai *et al.*, 1988; Forrester *et al.*, 1992; Ligas and Johnson, 1988; Roop *et al.*, 1993). Glycoproteins gE and gI, which form a complex, are also involved in cell-to-cell spread. These glycoproteins are not required for entry or for the production of infectious progeny. However, gE- and gI-negative HSV mutants yield significantly smaller plaques *in vitro*, and when injected into mice, spread poorly beyond the initial site of infection (Dingwell *et al.*, 1994). Moreover, HSV particles deleted for either gE or gI fail to spread efficiently within the nervous system (Dingwell *et al.*, 1995). A recent study by Dingwell and Johnson (1998) demonstrated that the gE-gI complex co-localizes with cell-junction components, supporting a model whereby gE-gI mediate transfer of virus to adjacent cells across cell junctions. This mode of transmission is particularly important to HSV pathogenesis, since the virus is therefore able to avoid neutralization by anti-HSV antibodies.

Entry of HSV into cells involves fusion of the virion envelope with the cell plasma membrane. In a similar process, newly synthesized proteins expressed on the plasma membrane of an infected cell induce fusion with neighboring uninfected cells. As mentioned previously, wild-type HSV infection causes limited amounts of cell fusion, although polykaryocytes have been observed in lesions of some infected individuals. This phenomenon is best observed when cells are infected with HSV syncytial mutants which yield large, multi-nucleated cells (Hutchinson *et al.*, 1992). A syncytial (*syn*) mutation has been mapped to the cytoplasmic tail of gB; an Arg-to-His alteration at residue 858 accounts for the syncytial phenotype (DeLuca *et al.*, 1982). HSV-1 strains ANG and ANG-path possess this *syn* mutation in gB, along a second mutation at residue 553, termed a fast rate of entry (*roe*) determinant in gB (Saharkhiz-Langroodi and Holland, 1997). Together, these mutations allow for a unique type of cell fusion in tissue culture called fusion-from-without (FFWO). FFWO is the rapid induction of cell fusion that is observed at a high m.o.i. and occurs without viral protein synthesis.

Additionally, gD and gH are involved in cell fusion, as antibodies directed against these glycoproteins will inhibit syncytia formation (Gompels and Minson, 1986;

Highlander *et al.*, 1987). Recently, Turner and colleagues (1998) demonstrated that transient expression of gB, gD, gH-gL of HSV-1 in cells was necessary and sufficient to induce cell fusion. Omitting any one of the glycoproteins reduced polykaryocyte formation. Finally, many *syn* mutations have been mapped to HSV-1 gK (Dolter *et al.*, 1994). gK has been detected in the nuclear and perinuclear space, but not in the viral envelope or in the plasma membrane (Hutchinson *et al.*, 1992a; Hutchinson *et al.*, 1995b). Because it is not present in the plasma membrane, it is unlikely that gK could directly influence cell fusion. Recently, however, HSV-1 gK was shown to be required for efficient viral envelopment and egress of infectious virions from the cytoplasm to the extracellular space (Hutchinson and Johnson, 1995a; Jayachandra *et al.*, 1997). Further studies are necessary to determine whether altered virion cell surface transport could influence cell-cell fusion.

Cellular factors have also been shown to influence viral-induced cell fusion. Shieh and Spear (1994) demonstrated that herpesvirus-induced cell fusion was dependent on cell surface heparan sulfate. Infectious DNA from the HSV-1 syncytial mutant HSV-1 (MP) was transfected into heparan sulfate-deficient mutant CHO cells. The ability of HSV-1 (MP) DNA to induce syncytia on the mutant cell line was reduced by 10-fold when compared to syncytia formation in cells expressing heparan sulfate. Soluble heparin was also shown to be able to substitute for cell surface HS in promoting fusion of transfected CHO cells. What is the role of HS in viral-induced cell fusion? The interaction of GAG with a GAG-binding protein can significantly alter the activity of the protein (Jackson *et al.* 1991). The role of HS/heparin in viral-induced cell fusion could, therefore, be to interact with a heparin-binding protein and thereby trigger fusogenic activity (Shieh and Spear, 1994). Given its role in viral penetration and cell-cell fusion, the heparin-binding glycoprotein gB is a possible candidate for HS activation of fusion. More recently, Terry-Allison and colleagues (1998) demonstrated that HveA also mediates cell-cell fusion. Cells resistant to HSV-1-induced cell fusion became susceptible when they expressed HveA.

### 1.5.6 HSV Infection of Mouse Cell lines Defective in GAG synthesis.

The approach our lab has taken to investigate the HSV entry pathway has involved the isolation of mouse L cell mutants defective in GAG synthesis. Gruenheid and colleagues (1993) infected mouse cells with HSV-1 and subsequently harvested any cells that survived to form colonies. This led to the isolation of the mutant cell line gro2C, which synthesizes chondroitin sulfate but not heparan sulfate. Gro2C cells are 90% resistant to HSV-1 infection relative to parental control L cells. The observation that gro2C cells were susceptible to HSV-1 infection, despite being deficient in HS GAGs, suggested that HSV-1 could interact with cell surface CS to mediate infection (Banfield *et al.*, 1995b). This was supported by the observation that soluble CS inhibited HSV-1 infection on gro2C cells. The sog9 cell line was derived from gro2C cells (Banfield *et al.*, 1995b) and exhibits a three-order of magnitude decrease in susceptibility to HSV-1 infection compared with control L cells. This observation can be explained by the fact that the sog9 cell line has additional defects in the GAG synthesis pathway and fails to synthesize any GAGs. Importantly, HSV-1 infection of sog9 cells is not significantly reduced by soluble HS, indicating that infection is GAG independent. Thus glycosaminoglycans do not appear to be essential for HSV infectivity. However, in the absence of GAGs, the ability of the virus to bind to the cell surface is significantly reduced. Table 2 shows the relative infectivities of HSV-1 and HSV-2 on the L, gro2C and sog9 cell lines.

## 1.6 GLYCOSAMINOGLYCANS

A glycosaminoglycan is a linear heteropolysaccharide consisting of specific repeating disaccharide units (Jackson *et al.*, 1991). Usually one monosaccharide of the disaccharide repeat is a hexuronic acid, either glucuronic acid or iduronic acid, and the

**Table 2.** Relative infectivities of HSV virus on control and mutant cell lines

Virus	Relative Infectivity (%)		
	L(control)	gro2C	sog9
HSV-1(KOS)	100	10	0.3
HSV-1(F)	100	20	0.8
HSV-2(G)	100	3.3	1

(From Banfield *et al.*, 1995).

other sugar is a hexosamine, either N-acetylglucosamine or N-acetylgalactosamine. One or both of the sugars are variably N- and O-sulfated, which contributes to the diversity of these structures and their high negative charge density. The most common GAGs include heparin, heparan sulfate (HS), dermatan sulfate (DS) and chondroitin sulfate (CS). These can be linked through an O-glycosidic linkage to a serine residue in a protein core. A GAG covalently linked to a protein in this manner is termed a proteoglycan. Proteoglycans and their attached GAGs have a variety of roles in cell-cell interactions and serve as activators of growth and anti-coagulation factors.

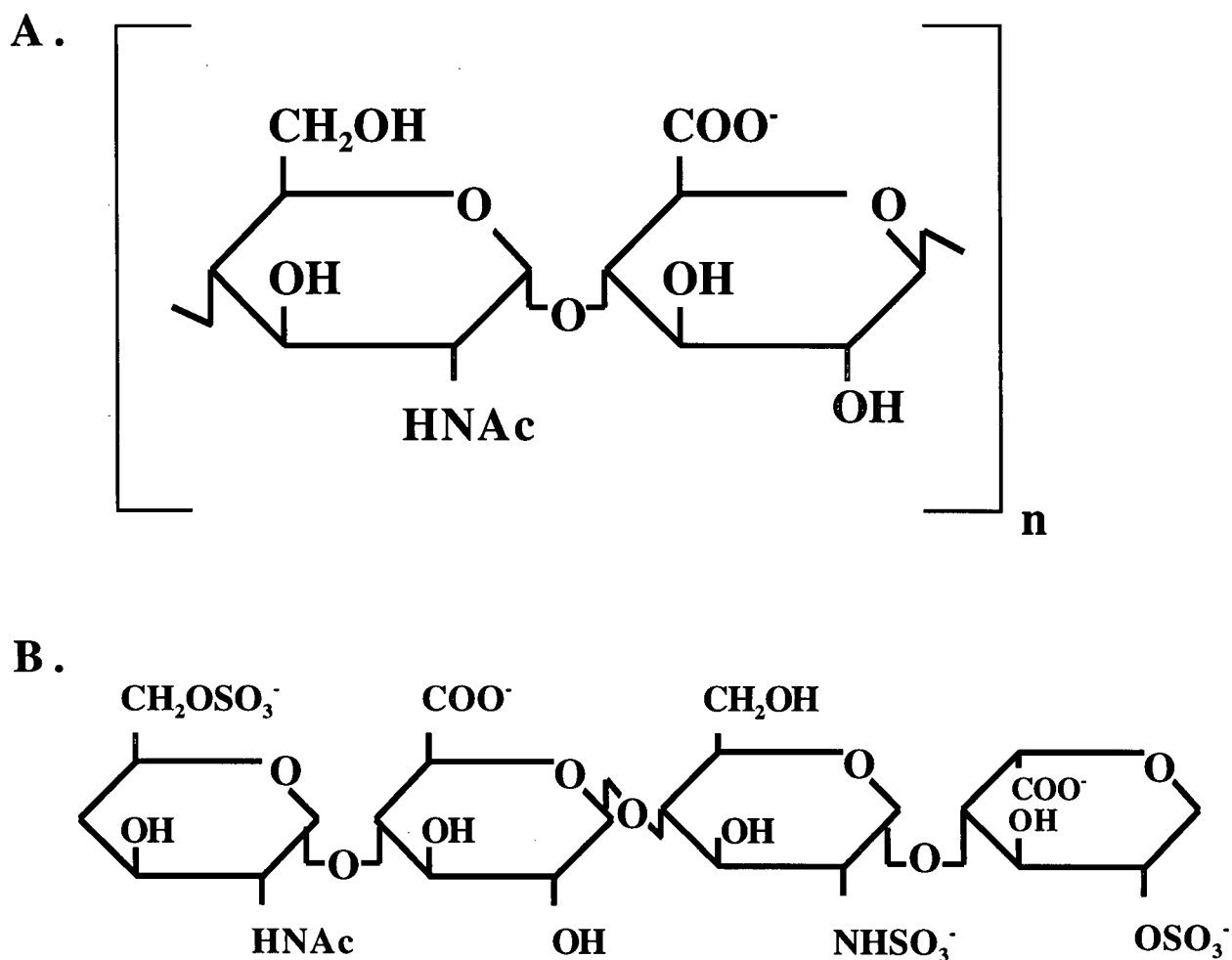
### 1.6.1 Glycosaminoglycan Structure

Heparan sulfate, which is produced in nearly all cell types studied, and heparin, which is synthesized by connective tissue-type mast cells, are both synthesized as proteoglycans (Lind *et al.*, 1993). Biosynthesis of HS and heparin proteoglycans involves the initial formation of a GAG structure composed of alternating D-glucuronic (GlcA) and N-acetylglucosamine (GlcNAc) units (Fig. 1.9). As discussed later, this initial structure is modified through a series of reactions, which leads to the formation of polymers with extensive structural diversity. By comparison, the modifications of CS are much less extensive, resulting in a relatively homogeneous molecule. Chondroitin sulfate is synthesized from an initial polymerization product consisting of alternating GlcA and N-galactosamine residues (GalNAc) (Fig. 1.10). Ester-linked sulfate groups are added typically at the C-4 and/or C-6 positions on GalNAc residues. In some instances, the GlcA residue epimerizes to iduronic acid (IdoA). In this case, the polysaccharide is called dermatan sulfate or chondroitin sulfate B.

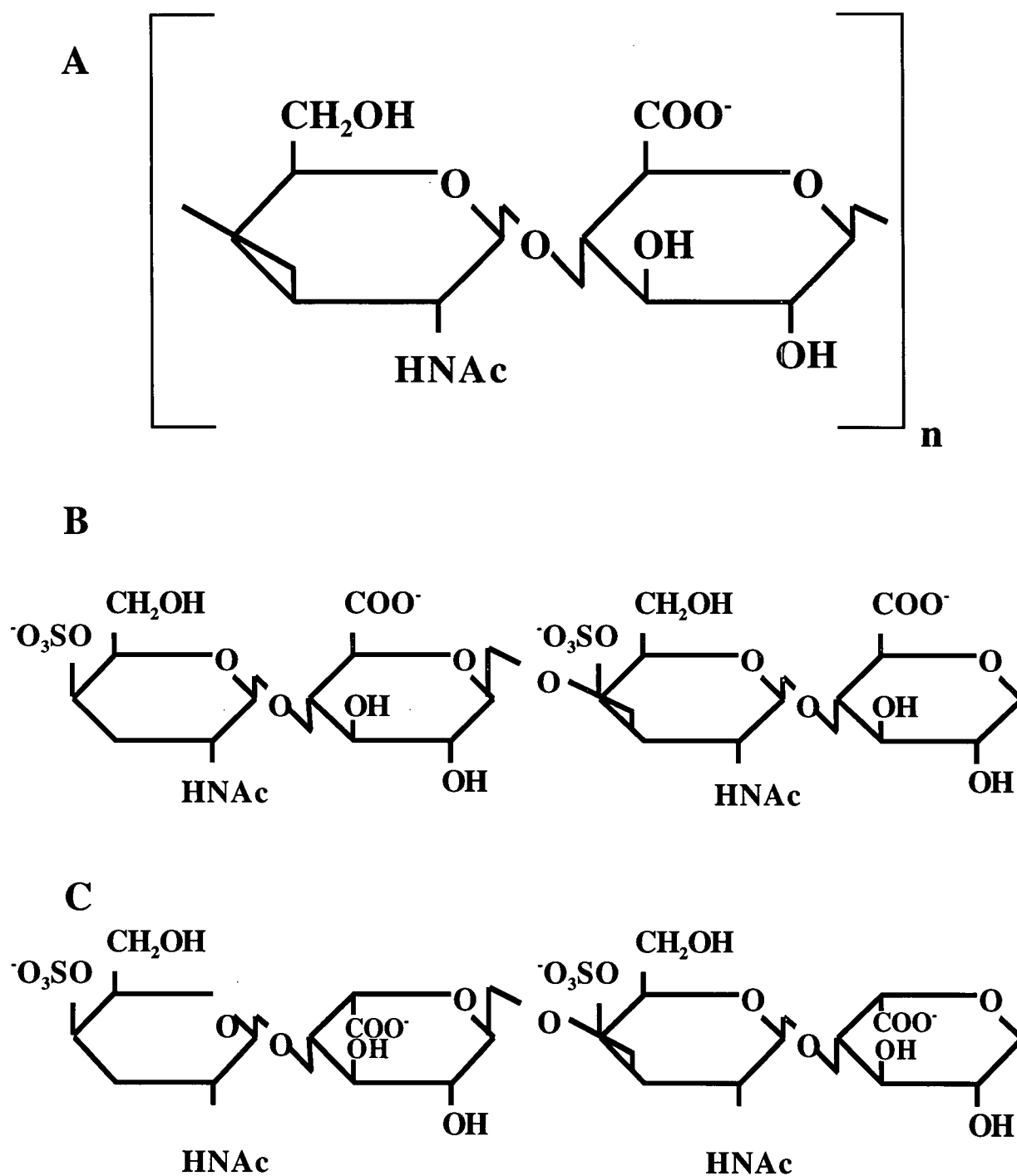
### 1.6.2. Chain Initiation

Synthesis of CS and HS begins after translation of the core protein and transfer of xylose to specific serine residues in the core protein (Kjellen and Lindahl, 1991).





**Figure 1.9** Structure of heparin/heparan sulfate. (A) The initial polymerization product consists of a repeating disaccharide unit containing D-glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc). (B) Structure of heparin/HS following N-deacetylation/N-sulfation, epimerization of GlcA to IdoA, and O-sulfation.



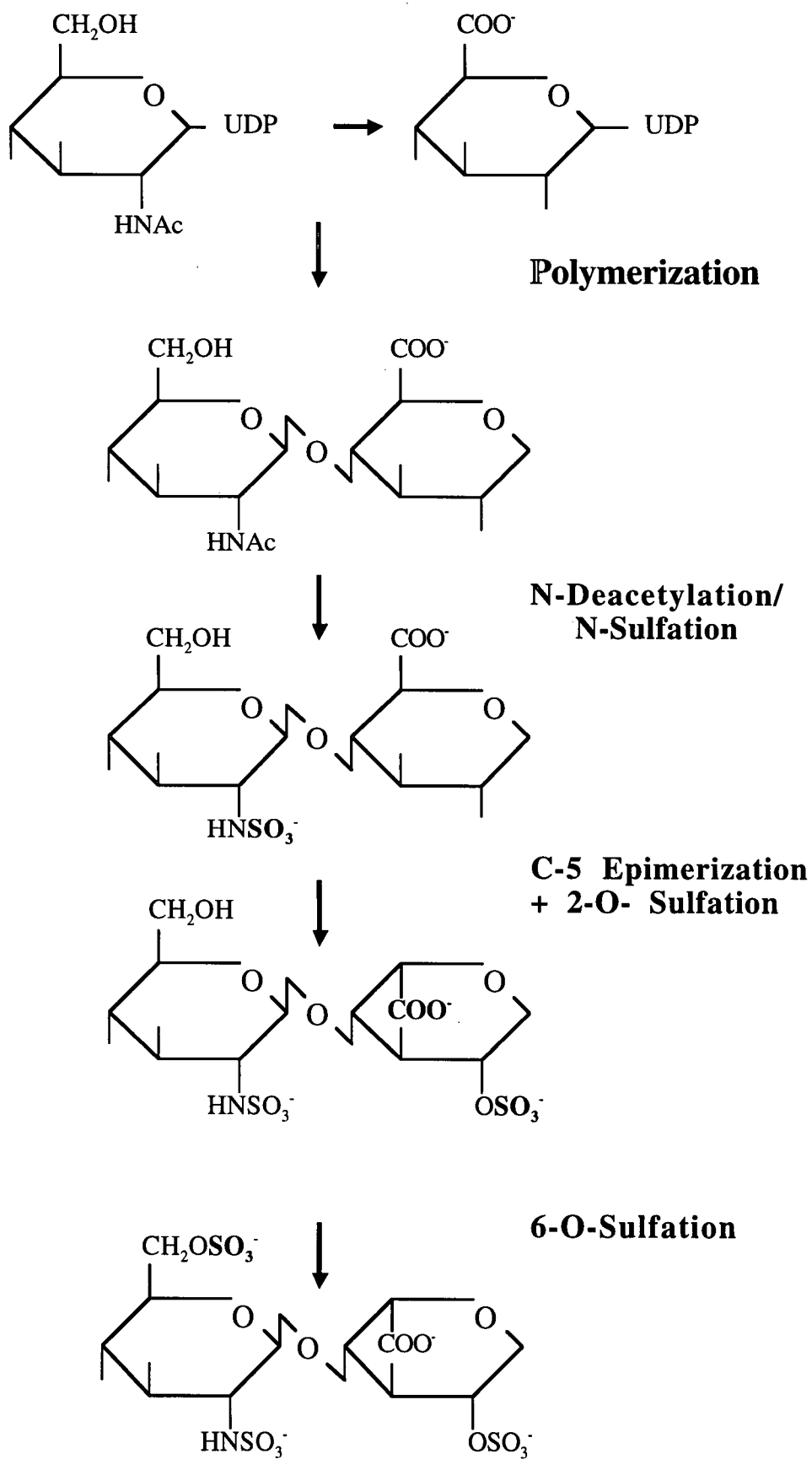
**Figure 1.10** Structure of chondroitin sulfate. (A) The initial polymerization product consists of a repeating disaccharide unit of N-galactosamine (GalNAc) and D-glucuronic acid. (B) Structure of chondroitin-4-sulfate (chondroitin sulfate A). (C) Structure of dermatan sulfate (chondroitin sulfate B).

Xylosylation is catalyzed by xylosyltransferase using UDP-xylose as the sugar donor and there is some evidence that this begins in the ER and continues in the Golgi (Silbert and Sugumaran, 1995). In several proteins, xylosylation occurs on S-G-X-G tetrapeptide sequences (where X is variable). The existence of a consensus sequence for GAG attachment is, however, controversial. The formation of polysaccharide chains continues in the cis and medial Golgi by the addition of galactose (Gal) and glucuronic acid (GlcA) residues. This results in the formation of what is termed the tetrasaccharide linkage region (-glucuronic acid-galactose-galactose-xylose-Ser), which is common to CS, heparin and heparan sulfate proteoglycans. The pathway diverges after the formation of the linkage tetrasaccharide. At this point, the addition of GalNAc or GlcNAc commits the structure to be synthesized either as CS or HS/heparin, respectively. Kitagawa and colleagues (1999) recently demonstrated that the gene *EXTL2* encodes an enzyme which transfers GalNAc and GlcNAc to the common GAG-protein linkage region. Thus, *EXTL2* is likely the critical enzyme that determines whether the polysaccharide chain is to be heparan sulfate or chondroitin sulfate.

### 1.6.3. Biosynthesis of Heparin and HS

HS GAGs have considerable structural diversity. As is relevant to this thesis, it is important to consider the means by which the structural heterogeneity of HS arises. Considered here is the synthesis of HS/heparin, which is illustrated in Figure 1.11. Polymerization of these GAGs begins after the formation of the tetrasaccharide linkage (Silbert and Sugumaran, 1995). In the case of heparan sulfate, polymerization is catalyzed by heparan sulfate polymerases, including those encoded by *EXT1* and *EXT2* (Lind *et al.*, 1998). The identification of *EXT1* as a component of the GAG synthesis pathway is described later in Section 1.6.4. The GAG chain is formed by the transfer of alternating GlcA and GlcNAc monosaccharides from UDP-sugar nucleotides. Coupled with polymerization is the first modification of the [GlcA-GlcNAc]<sub>n</sub> polymer, N-deacetylation, with subsequent N-sulfation of the GlcNAc units (Salmivirta *et al.*, 1995).

**Figure 1.11.** Polymer modification reactions involved in the biosynthesis of heparin and heparan sulfate. N-deacetylation/N-sulfation is the first modification. Regions that remain N-acetylated will not be further modified. N-sulfated regions can be further modified by C-5 epimerization of Glc A residues to Ido A, which is often concomitant with 2-O-sulfation. The final modification shown here is 6-O-sulfation of GlcNSO<sub>3</sub> residues.



This is followed by C-5 epimerization of GlcA units to IdoA, which is coupled with O-sulfation at C2. Finally, GlcNSO<sub>3</sub> units are modified by O-sulfation at C-6.

The stepwise nature of this process should be emphasized, as the product of any given reaction will be the substrate for the next series of modifications (Salmivirta *et al.*, 1995). This is significant since the enzymes catalyzing these reactions have substrate specificity. Therefore, the polymer product of one reaction may only contain a limited number of monosaccharide units that satisfy the substrate specificity for the next enzyme in the pathway. Throughout the modification process, therefore, the heterogeneity and complexity of the polysaccharide will increase. For example, the initial N-deacetylation/N-sulfation reaction will determine which GlcA units will epimerize to IdoA. N-sulfate groups are required for substrate recognition by enzymes catalyzing the C-5 epimerization and O-sulfation reactions. Regions that remain N-acetylated, therefore, are not further modified and will not contain IdoA and O-sulfate residues. At least 50% of the HS polymer consists of such unmodified domains, which alternate with heterogeneous N-sulfate domains. Heparin, by contrast, consists of long stretches of highly modified domains occasionally interspersed by short unmodified regions. The reasons why particular regions of a polysaccharide remain N-acetylated or why other regions are N-deacetylated/N-sulfated remain elusive. It will be important to understand how polymer modification is regulated, since it is by this process that protein-binding domains in the HS chain are initially formed.

#### **1.6.4. *EXT1*: A novel enzyme of the GAG synthesis pathway**

There are obviously numerous enzymes involved in proteoglycan biosynthesis, not all of which have been purified and cloned. A review of the accumulating information with regards to these enzymes is not within the scope of this thesis. Nonetheless, it relevant to the work conducted in this study to consider one of the enzymes involved in the GAG synthesis pathway, namely EXT-1. Our laboratory isolated EXT-1 using a

functional assay based on the ability of HSV-1 to infect cells by attaching to cell surface HS (McCormick *et al.*, 1998). As previously mentioned, sog9 cells do not synthesize any glycosaminoglycans. To determine the defect in the sog9 cell line, sog9 cells were transfected with pools of HeLa cell cDNAs, followed by incubation with HSV-1 (McCormick *et al.*, 1998). Several rounds of screening resulted in the isolation of a clone encoding the gene *EXT1*, which restored wild-type levels of susceptibility to HSV-1 infection in sog9 cells. To determine whether HSV-1 attachment in sog9-EXT1 cells was mediated by an interaction with a cell surface GAG, HSV-1 infection assays on sog9 cells expressing EXT1 (sog9-EXT1) were performed in the presence of soluble heparin. HSV-1 infection of sog9-EXT1 cells was effectively inhibited by the presence of the polyanion, suggesting that sog9-EXT1 cells resembled normal L cells with regard to HSV-1 infection and were decorated with HS GAGs. This was confirmed by anion-exchange HPLC analysis, which revealed that sog9-EXT1 cells synthesized a single HS GAG peak. Recent work by Craig McCormick in our laboratory has demonstrated that *EXT-1* encodes a heparan sulfate polymerase (McCormick *et al.*, 1999). Sog9 cells harbour a 322 bp deletion in the *EXT1* gene. This creates a premature stop codon which results in the production of a 335 amino acid protein, instead of the normal 746 amino acid protein product (Dr. F. Tufaro, personal communication)

*EXT1* is one of three genetic loci for hereditary multiple exostoses (HME). HME is an autosomal dominant disorder characterized by the formation of cartilage-capped tumours (exostoses). This condition can lead to short stature, skeletal abnormalities and malignant transformation of exostoses to chondrosarcomas (Hennekam, 1991) and osteosarcomas (Luckert-Wicklund *et al.*, 1995). The *EXT* gene family includes *EXT1* on 8q24.2 (Ahn *et al.*, 1995), *EXT2* on 11p11-13 (Stickens *et al.*, 1996) and *EXT3* on 19p (Le Merrer *et al.*, 1994). This family has recently been extended to include the EXT-like genes *EXTL1*, *EXTL2* and *EXTL3*, although none of these have been linked with HME (Kitagawa, 1999).

It is becoming increasingly evident that EXT family members play pivotal roles in the GAG synthesis pathway. As mentioned earlier, both *EXT1* and *EXT2* encode an HS polymerase (Lind *et al.*, 1998). *EXTL2* encodes a glycosyltransferase that transfers GlcNAc/GalNAc to the GAG-protein linkage region (Kitagawa, 1999). We previously demonstrated that EXT1 is an ER-resident transmembrane glycoprotein that has a type II configuration (that is, it has a cytoplasmic amino terminus and a luminal carboxy terminus) (McCormick *et al.*, 1998). Recent work by Craig McCormick in our laboratory has demonstrated that EXT1 forms a complex with EXT2 and in doing so is transported to the Golgi complex from the ER (Craig McCormick, personal communication). Our hypothesis is that EXT1 functions in the Golgi apparatus where it modifies proteoglycans transversing the secretory organelles *en route* to the cell surface. This hypothesis is now being tested.

#### 1.6.5. Core Proteins

Many distinct proteins serve as core proteins for proteoglycans. It is the combination of a core protein and specific GAG chain(s) that bestows a unique structure and function on any given proteoglycan. There are three general classes of proteoglycans. The first includes proteoglycans which are secreted and deposited into the extracellular matrix (Esko, 1991). These include PGs such as aggrecan and basement membrane proteoglycans. The second family comprises PGs which are anchored to the plasma membrane, such as syndecan. PGs can be anchored to the cell membrane either by a hydrophobic peptide region or by phosphatidylinositol (PI). The third group of PGs comprises those which are typically found intracellularly in secretory granules and include the PG serglycin. The primary sequences of known PGs range in molecular weight from 20,000 (serglycin) to greater than 200,000 (aggrecan) (Hascall *et al.*, 1994). Some core proteins contain only one or few GAG chains, while others can contain as many as 100 or more. Moreover, core proteins can contain more than one type of glycosaminoglycan.

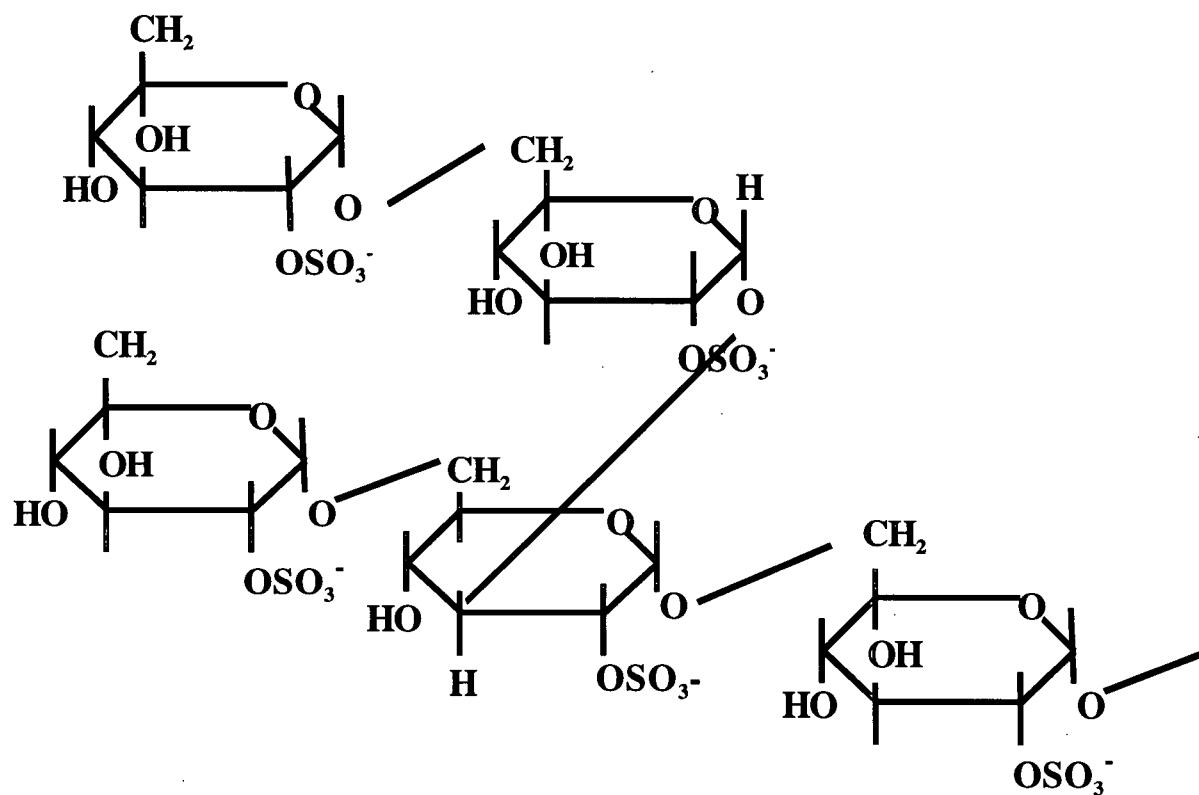


Which cell surface proteoglycans carry the HS moieties that HSV interacts with? Treatment of BHK cells with phosphatidylinositol-specific phospholipase C partially reduced HSV-1 binding to cells (Langeland and Moore, 1990). This suggests that PI-linked proteoglycans are among the PGs that can serve as receptors for the virus. Further studies are required to identify specific proteoglycans mediating viral attachment.

#### 1.6.6 Dextran Sulfate: A Glycosaminoglycan Analog

Dextrans are high molecular weight polymers of D-glucopyranose produced from sucrose by the bacterium *Leuconostoc mesenteroides* (Lindberg and Svenson, 1968). Dextran sucrase catalyzes the transfer of glucosyl groups from sucrose to the growing dextran chain, where they are linked by  $\alpha 1 \rightarrow 6$  linkages. Branching of the chain arises from  $\alpha 1 \rightarrow 3$  linkages. Dextran sulfate is a synthetic sulfated carbohydrate derived from the esterification of dextran. The structure of dextran sulfate is shown in Figure 1.12. Dextran sulfate is a highly sulfated glycosaminoglycan analog and as such is a useful reagent for studying GAG-ligand interactions. For example, dextran sulfate (DS) has been shown to be as active as heparin in binding fibronectin (Ruoslahti, 1988). This activity of dextran sulfate demonstrates that GAG-ligand interactions are not always specific and can be functions of charge density. Indeed, large glycosaminoglycan chains tend to interact more strongly with ligands than small ones (Klebe and Mock, 1982).

DS is an effective inhibitor of enveloped virus infection (Baba *et al.*, 1988). DS can inhibit viral infection by a number of mechanisms. For example, dextran sulfate causes certain strains of Coxsackie virus to aggregate, an effect which seems to inhibit plaque formation by repressing viral release (Totsuka *et al.*, 1981). Ohki and colleagues (1992) demonstrated that DS inhibits the binding and subsequent fusion of Sendai virus with erythrocyte ghosts. This may be due to the steric hindrance of a large polyanionic macromolecule adhered to the virion surface. DS has also been shown to inhibit human



**Figure 1.12** Structure of the glycosaminoglycan analog dextran sulfate.

immunodeficiency virus type 1 (HIV-1) binding, replication and formation of syncytia (Baba *et al.*, 1988a; Baba *et al.*, 1988b; Lederman *et al.*, 1989; Ida *et al.*, 1994).

Callahan *et al.* (1991) observed that DS interacted with one of the HIV glycoproteins, gp120. This interaction inhibits binding of HIV to the cell surface. Finally, DS inhibits HSV attachment and infection of cells (Banfield *et al.*, 1995a). The inhibitory action of DS on the virus can be attributed, in part, to a reduction in electrostatic binding. The positively charged sites on the proteins are likely sequestered by negative sulfate groups on dextran sulfate and are therefore be unavailable to interact with cell surface HS. In light of this, it was surprising that DS enhances HSV-1 infection of GAG-deficient sog9 cells (Dyer *et al.*, 1997; this study). The work described in this thesis supports a model whereby in the absence of HS, HSV-1 can use DS as a surrogate receptor to mediate viral attachment and infection.

## 1.7 VIRAL ENTRY AND TISSUE TROPISM

The initial interaction of a virus with its host cell is a key determinant of host range and tissue tropism. Broad host range viruses bind to widely distributed cell surface molecules. Influenza virus, for example, attaches to sialic acid moieties (Rossman, 1994). Narrow host range viruses, on the other hand, recognize very specific molecules, as exemplified by rhinoviruses which bind to intercellular adhesion molecule-1 (ICAM-1). Structural features of viral envelope glycoproteins are also key determinants of viral tropism. The host range of avian leukosis virus (ALV), for example, is defined by the *env* gene, which encodes two glycoproteins (Bova *et al.*, 1986). Nucleotide differences in the *env* genes of different ALV subgroups permit the virus to use different host cell receptors. Similarly, the conformation of the HIV-1 envelope glycoprotein has been shown to play a role in the host range of this virus (Stamatatos and Cheng-Mayer, 1993). Cellular proteinases, which can activate particular viral fusion proteins involved in penetration, also play a pivotal role in tissue tropism (Nagai, 1995). The availability of a

particular proteinase in the host, as well as the cleavability of the viral fusion protein, can determine the tropism of some viruses, such as influenza A.

For many years, it has been known that HSV-1 and HSV-2 have type-selective cell surface receptors. Vahlne and colleagues (1979) demonstrated that UV-irradiated HSV-1 interfered with the subsequent adsorption of HSV-1 but not HSV-2 particles to the cell surface. These findings correlated with those of Addison *et al.* (1984), who demonstrated that a temperature-sensitive HSV-1 mutant, which was defective in cell penetration at the non-permissive temperature, could block entry of wild-type HSV-1 but not HSV-2. It has also been observed that HSV-1 adsorption to human synaptosomes and glial cells is higher than that of HSV-2. Binding of HSV-1, but not HSV-2 to synaptosomes, is blocked by exposure to HSV-1, indicating the existence of type-selective receptors (Vahlne *et al.*, 1980). The identification of HveA, HveB, Hve C and HIgR as receptors for HSV, as well as the observation that strains of HSV-1 and HSV-2 display preferences for binding these receptors, has contributed significantly to our understanding of the different behaviours of the two serotypes with regard to tissue tropism (Geraghty *et al.*, 1998).

### 1.7.1 HSV Serotype Differences in Glycosaminoglycan-Binding

There is increasing evidence that the interaction of the HSV glycoproteins with GAGs may play an important role in the tissue tropism of the virus and that HSV-1 and HSV-2 use different surface glycoproteins to bind to GAGs and other receptors. The observation that HSV-1 gC-negative viruses are severely impaired in their ability to bind to HS GAGs, while HSV-2 gC-negative virions can bind to cells as efficiently as wild-type HSV-2 (Gerber *et al.*, 1995), has lead to the speculation that gB-2 may be the principle HSV-2 glycoprotein that interacts with GAGs. Several other studies have also provided indirect evidence that the GAG-binding functions of gB and gC may be different for the HSV serotypes. For example, neomycin, a cationic inositol lipid-complexing agent,

is able to inhibit binding of HSV-1 but not HSV-2 to the cell surface (Langeland *et al.*, 1987). The resistance of HSV-2 to neomycin was later shown to map to the N-terminal portion of gC-2 (Oyan *et al.*, 1993). Oyan and colleagues (1993) also demonstrated that in the presence of neomycin, inhibition of binding of gC-deficient HSV-2 virions approached that of wild-type HSV-1. The authors suggested that HSV-2 could use two adsorption pathways: one pathway is neomycin resistant and involves gC-2 binding to a specific receptor, while the second is neomycin sensitive and mediated by the HSV-1 receptor. The extent to which this may determine the epidemiology of HSV is unclear, although the findings are intriguing and suggest that the domains of gC involved in binding may be type-specific.

With regards to gB, our lab has demonstrated that the GAG-binding properties of gB-1 and gB-2 differ (Dyer *et al.*, 1997; this study). Soluble dextran sulfate is able to rescue HSV-1, but not HSV-2 infection of GAG-deficient sog9 cells. Using a panel of deletion mutants, the ability of HSV-1 to interact with dextran sulfate was shown to map to gB-1. The work presented in this thesis details the mechanism of DS stimulation, as well as attempts to identify the domains of gB-1 that interact with DS. Such domains may be important determinants of the interaction of HSV with cell surface GAGs.

Recently, Herold and colleagues (1996) identified the specific features of HS that are important for binding HSV-1 and HSV-2 by comparing the abilities of modified heparin compounds to inhibit plaque formation. Modified heparin compounds which structurally resembled the cell surface heparan sulfate receptor competitively inhibited viral attachment and infection. Key structural features for interactions of both HSV-1 and HSV-2 with cell surface GAGs included N-sulfate and carboxyl groups. Important determinants for HSV-1 infection in particular were 6-O and 2-,3-O sulfations. Taken together, these results suggest that differences in the interactions of HSV-1 and HSV-2 with cell surface HS may influence tissue tropism.

### 1.7.2 Protein-Glycosaminoglycan Interactions

In order to understand HSV attachment and tissue tropism, it is important to address the factors that govern protein-GAG interactions. The analysis of protein-GAG interactions, however, is complicated due to the structural heterogeneity of GAGs and the diversity of proteins which bind them. Thus, factors that influence one type of protein-GAG interaction may not be the same for an interaction involving different GAG-protein components. In general, interactions between HS/heparin and proteins are ionic in nature and depend on the presence of sulfate groups (Kjellen and Lindahl 1991; Salmivirta *et al.*, 1996). Negatively charged sulfate groups can interact with positively charged groups of amino acids on the protein. Frequently, therefore, binding can be of low specificity and affinity (electrostatic binding). In this instance, different polysaccharide sequences would be able to interact with a given protein. Conversely, binding can be of high specificity and affinity. For example, antithrombin binds with high affinity to a specific pentasaccharide sequence (Salmivirta *et al.*, 1996). Thus, the arrangement of sulfate groups and carboxyl groups, as well as the sugar composition of the polysaccharide can also influence protein binding.

The ability of proteins to bind to HS/heparin likely depends on the charge density, structure and conformation of defined heparin-binding domains (HBD) that are found in these proteins. Cardin and Weintraub (1989) analyzed various heparin-binding proteins and identified two consensus sequences for glycosaminoglycan recognition: [-X-B-B-X-B-X] and [-X-B-B-B-X-X-B-X], where B is a basic residue and X is a hydrophobic residue. Other proteins may, however, have different GAG-recognition motifs. Sobel *et al.* (1992) demonstrated that the heparin-binding domain of Human von Willebrand Factor (a plasma glycoprotein) consists of the sequence [X-B-B-X-X-B-B-B-X-X-B-B-X]. Some heparin-binding proteins do not contain any of these consensus motifs (Margalit *et al.*, 1993). Thus, Caldwell *et al.* (1996) examined the importance of non-basic amino acids in HS/heparin binding. Glycine, serine and proline were found to contribute to HS-peptide interactions. Glycine and serine, due to their small side chains,

minimize steric hindrance and when located between basic residues, can facilitate electrostatic interactions with anionic sulfate groups. Proline, on the other hand, introduces “kinks” in the protein and in this way may help to expose the critical binding site. Margalit and co-workers (1993) concluded that the spatial arrangement of basic amino acids within an  $\alpha$ -helix or  $\beta$ -sheet provided the critical secondary structure for the interaction with HS/heparin.

The heparin-binding domains of both HSV-1 gB and gC have been identified (Trybala *et al.*, 1994; Tal-Singer *et al.*, 1995; Laquerre *et al.*, 1998b). To study the heparin-binding properties of gB-1, Laquerre and colleagues (1998) constructed the HSV-1 mutant KgBpK<sup>-</sup>, which carried a deletion in the lysine-rich heparin-binding domain of gB-1 (amino acids 68 to 76) (Fig. 1.7). This virus showed reduced adsorption to cells. Moreover, solubilized gB-1 pK<sup>-</sup> protein had lower affinity for heparin-acrylic beads than wild-type gB-1. Taken together, these results demonstrated that the heparin-binding domain of gB-1 had been inactivated. The gB-1 heparin-binding domain has the sequence KPCKNKPK, which is similar to the consensus motifs predicted for other heparin-binding proteins (Cardin and Weintraub, 1989). The heparin-binding region of gB-2 has not been identified although clusters of basic amino acids are present in the N-terminus and have been proposed as the heparin-binding sites (Williams and Straus, 1997).

The interaction of gC-1 with HS has been extensively studied. A study by Trybala and colleagues (1994) identified two sites on the protein critical for gC-mediated attachment of HSV-1 to HS. One site included residues Arg-143, Arg-145, Arg-147 and Thr-150. A second site included Gly-247 (Fig. 1.7). The two regions are separated by 100 amino acids, although in a model proposed by the authors, these regions would be in close to one another in the three-dimensional structure of the glycoprotein, possibly forming a single attachment site. Another significant conclusion of this study was that the binding of HS to these regions of gC-1 was specific. Only synthetic peptides having the specified amino acid sequence bound irreversibly and with high affinity to HS. Synthetic peptides corresponding to other regions of gC-1 or which contained basic

residues but in a different sequence, bound weakly to HS. This clearly demonstrates that the topographical arrangement of amino acids is an important determinant of the HS-gC interaction. Finally, a study by Tal-Singer (1995) and workers demonstrated that there was an additional HBD in the N-terminus of gC-1, this one mapping to amino acids 33 to 123.

There is evidence that the heparin binding domains of HSV-1 gC and gB interact with different structural features of HS. Herold and colleagues (1995) compared the ability of modified heparin compounds to inhibit infection of wild-type HSV-1 as well as gC- and gB-negative viruses. 2-3-O- desulfated heparin and carboxyl-reduced heparin inhibited attachment of gC-positive viruses (wild-type and gB-negative) to cells, but minimally inhibited attachment of gC-negative HSV-1. These results suggest that carboxyl-reduced and 2-,3-O- reduced heparin compete with structural features of cell surface HS that bind to gC but not to gB. Using plasmon resonance-based biosensor technology, Williams and Straus (1997) directly examined the specificity of gB-2 binding to heparin. Heparin, which is more sulfated than heparan sulfate, was a better competitive inhibitor of gB-2 binding to immobilized heparin. Moreover, desulfated, N-acetylated heparin was unable to compete for gB-2 binding. Taken together, these results demonstrate that the degree of sulfation is an important determinant for gB-2 binding. Significantly, iduronic acid was also important for recognition by gB-2, since heparin, HS and chondroitin sulfate B (dermatan sulfate), which all contain IdoA, could inhibit binding of gB-2 to heparin, whereas chondroitin sulfate A, which lacks IdoA, was a weak inhibitor.

The structural requirements of HS for interaction with HSV-1 gC have been studied in detail. Using gC-1 affinity chromatography, Feyzi and colleagues (1997) demonstrated that the shortest HS sequence that could bind gC-1 comprised 10-12 monosaccharide units and contained at least one 2-O- and one 6-O-sulfate group. The authors concluded that these groups have to be arranged in a specific order within the polysaccharide sequence, since HS fragments which did not bind to gC-affinity columns



were of similar in charge and composition to those fragments which bound to gC. The HS-binding fragment also contained N-sulfate groups, although these sulfate groups were considered to be less important than the 2-O- and 6-O-sulfations. This was based on the finding that N-desulfated heparin reduced the inhibitory effect of heparin by 10-fold in a virus infectivity assay whereas removal of 2-O- or 6-O- sulfate groups reduced the antiviral activity of heparin by approximately 100-fold.

## 1.8 CONSTRUCTION OF HSV MUTANTS: TECHNIQUES FOR MANIPULATING THE HSV GENOME

It is evident that much of what is known about the biology of HSV has come from studies involving the construction of various deletion mutants or intertypic recombinant viruses. The present study is no exception, in that in order to understand better the interaction of HSV-1 with dextran sulfate, it was desirable to generate particular HSV recombinants (see Chapter 6). For this reason, it is worthwhile to review the various techniques that are used for the construction of viral mutants.

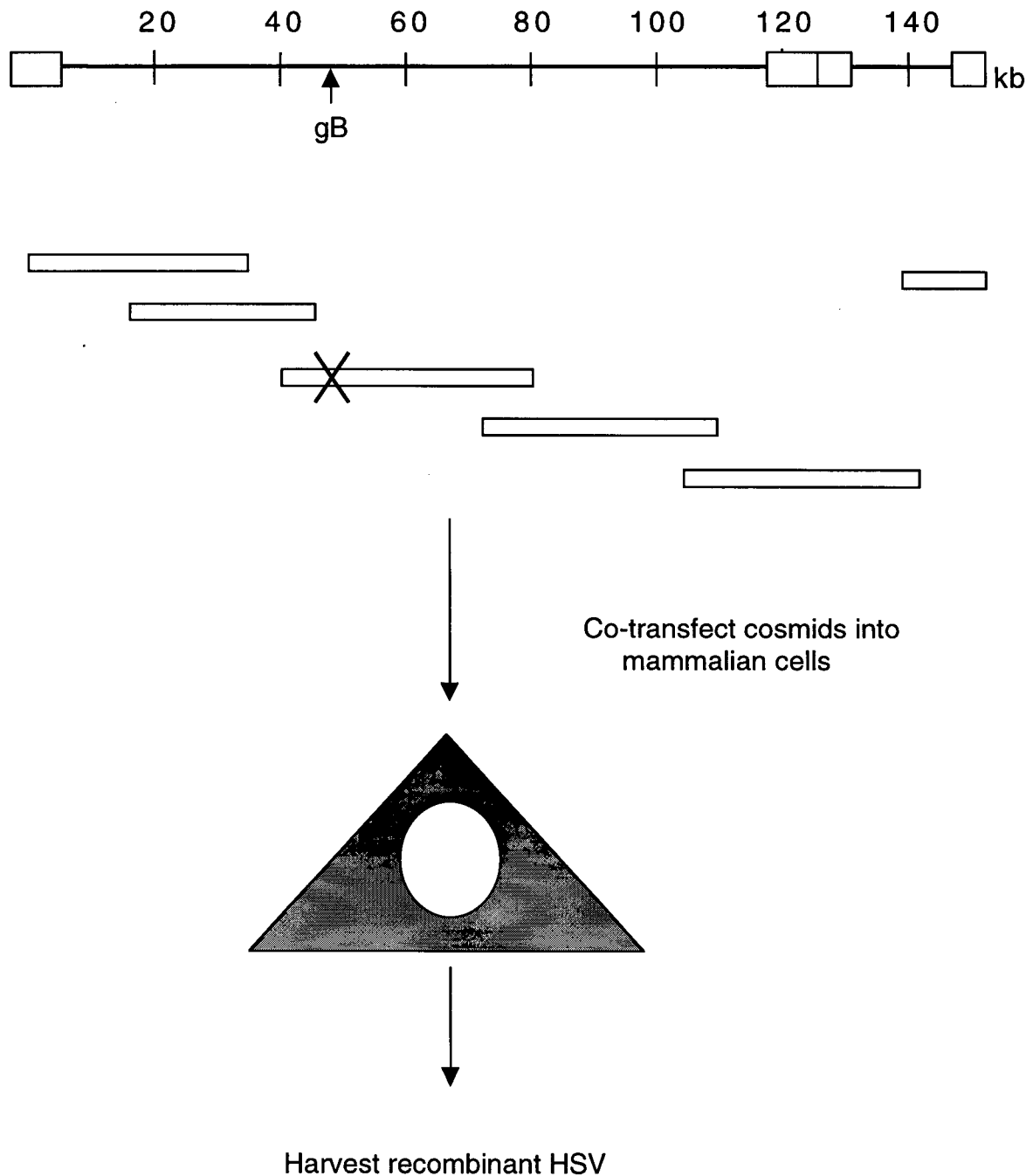
Until the complete DNA sequence of HSV-1 was published in 1988 by McGeoch and colleagues, HSV-1 recombinants were constructed with minimal knowledge of viral DNA sequences. Mutants could be selected on the basis of plaque morphology (Ejercito *et al.*, 1968), drug resistance (Kit and Dubs, 1963), or temperature sensitivity (Schaffer *et al.*, 1970). The drug resistant mutants contain lesions in genes encoding enzymes that are sensitive to these inhibitors. For example, HSV mutants containing lesions in the viral tk gene are sensitive to the drug ACV. Other mutants having defects in the envelope glycoproteins have been selected on the basis of resistance to monoclonal antibodies. These *monoclonal antibody-resistant (mar)* mutants have proven to be useful tools for identifying the functional domains of some of the HSV glycoproteins (Trybala *et al.*, 1994).

Current methods for constructing HSV mutants include the following: (i) a plasmid-based method, (ii) the use of HSV cosmid and (iii) the use of HSV bacterial-artificial chromosomes (HSV-BAC). The plasmid-based method relies on co-transfection of a plasmid bearing the mutated gene (which should also contain a marker) and the complete viral DNA genome. Markers can include the HSV-1 tk gene (Post *et al.*, 1981) or a detectable marker such as *E.coli lac Z* (Goldstein and Weller, 1988). Recombination between homologous regions in the plasmid and viral DNA will produce mutant progeny, which can be isolated from wild-type virus on the basis of expression of the marker gene. A limitation of this method, however, is the inability to isolate mutant progeny in the absence of wild-type virus. This problem is overcome using HSV cosmid and BAC technology to produce recombinants (Cunningham and Davison, 1993; Horsburgh and colleagues, 1999), both of which are described below.

### 1.8.1 HSV Cosmids

Cosmid sets that contain the genomes of other herpesviruses, including varicella-zoster virus, cytomegalovirus and Epstein-Barr virus, have been created (Cohen and Seidel, 1993; Tompkinson *et al.*, 1993; Kemble *et al.*, 1996). The method is illustrated in Figure 1.13. Briefly, cosmids containing HSV-1 fragments are constructed. Cosmid sets containing 4 to 5 cosmids, whose viral inserts overlap and represent the entire genome, are then identified. The viral inserts are co-transfected into cells and subsequent recombination between overlapping viral sequences yields infectious progeny. The cosmid system has been successfully used to produce recombinant HSV-1 particles (Cunningham and Davison, 1993; Fraefel *et al.*, 1996). This entails manipulation of cosmid DNA such that wild-type cosmids are replaced with cosmids containing the desired mutation(s). Co-transfection of these cosmids will produce the desired recombinant in the absence of any wild-type progeny.

Despite its advantages, the cosmid-based system does have several drawbacks.



**Figure 1.13.** Strategy for the generation of HSV recombinants using a cosmid - based system. The HSV genome is depicted as two unique regions (horizontal lines), the larger  $U_L$  and the smaller  $U_S$ , flanked by inverted repeats (rectangles). Overlapping cosmid clones are represented by open bars. The cosmid carrying a mutation in the gene of interest (gB) is co-transfected with the other cosmid clones. Recombination between overlapping cosmid sequences will form a complete HSV genome which can undergo replication.

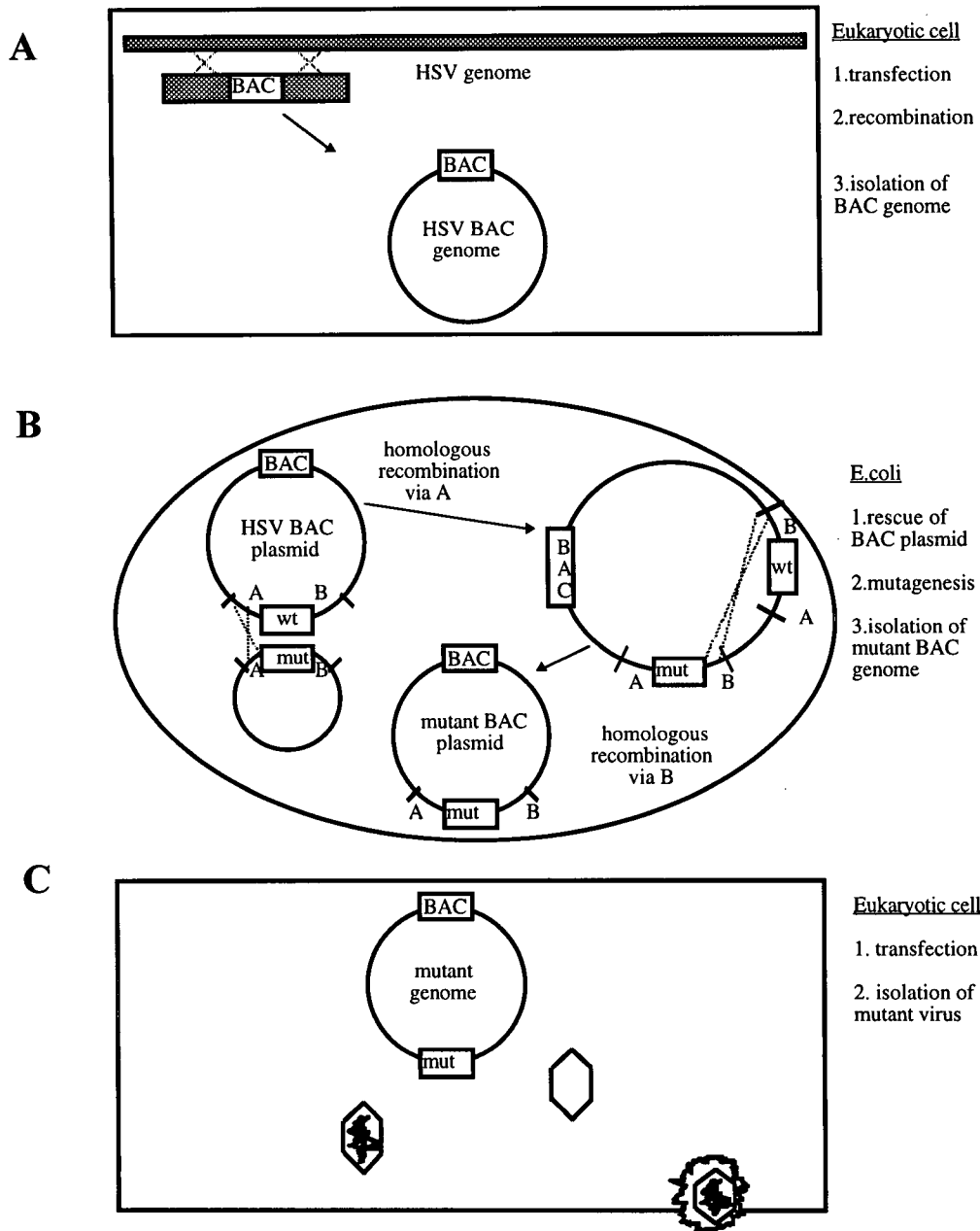
First, mutating viral sequences in cosmids is not necessarily a trivial task due to the large size of the cosmid inserts. In some instances, it may be necessary to perform a series of subcloning steps in order to obtain a manageable size of DNA before mutating the gene of interest. The cosmid then has to be reconstructed to contain the altered sequence. This can obviously be a tedious process. Finally, HSV cosmids are not stable in bacteria. HSV cosmids maintained in *E.coli* are prone to deletion, recombination and rearrangement and are, thus often heterogeneous in nature (Van Zigl *et al.*, 1988; Cunningham and Davison, 1993; Horsburgh *et al.* in press).

### 1.8.2 HSV Bacterial Artificial Chromosomes

Some of the difficulties experienced with HSV cosmids can be overcome using HSV-BACs, in which the entire HSV genome is cloned into a single bacterial artificial chromosome (Stavropoulos and Strathdee, 1998; Horsburgh *et al.*, 1999). HSV-BAC DNA is stable in bacteria. (Horsburgh *et al.*, 1999). This may be because the copy number of BACs in bacteria is only approximately one copy per cell which prevents recombination. Moreover, HSV-BAC technology exploits bacterial genetics for the construction of recombinant herpesviruses, allowing rapid and direct cloning of viral recombinants.

The HSV-BAC mutagenesis procedure is illustrated in Figure 1.14. Two starting reagents are required, the first being an HSV-BAC. The HSV-BAC is constructed by integration of a BAC vector into viral DNA (Horsburgh *et al.*, 1999). The BAC vector contains BAC sequences and a marker gene, such as chloramphenicol. The site of integration of the BAC vector into the viral genome needs to be at a locus not essential for viral replication. The viral tk gene is one such locus and also allows for selection of infectious HSV-BAC on the basis of ACV resistance. Recombinant HSV-BAC viruses are harvested and circular viral DNA is isolated and electroporated into *E.coli*. Colonies containing HSV-BAC are selected on the basis of resistance to chloramphenicol.

**Figure 1.14.** The BAC system for construction of HSV recombinants. (A) Viral DNA and a vector carrying BAC sequences flanked by viral sequences are co-transfected into mammalian cells. The BAC DNA is integrated into the viral genome by homologous recombination to generate HSV-BAC. (B) To generate mutants, a vector carrying the altered allele (mut) is transformed into bacteria containing HSV-BAC. The mutant allele is integrated into the HSV-BAC genome by a two-step replacement strategy. (C) The mutant HSV-BAC plasmid is transfected into mammalian cells to produce infectious recombinant virus.



The second reagent required for BAC mutagenesis is a gene replacement plasmid, which contains the desired mutated gene flanked by HSV sequences to target the replacement gene to a particular locus within the HSV-BAC. The gene replacement vector is transformed into *E.coli* that contain HSV-BAC. The mutated gene is transferred to the HSV-BAC using a 2-step homologous recombination strategy. Mutant HSV-BAC DNA is then isolated from positive clones and transfected into mammalian cells. HSV-BAC DNA is infectious in mammalian cells and cytopathic effects should be observed within 2 to 3 days post-transfection.

Using this procedure, Horsburgh and colleagues (1999) constructed a recombinant HSV-1 virus in as few as seven days. Moreover, all mutant viruses are produced in the absence of wild-type virus and thus, there is no need to purify recombinant virus. Since, all herpesvirus genomes circularize in the nucleus of infected cells, it should be possible to clone them as BACs and perform mutagenesis in bacteria. Murine CMV has also recently been cloned into a BAC (Messerle *et al.*, 1997). The BAC mutagenesis system should prove to be a powerful tool for manipulating the HSV genome, thereby facilitating construction of HSV mutants.

## 1.9 RATIONALE OF STUDY

At the time this project was initiated, the HSV entry pathway, and its role in determining HSV tropism, was not well understood. The approach our laboratory took to investigating HSV entry involved the isolation of HSV resistant cell lines (Gruenheid *et al.*, 1993; Banfield *et al.*, 1995a). It was previously demonstrated that HSV infection of sog9 cells, which do not synthesize any cell surface GAGs, was reduced by nearly three orders of magnitude relative to that of control mouse L cells. Surprisingly, however, the addition of the GAG analog dextran sulfate could enhance HSV-1 infection of sog9 cells

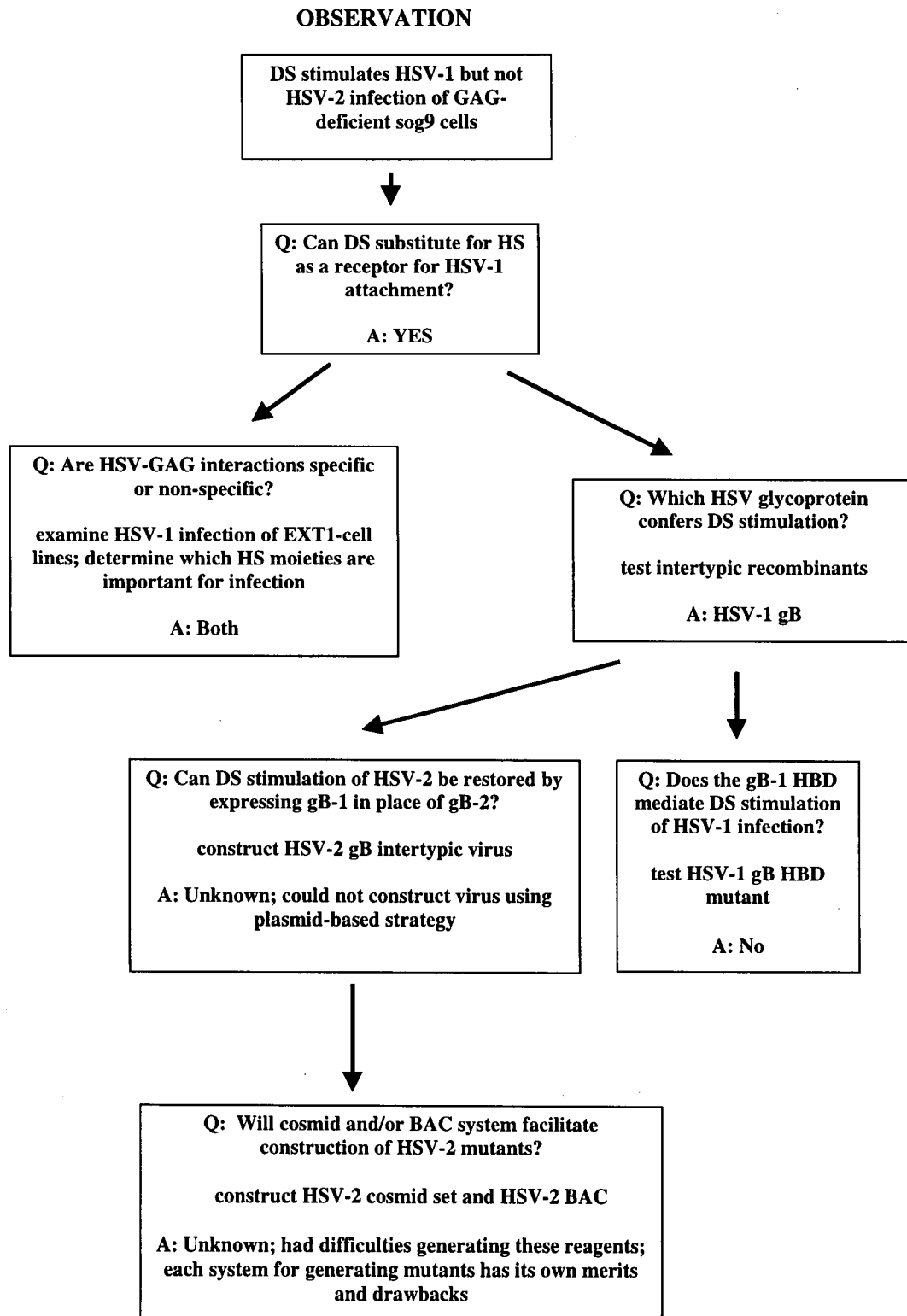
(Banfield et al., 1995). Moreover, DS stimulation of infection was type-specific, in that only HSV-1, but not HSV-2 infection, was enhanced in the presence of the GAG analog.

The overall rationale for this study is depicted in Figure 1.15. One of the objectives of this work was to determine the mechanism of DS stimulation of HSV-1 infection of sog9 cells. DS could act to tether the virus to the cell surface and thereby facilitate infection. Alternatively, DS could enhance infection by facilitating viral penetration and/or a post-entry process. Since DS stimulated HSV-1, but not HSV-2 infection, another aim of this study was to map the viral components that facilitated DS type-specific enhancement of infection. The differences between HSV-1 and HSV-2 in their interactions with DS might reflect differences in how the virions interact with HS GAGs. Mapping the DS activation site on the virus could potentially identify a virion component that may be a determinant of the different behaviours of HSV-1 and HSV-2 in the human host.

The observation of differences in the ability of HSV-1 and HSV-2 to use DS as a surrogate receptor on GAG-deficient sog9 cells, underscored the intricate nature of HSV glycoprotein interactions with glycosaminoglycans. The third objective of this work was to determine which viral and cellular determinants were important for interaction of HSV with its natural cell surface receptor, heparan sulfate (Chapter 4). One approach that was used was to test HSV-1 infection on control and *EXT1*-expressing cell lines in the presence of modified heparin compounds. This could help to identify the structural features of HS important for HSV infection of *EXT1*-expressing cells and of control cell lines. By testing HSV-1 heparin-binding mutants, the roles of gC-1 and gB-1 in mediating HSV-1 attachment to *EXT1* -expressing cell lines could also be assessed.

Finally, as part of the effort to characterize virion components mediating DS stimulation of HSV-1 infection, it was desirable to construct viruses bearing deleted and chimeric forms of particular HSV glycoproteins. As mentioned earlier, however, the construction of HSV recombinants is often a difficult and time-consuming process. Thus, the final objective of this work was to develop a system which would make the HSV





**Figure 1.15.** Rationale of study.

genome more amenable to genetic manipulation (Chapter 6). Specifically, I wanted to explore the possibility of using HSV-2 cosmids and HSV-2 BACs for constructing HSV-2 recombinants. Other research groups have used the cosmid and BAC technologies to successfully construct HSV-1 mutants (Cunningham and Davison, 1993; Horsburgh *et al.*, 1999). The development of similar systems for HSV-2 would greatly facilitate investigations of this virus.

## CHAPTER 2: MATERIALS AND METHODS

### 2.0 MATERIALS

All tissue culture reagents (media, fetal bovine serum (FBS), penicillin-streptomycin and trypsin), tissue culture dishes, LipofectAMINE, G418, T4 DNA ligase, electrophoresis grade agarose, acrylamide, Proteinase K, calf intestinal alkaline phosphatase, DNazol, DH10B *E. coli*, X-gal, Taq DNA polymerase, sequencing primers and most restriction enzymes, were from Canadian Life Technologies (Burlington, ON). The XLII Packaging System and the XL1 Blue strain of *E. coli* for generation of the HSV-2 cosmid library were from Stratagene (La Jolla, CA). Gelase and Gelase buffer were purchased from Epicentre Technologies (Madison, WI) and Nucleobond AX500 DNA purification kits were from the Nest Group (Southboro, MA). The T7 Sequenase v 2.0 7-deaza-dGTP sequencing kit and  $\alpha$ -[ $^{32}\text{P}$ ]-dATP were purchased from Amersham (Oakville, ON.). [ $^{35}\text{S}$ ]-methionine was from Dupont-NEN (Missassauga, ON). Bovine serum albumin (BSA), ampicillin, RNase A and acyclovir (ACV) were purchased from Sigma Chemical Co. (St. Louis, MO). Scintillation cocktail (Ready-Safe) and all ultracentrifuge rotors and tubes were from Beckman (Palo Alto, CA). Human IgG was purchased from ICN ( St. Laurent, PQ) while Qiagen DNA purification kits were from Qiagen (Missassauga, ON.) Pme I restriction enzyme was purchased from New England Biolabs (Missassauga, ON.) DS with a molecular weight (MW) of 500,000 (MW 500,000 DS) was from Pharmacia (Baie d' Urfe, PQ). MW 5000, 15,000, and 50,000 DS were from Sigma, as was heparan sulfate. Modified heparin compounds were a generous gift from Dr. U. Lindhal, Uppsala University, Sweden.

## 2.1 PLASMIDS

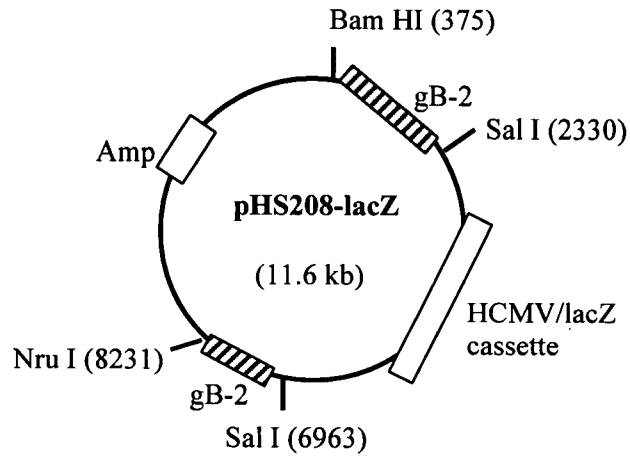
Standard molecular biology techniques were used in the course of this study. Small-scale plasmid DNA purification, restriction enzyme analysis and gel electrophoresis were performed according to "Methods in Molecular Cloning" (Sambrook *et al.* 1989). Large scale DNA purification was performed using either Nucleobond or Qiagen purification kits according to the manufacturer's specifications.

The plasmid pHS208 contains the gene encoding for gB-2 in the plasmid vector pBR322 and was a gift from Chiron Inc. The plasmid pHS208-*lacZ* (Fig.2.1A) is derived from pHS208. In pHS208-*lacZ*, the gB-2 gene is disrupted by a *lacZ* gene cassette. To construct this plasmid, the plasmid pUC249, a gift from Neurovir Inc., was digested with *Sall* to yield a 4.6 kb fragment containing *lacZ* driven by the hCMV promoter. The hCMV-*lacZ* cassette was then ligated into *Sall* digested pHS208 to produce pHS208-*lacZ*.

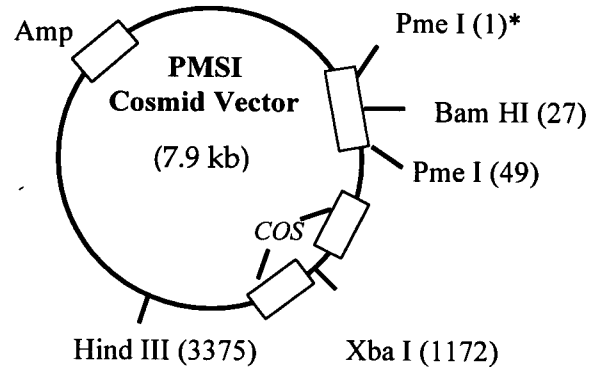
The PMSI cosmid vector (Fig. 2.1 B) was a kind gift from Dr. Bernard Roizman (University of Chicago, IL.). This vector was derived from the Stratagene SuperCos 1 cosmid vector and was generated by replacing the cloning site of SuperCos 1 with one which was flanked by two *PmeI* sites. The plasmid pBAC-TK (Fig. 2.1C), which contains HSV tk sequences flanking bacterial artificial chromosome (BAC) elements and the chloramphenicol resistance gene, was a gift from Neurovir Inc. The BAC elements and the chloramphenicol marker gene in pBAC-tk were derived from pBeloBAC (Shizuya *et al.*, 1992).

**Figure 2.1.** Schematic diagram of the pHS208-lacZ plasmid, the PMSI cosmid vector, and the pBAC-tk plasmid. (A) The pHS208-lacZ plasmid. pHS208-lacZ contains the *E. coli lac Z* gene driven by the hCMV promoter. The hCMV/lacZ cassette is flanked by HSV-2 gB-2 sequences. pHS208-lacZ also contains an ampicillin resistance gene (amp). (B) The PMSI cosmid vector. PMSI was constructed by C. Van Sant in the laboratory of Dr. B. Roizman (University of Chicago) and is derived from the Stratagene Super COS 1 vector. PMSI has two *cos* sites that facilitate packaging of the vector into lambda phage heads. Two PmeI sites are also indicated in the illustration. Work presented in Chapter 6, however, demonstrates that one of these sites (indicated by the asterisk) is not functional. (C) The pBAC-tk plasmid. This plasmid was constructed by Neurovir Inc. and contains BAC sequences flanked by HSV-1 tk sequences. pBAC-tk also contains a chloramphenicol (CM) resistance gene. Relevant restriction sites for all the vectors are indicated.

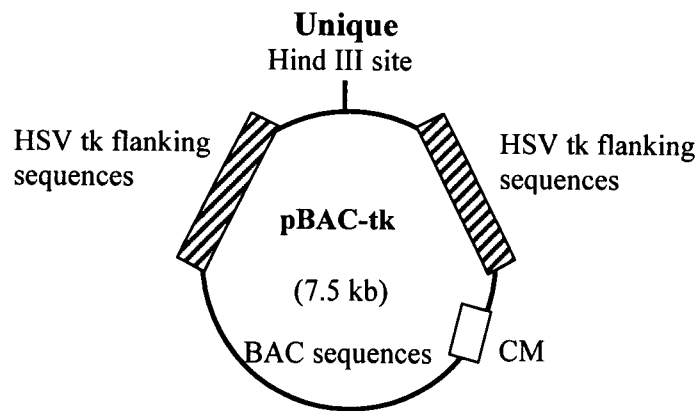
**A**



**B**



**C**



## 2.2 CELL LINES

The parental L cell line used for all experiments was the clone 1D line of LMtk<sup>-</sup> murine fibroblasts. The procedure for isolation of the mutant gro2C and sog9 cell lines was described previously (Gruenheid *et al.*, 1993; Banfield *et al.*, 1995a). L, gro2C and sog9 cells were grown at 37°C in Dulbecco Modified Eagle medium (DMEM) supplemented with 10% FBS in a 5% CO<sub>2</sub> atmosphere. Sog9-EXT1 and L-EXT1 cells were generated by liposome-mediated transfection with the plasmid pEXT1, followed by selection in media supplemented with 700 µg/ml G418 (McCormick *et al.*, 1998). Clonal versions of these cell lines were derived from individual transfection colonies. The parental CHOK1 and mutant CHO cell lines were a gift from J.D. Esko and were grown in Ham's F12 media supplemented with 10% FBS (Esko *et al.*, 1985). Vero cells were obtained from S. McKnight and grown in DMEM with 10% FBS. The D6 cell line, a Vero-derived cell line expressing HSV-1 gB-1, was a gift from S. Person (Cai *et al.*, 1987). D6 cells were grown in DMEM, supplemented with 10% FBS and 500µg/ml G418.

## 2.3 VIRUSES AND VIRAL STOCK PRODUCTION

Viruses used in this study, and the research labs in which these viruses were purified and/or constructed, are shown in Table 3. To produce viral stocks, Vero cells were grown overnight in T-flasks and infected with virus at a multiplicity of infection (m.o.i) of 0.1. After a 1 h adsorption period at 37° C, virus was removed and DMEM with 4% FBS was added. When general cytopathic effect was evident (approximately 96 h post-infection), cells were scraped off the dish and transferred, along with the supernatant, into a 50 ml conical tube. To release intracellular virus, harvested cells were subjected to two rounds of freeze-thawing. To pellet cellular debris, tubes were spun

**Table 3.** Viruses used in this study

Virus Strain	Description	Source
HSV-1 (F)	Wild type	Dr. B. Roizman (University of Chicago)
HSV-1 (KOS)	Wild type	Dr. D. Coen (Harvard Medical School)
HSV-2 (G)	Wild type	Dr. S. Sacks (UBC)
HSV-2 (333)	Wild type	Dr. J. Smiley (University of Alberta)
RSIG25	HSV-1;gC-2	Dr. B. Roizman
R7015	HSV-1;gD-2, gE-2; gG-2	"
HSV-1 (MP)	HSV-1; gC-deficient syncytial strain	"
RHIG13	HSV-1; gB-2	"
UL10 <sup>-</sup>	HSV-1; gM deficient	"
R126	HSV-1; gB <i>mar</i> mutant	"
R233	HSV-1; gB <i>mar</i> mutant	"
HSV-1(KOS) ΔgC2-3	HSV-1; gC-deficient	Dr. B. Herold, (University of Chicago)
HSV-2(G)gC <sup>-</sup>	HSV-2; gC-deficient	"
KCZ	HSV-1(KOS); gC-deficient	Dr. J. Glorioso (University of Pittsburgh)
KgBpK <sup>-</sup>	HSV-1(KOS); gB heparin-binding domain deleted	"
KgBpK <sup>-</sup> gC <sup>-</sup>	HSV-1(KOS); gB heparin-binding domain deleted and gC-deficient	"
HSV-1 (ANG)	Wild type; gB <i>syn</i> mutation; rate of entry ( <i>roe</i> ) mutation in gB	Dr. G. Cohen (University of Pennsylvania)
HSV-1 (ANG-path)	HSV-1 ANG derivative; mutation in a.a 84 of gD; <i>roe</i> and <i>syn</i> mutation in gB	"



at 3000 rpm for 5 min at 4° C. The supernatant was collected, aliquoted and stored at -80° C.

## **2.4 VIRUS TITER DETERMINATION**

Vero cells were plated in 6-well dishes one day prior to inoculation. Confluent monolayers were rinsed with PBS and inoculated with diluted virus preparations in 100µl of DMEM. After a 1 h adsorption period at 37°C, the viral inoculum was removed and the monolayer was rinsed with PBS. Cells were overlaid with DMEM containing 0.1% pooled human IgG which neutralized any extracellular virus. Plaques were visualized and counted after 3 days by fixation and staining of the cells for 10 min with 5% methylene blue in 70% methanol.

## **2.5 DS STIMULATION ASSAYS**

Assays for DS stimulation were performed on sog9 cells plated in 6-well dishes. Confluent sog9 cell monolayers were pre-treated with DS in DMEM for selected lengths of time prior to infection or inoculated with virus in DMEM previously mixed with DS. The cells pre-incubated with DS were rinsed three times with PBS prior to infection. After a 60 min viral adsorption period at 37°C, the inoculum was removed and the cells were washed with PBS. The cells were then overlaid with DMEM containing 0.1% pooled human IgG. Plaques were visualized and counted 96 h post-infection. As a baseline measure of infectivity of HSV-1 on sog9 cells in the absence of DS, monolayers were infected with virus such that approximately 30 to 40 plaques were consistently counted.

## 2.6 PLAQUE INHIBITION ASSAYS

Confluent monolayers of L, sog9, sog9-EXT1, and L-EXT1 cells were rinsed once with PBS and then inoculated with HSV-1 (F) previously mixed with bovine heparin or with one of the following chemically modified heparin compounds: 6-O-desulfated heparin; 2-O-desulfated heparin; N-2 desulfated heparin, N-desulfated/N-reacetylated heparin. N-2,6- O-desulfated heparin; N-6-O-desulfated heparin; and 2,6-O desulfated heparin. The production of these modified heparin compounds has been previously described (Feyzi *et al.*, 1997). After a 60 min incubation at 37°C, the inoculum was removed and the cells were washed with PBS. As before, the cells were overlaid with DMEM containing IgG to facilitate plaque formation and plaques were counted 3 days postinfection by fixation and staining of the cells for 10 min with 5% methylene blue in 70% methanol.

## 2.7 VIRAL PENETRATION ASSAYS

Confluent monolayers of sog9 cells growing in 6-well dishes were rinsed with PBS and incubated with 1 ml of 100µg/ml DS in DMEM at 4°C for 30 min. The cells were rinsed three times with PBS and inoculated with HSV-1 at 4°C for 30 min. The dishes were then shifted to 37°C to allow penetration to proceed. At various times after the temperature shift, the wells were treated for 2 min with 2 ml of low-pH citrate buffer (40 mM sodium citrate, 10 mM KCl, 135 mM NaCl [pH 3.0]) to remove any bound virus that had not penetrated into cells. The monolayers were washed three times with PBS and overlaid with DMEM containing 4% FBS and 0.1% IgG. Alternatively cells were inoculated with virus in the presence of 10 µg/ml of DS at 37°C. At various times, the virus was removed and the monolayers were washed with citrate buffer. Plaques were visualized and counted 96 h postinfection by fixing and staining the cells as described above.

## 2.8 PREPARATION OF RADIOLABELLED VIRUS

Monolayers of L cells were infected with HSV at a M.O.I. of 10. At 2 h postinfection, the medium was changed to methionine-free labeling medium (methionine-free DMEM: 0.1 volume of DMEM/10% FBS-4% dialyzed FBS-500 $\mu$ Ci of [ $^{35}$ S] methionine. When a generalized CPE was evident, the medium was removed from the infected-cell monolayers and subjected to low-speed centrifugation to pellet cell debris. The supernatant was sedimented through a 30% sucrose pad formed in 50 mM NaCl-10 mM Tris (pH 7.8) for 2 h at 39,000 rpm in a Beckman SW41 rotor. Following centrifugation, the sucrose was removed by aspiration, and the virus pellet was suspended in PBS at 4°C. For determination of viral titers, a sample of virus was diluted serially with medium and used to inoculate monolayers of Vero cells growing in 6-well dishes. Plaques were scored after 3 days.

## 2.9 BINDING OF HSV TO IMMOBILIZED DS

A modified protocol by Leong *et al.* (1995) was used to examine binding of HSV-1 and HSV-2 particles to immobilized DS. Nunc-Maxisorp 96-well microtiter dishes were coated with 50  $\mu$ l of PBS containing 5 mg of DS. The dishes were incubated overnight at 4°C. The wells were rinsed twice with PBS and then blocked for 2 h in 3.5% bovine serum albumin (BSA) in 50 mM Tris (pH 7.5)-100 mM NaCl - 1 mM MgCl<sub>2</sub>-1 mM CaCl<sub>2</sub> at 20°C.  $^{35}$ S-labeled HSV was diluted in adsorption buffer (PBS plus 0.1% glucose). The blocking solution was removed, and 50  $\mu$ l of virus was added to each well. The microtiter dishes were centrifuged for 15 min at 1,100 x g at 4°C, and then rocked for 45 min at 20°C. The wells were washed three times with PBS, and the dishes were incubated for 10 min with 100  $\mu$ l of lysis buffer (10mM Tris-HCl [pH 7.4], 150mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate). The dishes were rinsed once more with lysis buffer and all rinses were added to scintillation vials. The radioactivity

## **2.10 TIME COURSE EXPERIMENT**

Sog9 cells grown in 35-mm dishes were inoculated with HSV-1 in DMEM containing 10 µg/ml of DS DMEM and incubated at 37°C. Control dishes were infected with virus in the absence of DS. At different times postinoculation, virus was removed and 2 ml of DMEM with 4% FBS and 0.1% IgG was added to cells after the monolayers were rinsed with PBS. Plaques were counted 3 days postinfection.

## **2.11 PREPARATION OF VIRAL DNA**

HSV-2 DNA for construction of the HSV-2 cosmid library was prepared as follows. Two roller bottles of Vero cells were infected with HSV-2 (G) at M.O.I = 1.0. Two days later, cells were collected from the roller bottles and centrifuged at 5000 rpm for 5 min. The cell pellet was washed with PBS and spun once more as before. Cells were resuspended in 20 ml of resuspension buffer (150 mM NaCl, 10 mM Tris [pH 7.4], 1.5 mM MgCl<sub>2</sub>). NP-40 was added to a final concentration of 0.1%, vortexed briefly, and the nuclei pelleted at 5000 rpm for 5 min. The supernatant was collected and adjusted to have a final concentration of 0.2% SDS, 5 mM EDTA and 50 mM β-ME. The lysate was extracted twice with an equal volume of 1:1 phenol:chloroform and each time centrifuged for 10,000 rpm for 10 min. Two volumes of ethanol were added and the lysate centrifuged again at 10,000 rpm for 10 min. The resulting DNA pellet was resuspended in 2 ml of TE and RNase was added to a final concentration of 20 µg/ml. The DNA was allowed to dissolve at 37°C for 15min. The viral DNA solution was then loaded on top of a linear 5-20% potassium acetate gradient in TE (10 mM Tris-HCl [pH 8] and 5mM EDTA) which was centrifuged in an SW41 rotor at 27,000 rpm for 16 h at 4°C. The supernatant was gently removed and 3 ml of TE was added to the pellet. The DNA was ethanol precipitated once more and redissolved in 0.5 ml TE [pH 7.5].

For all other experiments, viral DNA was prepared by infecting Vero cells grown in 10 cm dishes at a M.O.I. of 0.1. When generalized CPE was evident, cells and medium were collected in a 15 ml conical tube. Cells were spun down for 5 min at 3,000 rpm, the supernatant was removed and the cells washed by resuspending in 5 ml PBS. The cells were pelleted again and the PBS removed. The cells were resuspended in 1 ml of DNA extraction buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8], 25 mM EDTA [pH 8], 0.5% SDS) and proteinase K was added to a final concentration of 0.1 mg/ml. The cells were incubated overnight at 65°C, following which the lysate was transferred to a 1.5 ml vial. The DNA was extracted once with an equal volume of phenol and extracted once more with an equal volume of 1:1 phenol:chloroform and then again with an equal volume of chloroform. The viral DNA was precipitated with 1/2 volume of 7.5M ammonium acetate and 2 volume of ethanol. The pellet was resuspended in 50 µl of TE and the DNA later analyzed by restriction digest.

## 2.12 CONSTRUCTION OF HSV-2 gB<sup>-</sup>(*LACZ*) VIRAL MUTANT

Construction of the HSV-2 gB<sup>-</sup> *lacZ* virus involved co-transfection of pHS208-*lacZ* with HSV-2 DNA into gB-1 complementing D6 cells. The day prior to transfection, D6 cells were plated in 60 mm dishes at a density of  $1 \times 10^6$  cells per dish. pHS208-*lacZ* was linearized by overnight digestion with XhoI. One microgram of linearized pHS208-*lacZ* was mixed with 4 µg of HSV-2 genomic DNA. 100 µl of sterile dH<sub>2</sub>O and 200 µl of serum-free, antibiotic-free DMEM were added to the DNA solution. In a separate tube, 10 µl LipofectAMINE was diluted in 300 µl serum-free antibiotic-free DMEM. The DNA and LipofectAMINE solutions were then mixed together and kept at room temperature for 15 min. In the meantime, the D6 cells were rinsed with serum-free, antibiotic-free DMEM. The transfection mixture was combined with 2.4 ml of serum-free, antibiotic-free DMEM and was then added to the cells. Following an incubation period of 18 h at 37°C, the transfection mixture was removed and regular DMEM/10%

FBS was added to the monolayers. When generalized CPE was evident, infected cells were scraped off the dishes and virus was harvested as previously described.

To isolate HSV-2 gB<sup>+</sup>*lacZ* virus, confluent monolayers of D6 cells in 6-well dishes were infected with serially diluted virus stock. After a 1 h incubation period at 37°C, virus was removed and monolayers were rinsed with PBS. Cells were then overlaid with 0.8% agarose in DMEM/4% FBS. When plaques were observed (3 days post-infection), cell monolayers were stained with 0.1% X-gal in X-gal buffer (10 mM potassium ferricyanide, 10mM potassium ferrocyanide, 1 mM MgCl<sub>2</sub> in a final volume of 200 ml in PBS). Plaques expressing *lacZ* (those which stained blue) were picked and agar plugs containing virus were added to D6 cells growing in 96-well dishes. Infection was allowed to proceed for 3-6 days. If CPE was evident, cells were scraped and virus harvested. To confirm the gB2<sup>+</sup>*lacZ* genotype, isolated virus was once more used to infect D6 cells grown in 6-well dishes. Following a 1 h incubation at 37°C, virus was removed and DMEM containing 4% FBS and 0.1% pooled human IgG was added to the cell monolayers. Plaques were stained with X-gal to detect *lacZ* expression.

## 2.13 CONSTRUCTION OF HSV-2 VIRAL COSMID LIBRARY

### 2.13.1 Preparation of viral DNA

HSV-2 DNA was prepared as described above. Viral DNA was partially digested with Sau3AI. One unit of Sau3AI was added to 40 µg of DNA at room temperature and samples were collected at 7, 9 and 11 min. The enzyme was inactivated by adding 50 mM EDTA and then heat inactivated for 20 min at 65°C. The samples were loaded onto a 0.6% low melt gel and run with PacI digested HSV-1 cosmid as a 40 kb control. The 40 kb HSV-2 fragment was excised from the gel and purified using Gelase. For Gelase purification, the gel fragment was incubated in 1 ml Gelase buffer for 30 min on ice. After 30 min, the remaining buffer was removed and the gel melted at 65°C for 20 min. The

sample was then incubated at 40°C for 20 min and 20 µl of Gelase was added to each sample. This was allowed to incubate at 40°C for 2 hours after which 0.1 volume of sodium acetate was added. The sample was chilled on ice for 15 min and then centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was transferred to a new tube to which 2 volumes of cold isopropanol was added. The sample was gently mixed and then centrifuged for 15 min at 10,000 rpm, 4°C. The pellet was allowed to dry in the fumehood and then resuspended in 15 µl dH<sub>2</sub>O.

### **2.13.2 Cosmid vector preparation**

20 µg of the cosmid vector PMSI was digested with XbaI at 37°C overnight. The enzyme was heat inactivated at 65°C for 20 min and the sample dephosphorylated with 1 µl of calf alkaline phosphatase for 30 min at 37°C. The sample was heat inactivated again and then digested with BamHI at 37°C overnight.

### **2.13.3 Ligation of HSV-2 Viral DNA Fragments and PMSI Cosmid Vector**

A ligation mix of 20 µl total, containing 10X T4 DNA ligase buffer, the entire volume of partially digested HSV-2 viral DNA, 0.5 µg of prepared PMSI vector and 1.0 unit of T4 DNA ligase, was used. The ligation was performed overnight at 2-8°C. Once ligated, 30 µl of water was added and the sample was gently mixed with 500 µl of 1-butanol to precipitate the DNA. The tube was spun for 10 min, 10,000 rpm at 4°C and the pellet resuspended in 5 µl of dH<sub>2</sub>O. The sample was then ready to be packaged into lambda phage.

#### 2.13.4 Packaging HSV-2 Cosmids into Lambda Phage

Stratagene's XL II packaging system was used to package ligated DNA. Packaging was done according to the manufacturer's specifications. Packaged cosmid DNA was stored in 500  $\mu$ l of SM buffer (5.8 g NaCl, 2.0 g  $\text{MgSO}_4$ , 7  $\text{H}_2\text{O}$ , 50 ml 1M Tris-HCl [pH7.5], 5.0 ml 2%w/v gelatin per 1L  $\text{dH}_2\text{O}$ ) at 2-8°C until infection.

#### 2.13.5 Infection

XL-1 Blue MR strain *E.coli* (Stratagene) was streaked onto an LB plate the day before infection. One colony was picked and incubated for 5 h at 37°C in 50 ml of LB supplemented with 0.2% maltose and 10 mM  $\text{MgSO}_4$ . The culture was centrifuged at 4000 x g for 5 min after which the supernatant was removed. The pellet was re-suspended in 1 ml of cold  $\text{MgSO}_4$  and the adsorbance (A) of the culture was adjusted to  $A_{600}=1.0$  in 10 mM  $\text{MgSO}_4$ . For infection, 50  $\mu$ l of bacteria was combined with 50  $\mu$ l of packaged DNA and incubated at room temperature for 30 min. Following this incubation, 400  $\mu$ l of LB was added to the tube and the infection allowed to continue for 60 min at 37°C. The tube was gently inverted every 15 min. Finally, the tube was centrifuged and the bacterial pellet resuspended in 50  $\mu$ l of LB. This was followed by plating the sample onto LB plates containing 100 $\mu$ g/ml ampicillin and then incubating overnight at 37°C.

#### 2.13.6 Restriction mapping HSV-2 Cosmid Clones

Using standard alkaline lysis, cosmid DNA was prepared from 200 individual colonies. Cosmids were digested with Bgl II and where necessary, Kpn I, to map the viral DNA fragments contained in each cosmid. Cosmid DNA was digested as follows: 2 $\mu$ l cosmid DNA in 2  $\mu$ l enzyme buffer and 15.5  $\mu$ l  $\text{dH}_2\text{O}$ , was digested with 0.5  $\mu$ l restriction enzyme for 6-18 hours at 37°C. For controls, parental HSV-2(G) viral DNA was digested with Bgl II and Kpn I, respectively. For these digests, 1  $\mu$ g viral DNA was



0.5 µl of enzyme in a 50 µl reaction for 6-18 h at 37°C. Digested cosmid and control DNA was electrophoresed on a 0.5% agarose gel.

### 2.13.7 Cosmid Sequencing

To establish an overlapping set of cosmids that spanned the entire HSV-2 genome, it was necessary to sequence the ends of selected viral inserts. Sequencing was done according to the Sanger dideoxy method using the T7 Sequenase v 2.0 7-deaza-dGTP sequencing kit. Cosmid DNA to be sequenced was prepared using Nucleobond 500X columns. Approximately 10 µg of cosmid DNA was denatured in 0.1 volume of 2M NaOH, 2 mM EDTA for 30 min at 37°C. Denatured DNA was precipitated by adding 0.1 volume sodium acetate and 2 volumes of ethanol and transferred to -30°C for a minimum of 15 min. Precipitated DNA was washed two times with 70% ethanol and resuspended in 7 µl dH<sub>2</sub>O, 2 µl T7 Sequenase Reaction buffer and 1 µl (10ng) of primer. The forward primer had the sequence 5'AAAGTGCCACCTGACGTC and initiated at position 7,841 in the PMSI cosmid vector. The reverse primer had the sequence 5'CCGCTTATCATCGATAAG and initiated at position 80 in the cosmid vector. Primers were allowed to anneal for 2 min at 65°C, after which the reaction was allowed to cool for 30 min at room temperature. The samples were briefly centrifuged and stored on ice. In the meantime, 2.5 µl of each chain terminator (ddG, ddA, ddT, ddC) was aliquoted to wells in a multi-well dish. The labelling mix (7.5 µM 7-deaza-dGTP, 7.5 µM dCTP, 7.5 µM dTTP) was diluted 1:5 in dH<sub>2</sub>O and the Sequenase was diluted 1:8 in Sequenase dilution buffer. To annealed DNA, 1 µl 0.1M DTT, 2 µl diluted labelling mix, 0.5 µl [ $\alpha$ <sup>32</sup>P]-dATP and 2 µl of diluted Sequenase was added. DNA was labelled at room temperature for 5 min. To terminate the reaction, 3.5 µl of the labelling reaction was added to each termination mix in the multi-well dish, which had been pre-warmed to 37°C. The reactions were kept at room temperature for an additional 15 min. Finally, 4 µl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05%

xylene cyanol) was added to each reaction followed by an incubation at 75°C for 2 min. The reactions were then run on a 6% polyacrylamide gel (5.7 g acrylamide, 0.3 g bis-acrylamide, 48 g urea, 10ml 10X TBE buffer, 40 ml dH<sub>2</sub>O, 10% ammonium persulfate, 50µl TEMED). The gel was transferred to Whatman 3MM paper and dried for 1 h on a gel dryer at 70°C. The gel was placed in an X-ray cassette with Kodak X-OMAT AR film and kept at room temperature overnight. The following day, the X-ray film was removed and processed.

## **2.14 CO-TRANSFECTION OF HSV COSMID CLONES**

Vero cells were plated at a density of  $1-1.2 \times 10^6$  cells per dish in 60 mm dishes the day prior to transfection. Cosmids were first linearized by overnight digestion with Pme I. For each transfection, 5 µl of cosmid mix (which consisted of 250 ng/µl of each linearized cosmid) was combined with 95 µl of antibiotic-free OptiMEM. This was then mixed with 100 µl of LipofectAMINE stock solution (12 µl of LipofectAMINE plus 88 µl of OptiMEM) and incubated at room temperature for 45 minutes. Prior to the addition of the transfection mixture, the cells were washed with antibiotic and serum-free OptiMEM to remove any FBS present. The transfection mix was mixed thoroughly with 1.8 ml of OptiMEM and added to the dish. The cells were incubated at 37°C with 5% CO<sub>2</sub> for 2-4 h. Cells were washed once with DMEM to remove all LipofectAMINE and then overlaid with 3 ml of 5% FBS DMEM and placed at 34°C with 5% CO<sub>2</sub>. Three to four days post-transfection, plates were examined for evidence of CPE.

## 2.15 CONSTRUCTION OF HSV2-BAC

### 2.15.1 Production of BAC Virus

The day prior to transfection, 60mm dishes were seeded with  $1 \times 10^6$  Vero cells per dish. pBAC-TK was linearized by digestion with Hind III overnight. For transfection, 1  $\mu$ g of linearized pBAC-TK and 4  $\mu$ g of HSV-2 viral DNA were mixed together. To this DNA mix was added 100  $\mu$ l of dH<sub>2</sub>O and 200  $\mu$ l of serum-free, antibiotic-free Opti-MEM. In a separate tube, 10  $\mu$ l LipofectAMINE and 300  $\mu$ l Opti-MEM were combined. The DNA and LipofectAMINE solutions were mixed together and allowed to sit at room temperature for 15 min. Following this incubation, 2.4 ml of Opti-MEM was added to the mixture, which was then overlaid on cells which had been previously rinsed with Opti-MEM. The cells were incubated with the transfection mixture for 18 h at 37°C, after which the mixture was removed and replaced by DMEM/10% FBS. When cells showed CPE, cells were scraped into sterile tubes and the mixture was frozen and thawed to release virus. The virus titer was determined on fresh Vero cell monolayers. TK-deficient virus were identified by infecting dishes of cells at an M.O.I. of 0.01 and adding 100 mM ACV. ACV resistant plaques arising within several days are tk-deficient. To concentrate tk-deficient virus, viral infection was allowed to continue until the cell monolayer was completely infected. The virus was then harvested.

### 2.15.2 Confirmation of pBAC-TK Sequences in HSV2-BAC

Viral DNA was purified from the ACV-resistant viral stock. To confirm that ACV-resistance was due to incorporation of pBAC-TK into the HSV-2 tk locus, PCR was performed to amplify tk sequences. The primers used were 5'AGGCCGGATAGCTTGTGC and 5' CGGAACAGAGAGCGTCACA. PCR reactions were performed in a 50  $\mu$ l volume and contained the following: 5  $\mu$ l 10X PCR

buffer, 1.5  $\mu$ l 10mM dNTP mixture, 1.5  $\mu$ l 50mM MgCl<sub>2</sub>, 2  $\mu$ l primer mix (10 $\mu$ M each) 10  $\mu$ l viral DNA, 5 $\mu$ l DMSO and 24.5  $\mu$ l dH<sub>2</sub>O. As a control reaction, 1  $\mu$ g pBAC-TK DNA was used as template DNA. The tubes were incubated in a thermocycler at 94°C for 5 minutes to completely denature the template DNA. Midway during this incubation, 0.5  $\mu$ l Taq DNA polymerase was added to the tubes. The PCR amplification was then allowed to proceed for 25 cycles which comprised the following steps: denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 1 min. Tubes were incubated for an additional 10 min at 72°C and then maintained at 4°C. The amplification products were analyzed by gel electrophoresis.

### **2.15.3 Plaque purification of ACV-resistant HSV2-BAC**

To plaque purify ACV-resistant HSV2-BAC, the ACV-resistant HSV2-BAC stock was serially diluted and used to infect confluent monolayers of Vero cells grown in 6-well dishes. After a 1 h adsorption period at 37°C, the virus was removed and the monolayers were washed with PBS. To facilitate plaque formation, monolayers were overlaid with 0.8% agarose in DMEM/4% FBS containing 100 mM ACV. Approximately 3 days post-infection, plaques were picked and agarose plugs containing virus were used to infect Vero cells grown in 96-well dishes. Once again, the infection was performed in DMEM/4% FBS supplemented with 100mM ACV. Viral isolates were harvested when CPE was evident and analyzed by PCR for virus containing BAC sequences (Section 2.15.2).

### **2.15.4 Isolation of HSV-2 recombinant as a BAC plasmid**

To isolate the HSV-2 recombinant as a BAC plasmid in *E.coli*, 10 cm dishes of confluent Vero cells were infected with HSV2-BAC isolates at an M.O.I. of 3. Two hours later, the medium was removed and 1 ml of DNAzol was added per 10 cm plate. The cells were lysed by gentle pipetting and the resulting DNA precipitated from the cell

lysate by adding 0.5 ml ethanol per ml of DNAzol. Samples were gently mixed and centrifuged for 2 min at 12,000 x g. The supernatant was removed and the DNA pellet was washed with 70% ethanol. After centrifugation, the pellet was allowed to air dry and the DNA resuspended in 100  $\mu$ l of TE (10 mM Tris-Cl, 1 mM EDTA [pH8.0]).

One  $\mu$ l of purified DNA was added to an equal volume of dH<sub>2</sub>O and electroporated (2.5 kV, 200 $\Omega$ ) into 40  $\mu$ l electrocompetent DH10B *E.coli*. After electroporation, 1 ml LB media was added to the cells and the mixture was transferred to a conical tube. The tube was shaken at 200 rpm at 37°C for 1 h, after which the cells were plated onto LB plates containing 20 $\mu$ g/ml chloramphenicol. The plates were incubated overnight at 37°C to allow bacterial colonies to grow.

## CHAPTER 3: DEXTRAN SULFATE CAN ACT AS AN ARTIFICIAL RECEPTOR TO MEDIATE HSV-1 INFECTION

### 3.0 INTRODUCTION

To investigate the HSV entry pathway, our laboratory isolated and characterized HSV resistant mouse cell lines (Chapter 1, Table 2). Parental L cells, which express both HS and CS GAGs on their cell surface, were infected with HSV-1 at a low MOI (Gruenheid *et al.*, 1993). Cells which survived to form colonies were harvested and plated. In this manner, the gro2C cell line was established. Gro2C cells are 90% resistant to infection compared to parental L cells. HPLC analysis demonstrated that gro2C cells synthesized chondroitin sulfate as efficiently as control L cells but generated little, if any, heparan sulfate, a known receptor for HSV attachment. Despite the lack of HS moieties, gro2C cells are still partially susceptible to HSV infection, and further analysis indicated that CS can act as receptor for viral binding in the absence of HS. Sog9 cells were sequentially isolated from gro2C cells and exhibited a three-order-of-magnitude reduction in susceptibility to HSV-1 compared to L cells. This resistance to infection is likely due to the fact that sog9 cells do not synthesize any cell surface HS or CS GAGs (Banfield *et al.*, 1995a). Sog9 cells however, retain some susceptibility to infection, providing evidence for a GAG-independent entry pathway. Taken together, these studies demonstrated that HSV-1 need not interact specifically with HS to gain entry into mouse fibroblasts, although the HSV-HS interaction significantly enhances infection efficiency.

To characterize further the mutant cell lines, Banfield *et al.* (1995a) compared the inhibitory effects of sulfated polyanions on HSV-1 infection. Parental L cells were infected with HSV-1 in the presence of soluble HS to test if virus used cellular HS as a receptor for viral binding. Soluble HS inhibited L cell infection, demonstrating that it could act as a competitive inhibitor of virus attachment to cell surface HS. Due to its high negative charge density, soluble HS also inhibited infection of gro2C cells by competing

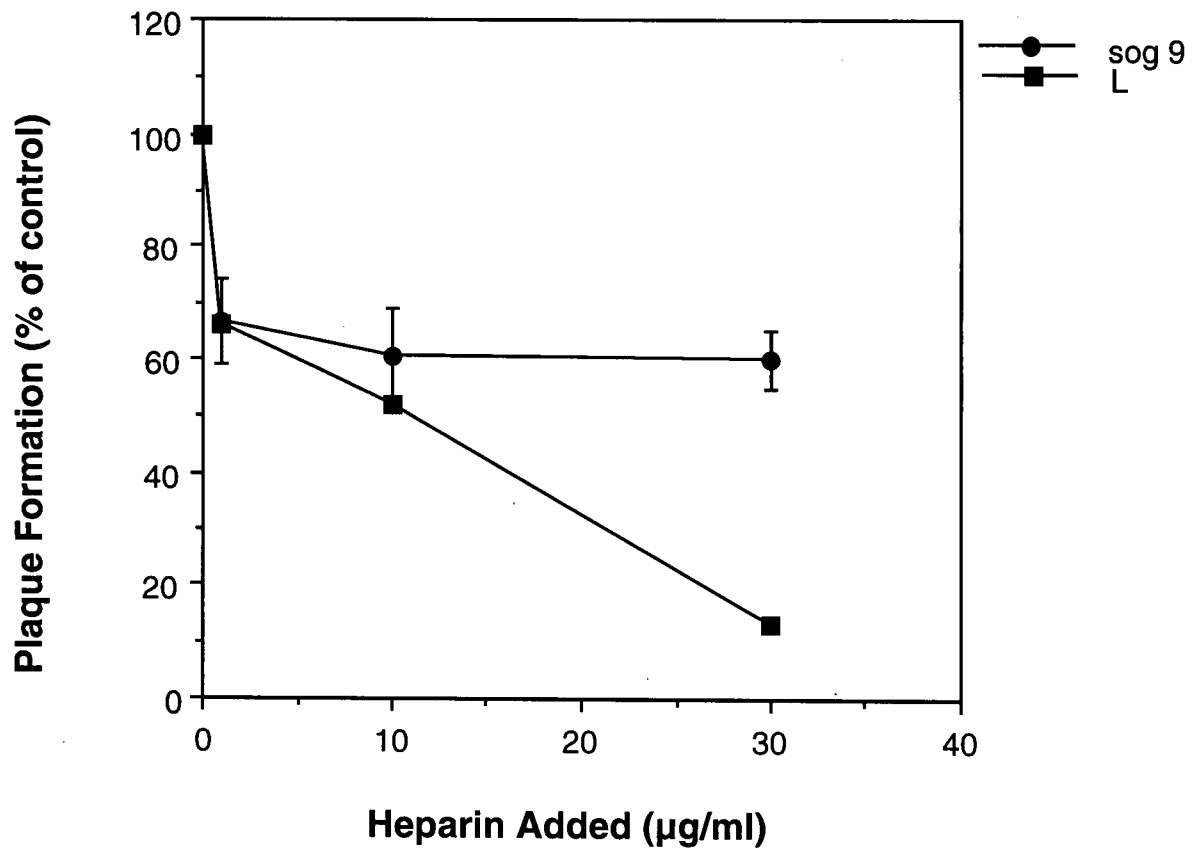
with viral attachment to CS GAGs. Sog9 cells, however, do not express any GAG moieties, and thus, it was not surprising that HSV-1 infection of sog9 cells was resistant to inhibition by soluble HS. To assess whether the inhibitory effects of HS on L and gro2C cell infection were specific or ionic in nature, cells were infected with HSV-1 in the presence of the highly sulfate GAG analog dextran sulfate. DS inhibited infection of L cells, suggesting that some of the inhibitory effect of HS on L cell infection was, in part, due to electrostatic forces. Gro2C cell infection was neither enhanced nor inhibited in the presence of DS. Surprisingly, however, DS stimulated HSV-1 infection of sog9 cells by up to 25-fold. Moreover, this effect was type-specific, in that HSV-2 infection of sog9 cells was not enhanced by the addition of DS.

I decided to investigate further the effects of dextran sulfate on sog9 cell infection for two reasons. First, understanding how DS could enhance viral infection on a GAG-deficient cell line might provide further insight into the initial interactions of the virus with cell surface molecules. Second, little is known of the differences between HSV-1 and HSV-2 and their interactions with cell surface GAGs. Differences in the interactions of the HSV serotypes with DS might reflect variations in the manner in which these viruses normally recognize and attach to GAGs on the host cell. This chapter summarizes experiments that were conducted to determine the mechanism by which DS enhances HSV-1 infection.

## 3.1 RESULTS

### 3.1.1 Effect of sulfated polyanions on HSV infection of L and sog9 cells

To expand on the earlier findings of Banfield *et al.* (1995a), monolayers of L and sog9 cells were infected with HSV-1 in the presence of increasing concentrations of either soluble heparin or DS (0-300  $\mu\text{g/ml}$ ). HSV-1 infection of control L cells was sensitive to inhibition by soluble heparin, as 30  $\mu\text{g}$  heparin/ml reduced L cell infection by 90% (Fig. 3.1). By contrast, HSV-1 infection of sog9 cells was only decreased by 28% at the highest



**Figure 3.1.** Effect of soluble heparin on HSV-1(F) plaque formation. Monolayers of L and sog9 cells were inoculated with HSV-1 (F) diluted in various concentrations of heparin. After a 1 h adsorption period, the virus was removed and medium containing 0.1% pooled human IgG was added to the monolayer to allow for plaque formation. Data are expressed as percents of the number of plaques that form in the absence of heparin. Values represent the average  $\pm$  range from two independent experiments.



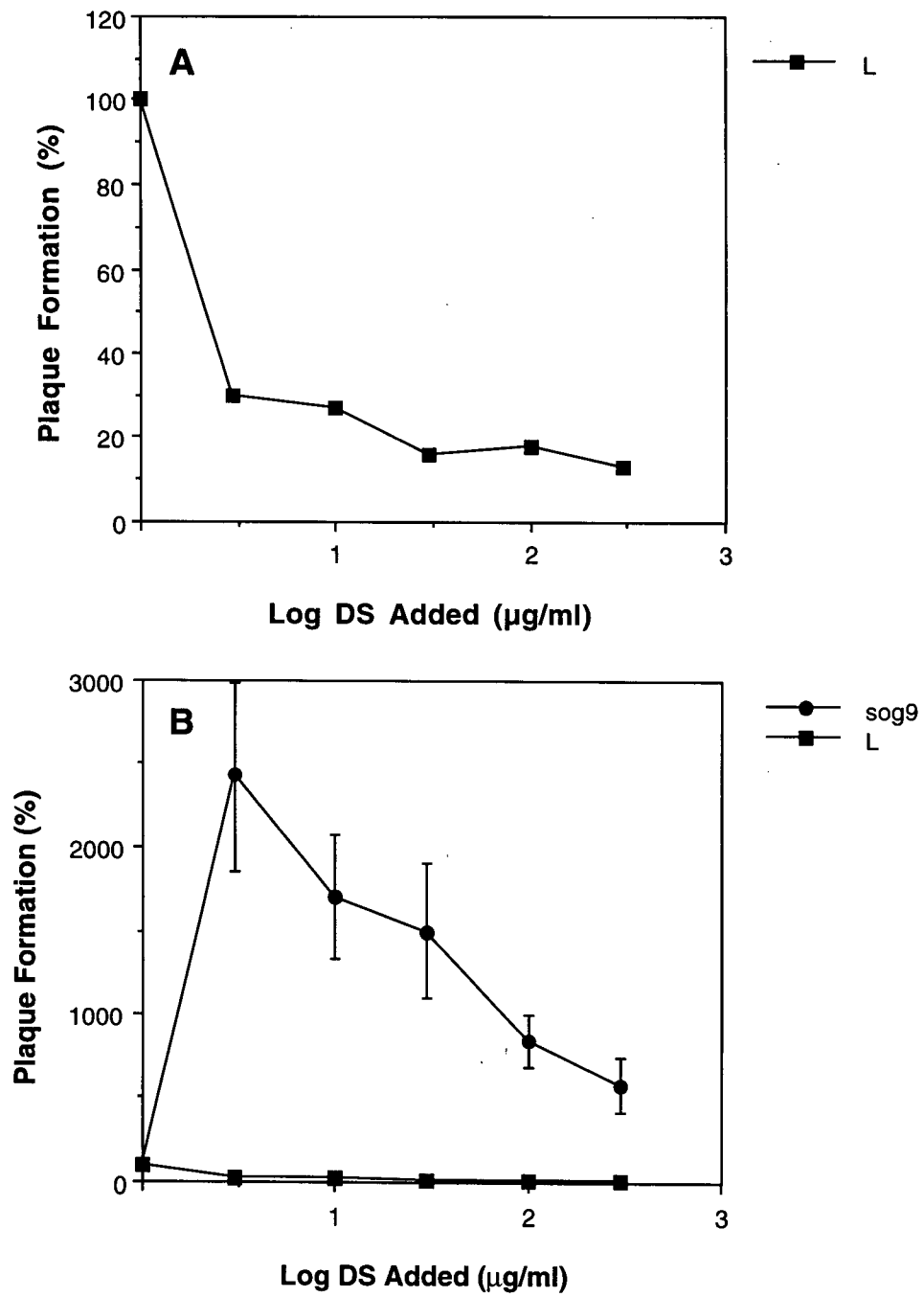
concentration tested. Next, L and sog9 cells were infected in the presence of DS (Fig 3.2 A and B). DS was a strong inhibitor of HSV-1 infection of L cells, since as little as 3  $\mu\text{g/ml}$  DS could reduce infection by 70%. By contrast, low concentrations of DS stimulated HSV-1 infection on sog9 cells by as much as 25-fold. This effect was dose-dependent as increasing DS concentration reduced the level to which infection was enhanced. This effect was likely caused by the propensity of soluble DS to bind virus in solution. Nevertheless, infection was still enhanced using 300  $\mu\text{g/ml}$  DS, the highest concentration tested. It is worthwhile to note that in Figure 3.2, only the results from a single determination are shown for DS inhibition of L cell infection, despite the fact that this experiment was repeated three to four times with similar results. Other data throughout this thesis have been presented in a similar fashion. The reason for this is that different stocks of virus and/or polyanion were used in some of these experiments, introducing variation in the absolute number of plaques formed. In these instances, it was not possible nor appropriate to average the data from different experiments. Despite this variation in absolute plaque number, however, the overall results for different experiments were the same. Therefore, the experiments presented are representative experiments.

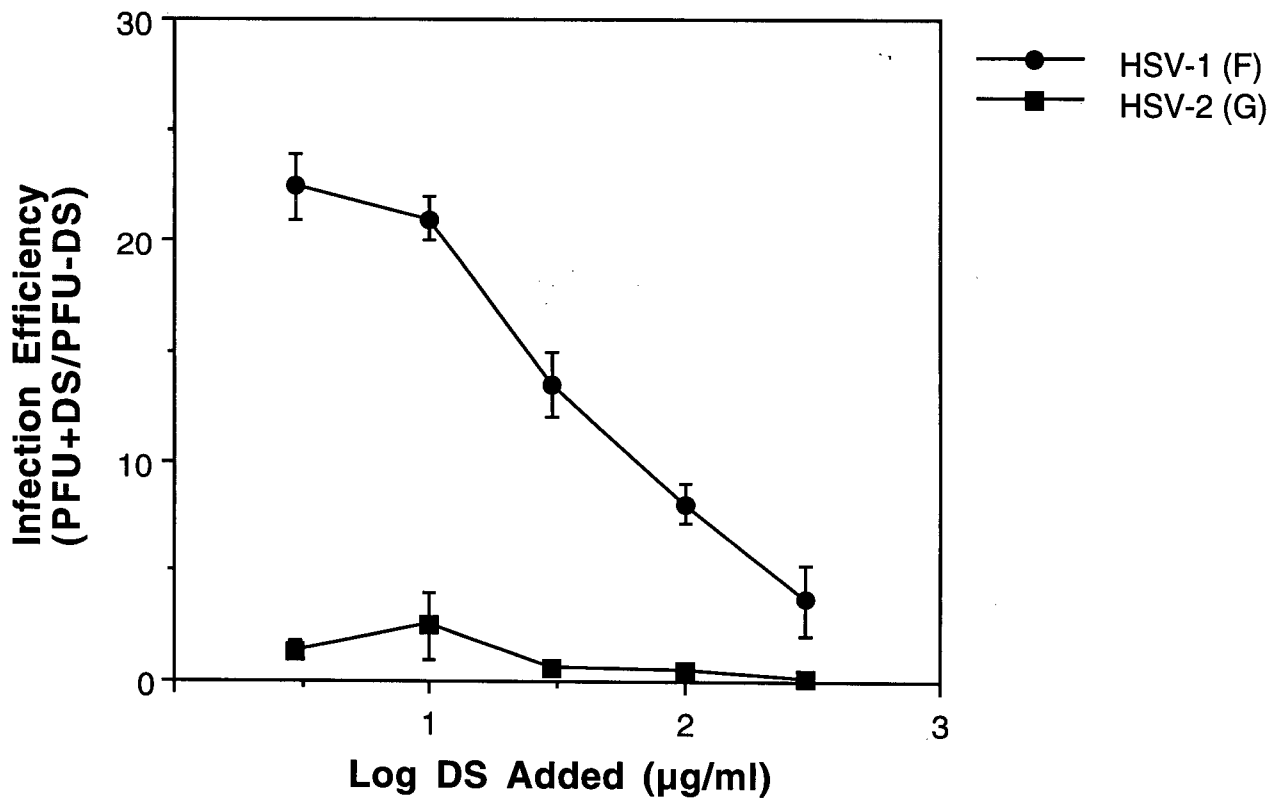
To confirm that DS stimulation was type-specific, plaque assays were done with HSV-2 (G). DS did not significantly enhance HSV-2 infection (less than four-fold) (Fig. 3.3). These results are consistent with the earlier findings of Banfield *et al.* (1995a) and confirmed that DS promoted infection of sog9 cells in a manner that was dependent on both the virus strain and the cell phenotype.

### 3.1.2 Characterization of the Interaction of Dextran Sulfate with sog9 Cells

There were several possibilities as to how dextran sulfate could enhance HSV-1 infection: (a) DS could act to tether the virus at the cell surface, (b) it could enhance viral penetration and/or (c) it might impinge on a post-entry step. It was reasoned that if (a),

**Figure 3.2.** Effect of soluble dextran sulfate on HSV-1 infection. (A) Effect of soluble dextran sulfate on HSV-1 (F) plaque formation on control L cells. (B) Effect of soluble dextran sulfate on infection of sog9 cells (and for comparison, control L cells). It should be noted that the scales for each graph are different. For both experiments, monolayers of cells were inoculated with HSV-1 (F) was diluted in various concentrations of dextran sulfate. After a 1 h adsorption period, the virus was removed and medium containing 0.1% pooled human IgG was added. Data are expressed as percents of the plaque formation that occurs in the absence of DS. Three to four independent experiments were performed with similar results. Where error bars are shown, the value represents the average  $\pm$  range from two independent experiments. Where no error bars are shown, the value represents the results from a single experiment .



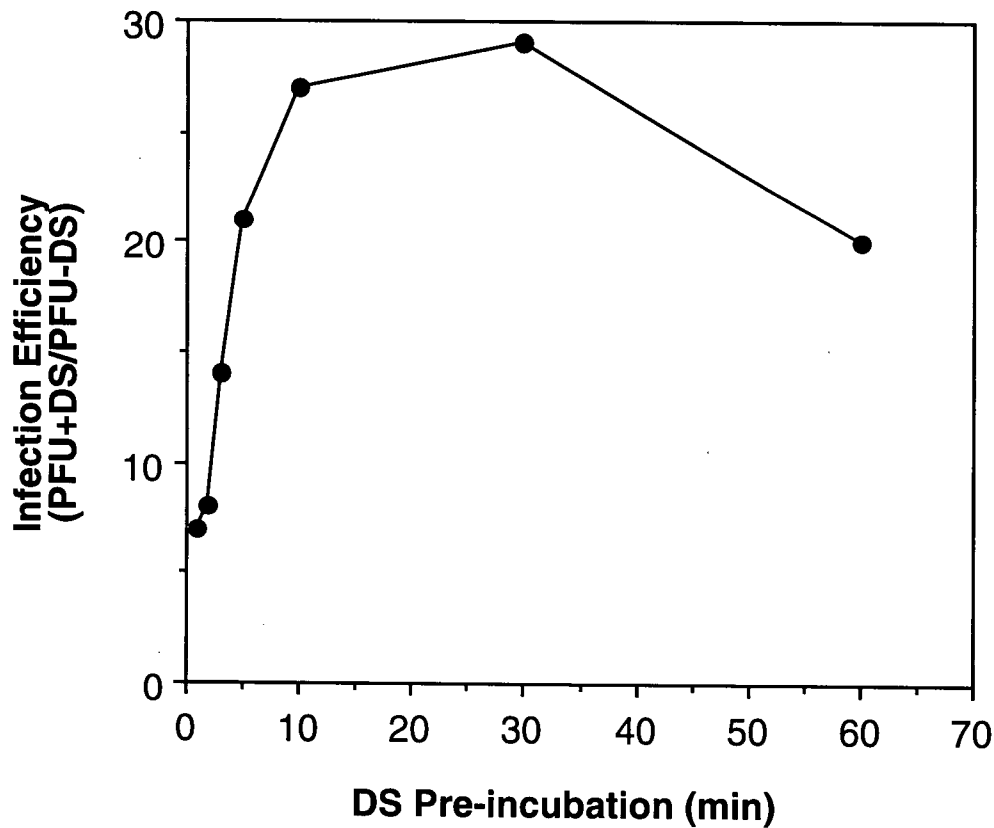


**Figure 3.3** Effect of soluble DS on herpes simplex virus infection. Fold stimulation of plaque formation observed when sog9 cell monolayers were inoculated with either HSV-1(F) or HSV-2(G) diluted in media containing various concentrations of DS. After a 1 h adsorption period at 37°C, the inoculum was removed and medium containing 0.1% pooled human IgG was added to the monolayers. Plaques were counted after three days. Each datum point represents the average of two determinations which did not vary by more than 4-fold at any point. Results are ratios of plaque formation on DS-treated monolayers to plaques formed on untreated controls. Values represent the average  $\pm$  range from two independent experiments.

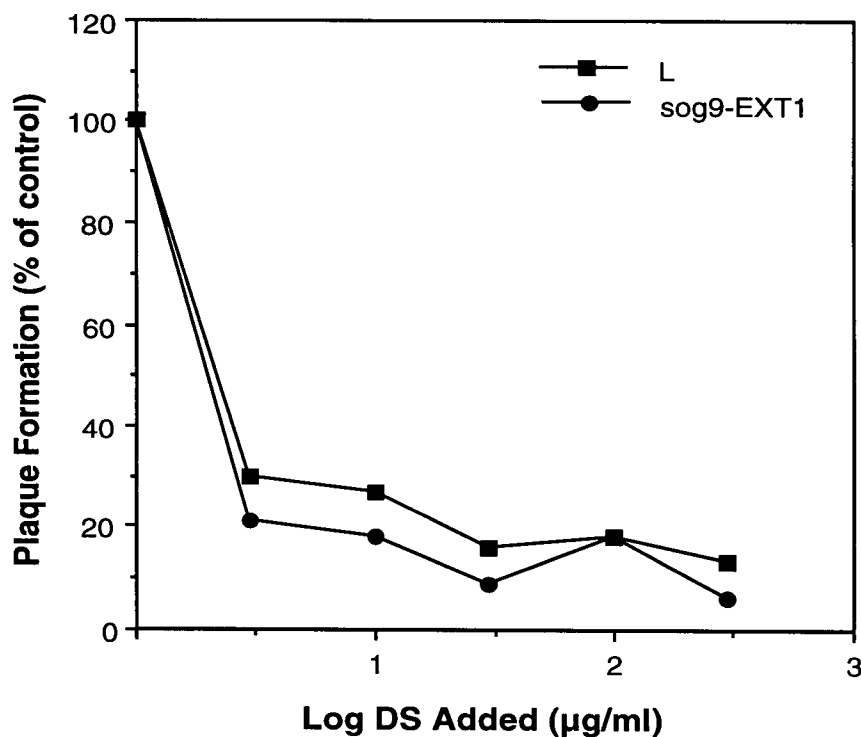
DS tethered the virus to the cell, then DS would likely interact at some level with the sog9 cell surface. To test this possibility, sog9 cell monolayers were incubated with DS for up to 60 min, rinsed, and incubated with HSV-1 for an additional 60 min. As shown in Figure 3.4, HSV-1 infection was stimulated by more than 25-fold after only a few minutes of pre-incubation. Longer incubations up to 1 h did not significantly increase infection efficiency. DS could likely interact with sog9 cells, in part, because of their lack of cell surface GAGs. To test this, sog9-EXT1 cells, which express cell surface HS GAGs, were pre-treated with 30 µg/ml DS for 10 min (Fig. 3.5). The monolayers were then rinsed and infected with virus. HSV-1 infection of sog9-EXT1 cells was clearly inhibited by the addition of DS and in a manner similar to that observed with parental L cells.

To establish optimum conditions for enhancing HSV-1 infection of sog9 cells, confluent monolayers of cells were treated with various concentrations of DS either before or during inoculation with HSV-1 (Fig. 3.6). Maximum enhancement (35-fold stimulation) was achieved by pre-incubating cell monolayers with low concentrations of DS. As expected, less stimulation was achieved with simultaneous DS treatment. Experiments also determined that there was a size dependency for DS enhancement of HSV-1 infection (Fig. 3.7). MW 5,000 DS and MW 15,000 DS were not active (less than two-fold) in stimulating HSV-1 infection, whereas up to 14-fold stimulation was observed with MW 50,000 DS. MW 500,000 DS possessed the most stimulatory activity (up to 35-fold). It appears from these data that relatively long-chain DS is required for efficient stimulation of HSV-1 infection.

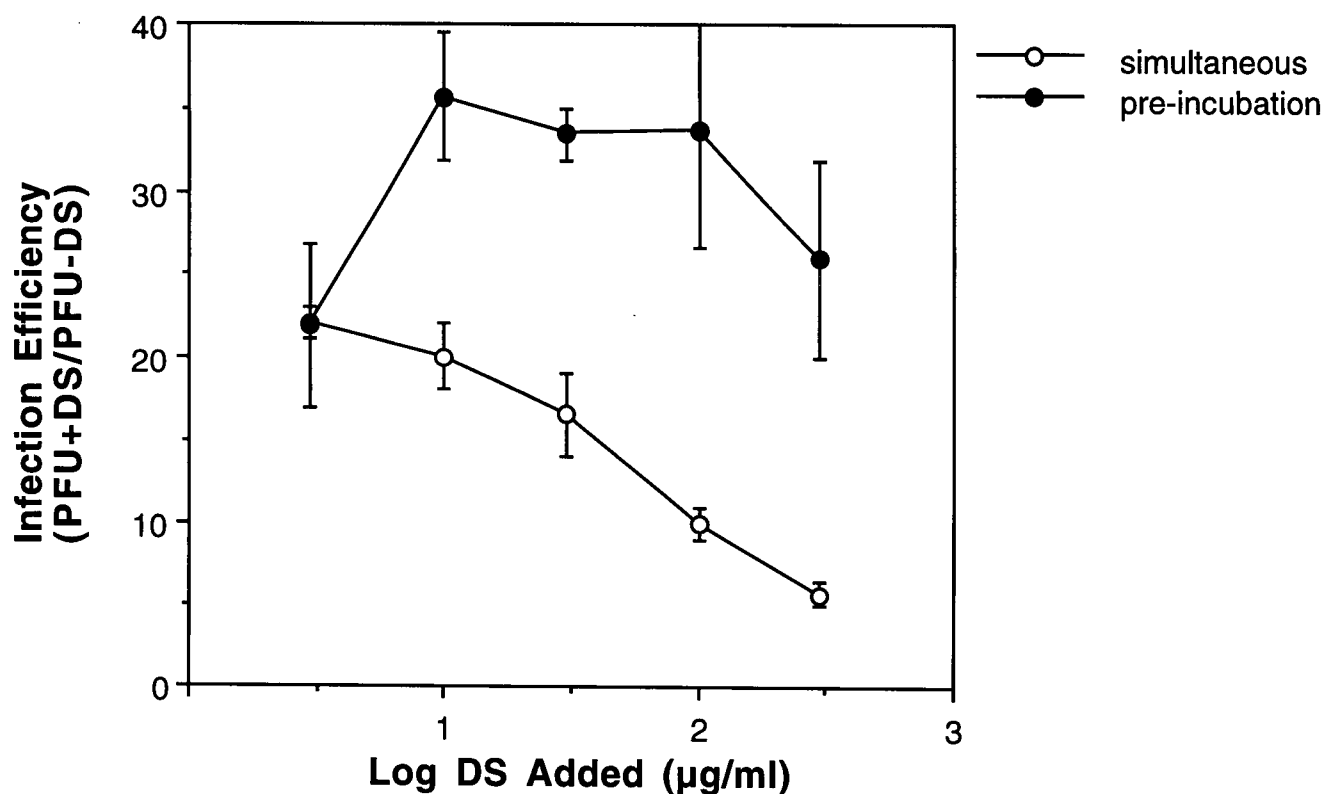
To assess the stability of DS on the sog9 cell surface, cell monolayers were treated with two different concentrations of DS for 10 min, incubated in DMEM for various times (pre-treatment window), and then infected with HSV-1 (Fig. 3.8). With 10 µg/ml of DS, stimulation declined rapidly as the length of the pre-treatment window was increased, and by 2 h, stimulation was reduced to two-fold over that of controls. However, DS stimulation persisted for up to 60 min when 100 µg of DS/ml was used. At this



**Figure 3.4.** Effect of soluble DS on HSV-1 (F) infection of sog9 cells. Sog9 cell monolayers were incubated with 10 $\mu$ g /ml DS in DMEM at 37°C for various lengths of time prior to inoculation with virus. Following removal of DS, the cells were inoculated with HSV-1 and incubated for 1 h at 37°C. Virus was removed, and DMEM containing 0.1% pooled human IgG was added to the monolayers. Plaques were counted after three days. Each datum point represents the average of two determinations which did not vary by more than approximately 5-fold at any point. Results are ratios of plaque formation on DS-treated monolayers to that on control monolayers not exposed to DS. Two independent experiments were performed with similar results. The results from a single determination are shown.

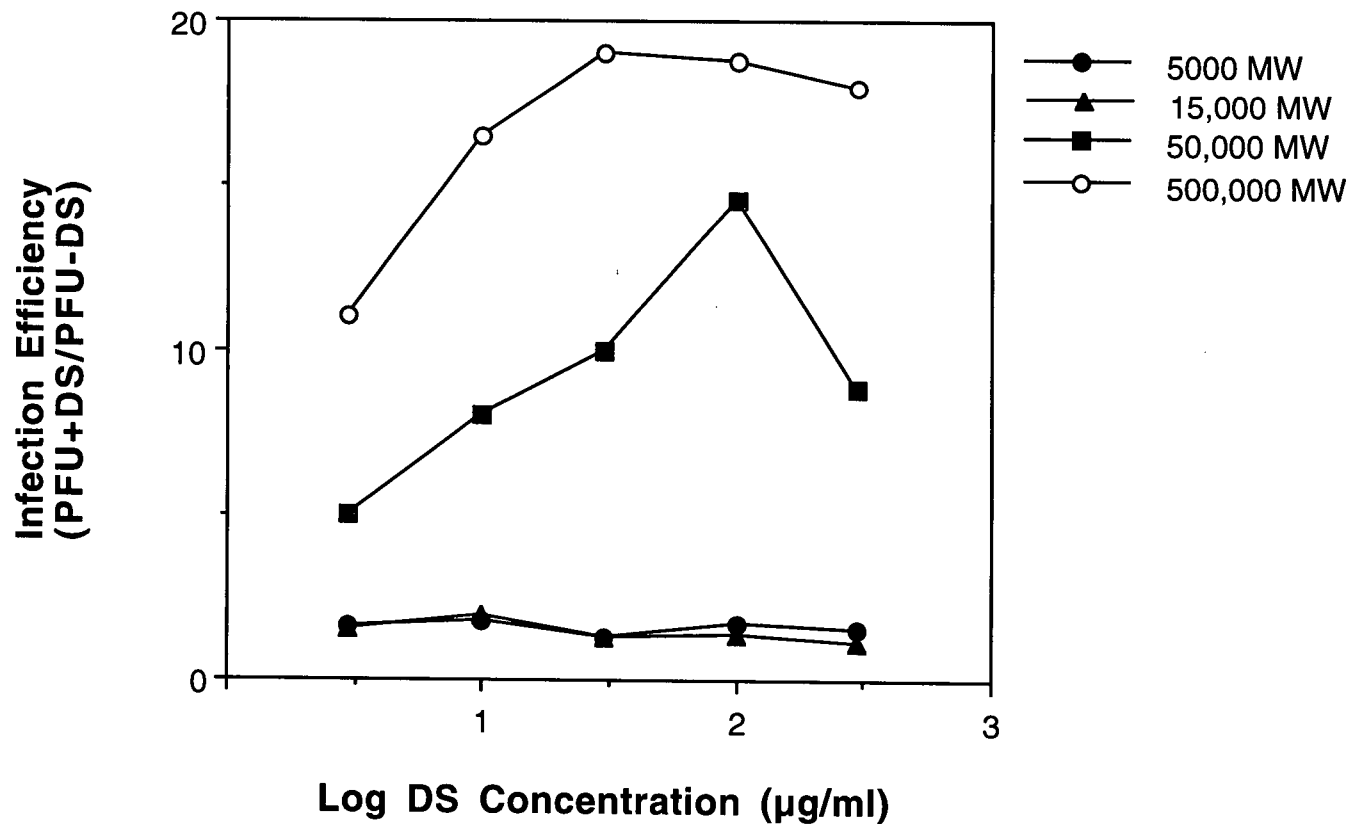


**Figure 3.5.** Effect of dextran sulfate on HSV-1(F) infection of sog9-EXT1 cells and L cells. Monolayers of L and sog9-EXT1 cells were incubated with various concentrations of DS for 10 min at 37°C. Following removal of DS, the cells were inoculated with HSV-1(F) for 1 h at 37°C. Virus was removed, and DMEM containing 0.1% pooled human IgG was added to the monolayers. Plaques were counted after three days. Two independent experiments were performed with similar results. The results from a single determination are shown.

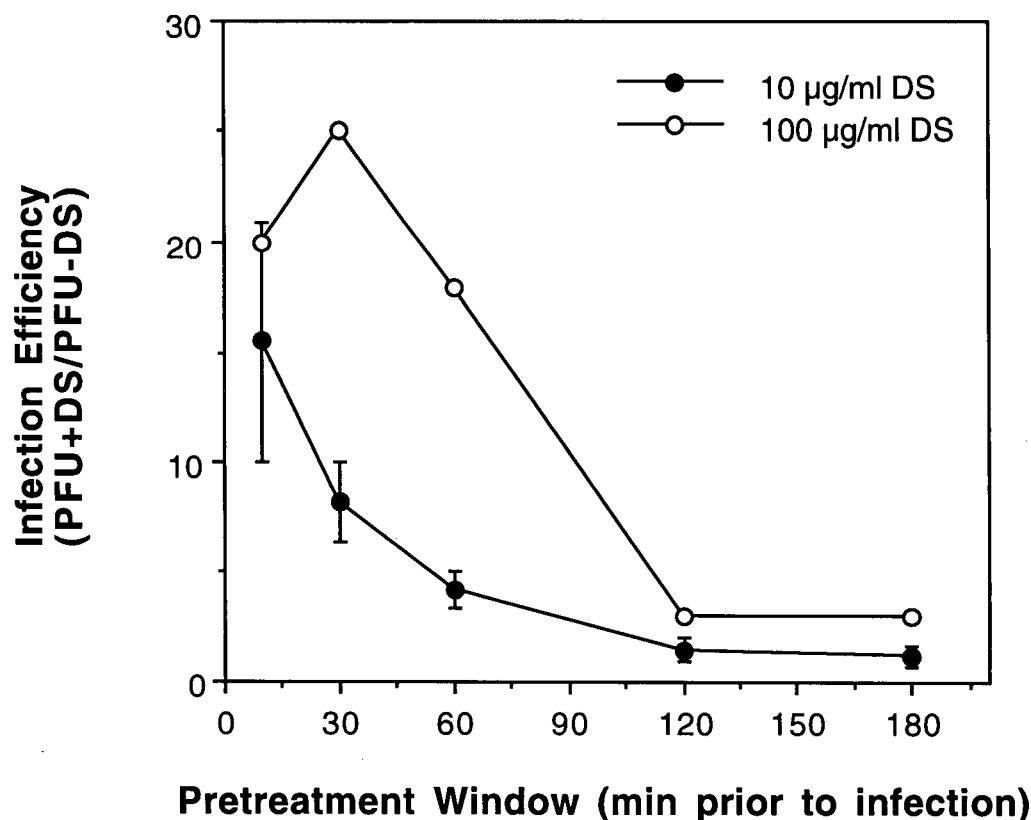


**Figure 3.6.** Effect of soluble DS on HSV-1 plaque formation. sog9 cell monolayers were either (i) incubated with DS in DMEM for 10 min at 37°C prior to infection or (ii) inoculated with virus in the presence of various concentrations of DS. Equivalent concentrations of HSV-1 were used for both experiments. After a 1h adsorption period at 37°C, the inoculum was removed and medium containing 0.1% pooled human IgG was added to the monolayers. Plaques were counted after three days. Results are the ratios of plaques formed on DS-treated monolayers to plaques formed on untreated controls. Values represent the average  $\pm$  the range from two determinations.





**Figure 3.7.** Size requirement for dextran sulfate. DS of different molecular weights was diluted in DMEM and then added to monolayers of sog9 cells for 10 min at 37°C prior to infection. DS was removed and HSV-1(F) was added for 1h at 37°C. The inoculum was removed and medium containing 0.1% pooled human IgG was added to the monolayers. The results from a single determination are shown. Data are ratios of plaque formation on DS-treated monolayers to that on control monolayers not exposed to DS. Two independent experiments were performed with similar results. The results from a single experiment are shown.



**Figure 3.8.** Effect of pretreating sog9 cells with DS on HSV-1(F) plaque formation. Monolayers of sog9 cells were incubated with 10 or 100 µg of DS/ml in DMEM at 37°C for 10 min at different times prior to inoculation with HSV-1 (pretreatment window). The monolayers were rinsed three times with PBS after treatment with DS, following which the cells were either (i) incubated with DMEM at 37°C until infection or (ii) infected immediately after a 10min pretreatment with DS in DMEM. The cells were rinsed once with PBS before a 1h adsorption period at 37°C, and the inoculum was removed and replaced with DMEM containing 0.1% pooled human IgG. Plaque numbers were determined after 3 days. Data are ratios of plaque formation on DS-treated monolayers to that on control monolayers not exposed to DS. The results shown are averages  $\pm$  the ranges from two independent experiments.

concentration, maximal stimulation was achieved when the cells were treated 30 min prior to infection. It was thus concluded that DS rapidly adsorbed to cells in active form and was then inactivated by either dissociation, degradation, or both. To determine whether DS was toxic to cells, which could account for the loss of DS stimulation when cells were treated hours in advance of infection, cells incubated with DS 3 h prior to infection were treated a second time just before inoculation. DS stimulation was normal in these cells, which showed that DS pretreatment did not reduce cell viability or susceptibility to infection (data not shown).

### **3.1.3 Analysis of Viral Attachment in the Presence of Dextran Sulfate**

On the basis of these results, it appeared that DS stimulated infection by binding stably to the host cell, where it acted as a matrix for subsequent virus adsorption and entry. This possibility was tested directly by experiments in which inoculation of cell monolayers with HSV-1 preceded DS treatment (Table 4, experiments A, B, and C) or in which cells were incubated with DS before inoculation (Table 4, experiment D). These assays took advantage of the fact that viral adsorption can be experimentally separated from penetration simply by keeping the temperature of incubation at 4°C. The temperature can then be shifted to 37°C to allow adsorbed virus to penetrate into cells. These experiments demonstrated that DS did not affect virus already bound to the cell surface (Table 4, experiment C), nor did it influence the internalization of bound virus (Table 4, experiment A). Experiment B (Table 4) showed that DS did not influence virus once it had been internalized, eliminating the possibility that DS impinged on a post-entry step. Only in experiment D (Table 4), in which cells were incubated with DS at 4°C prior to inoculation, was infection stimulated. From these results, it can be concluded that DS stimulates infection only when it is present prior to or during viral inoculation and that this activity is energy independent.

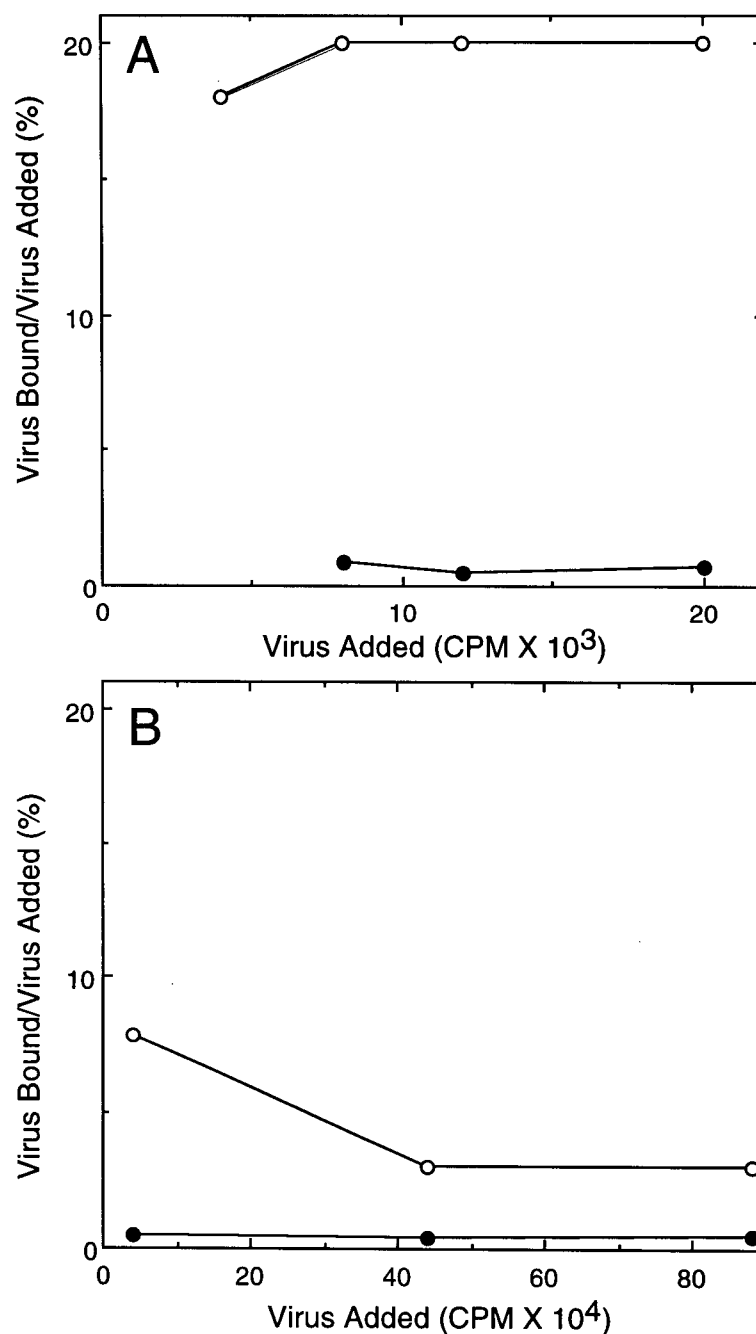
TABLE 4. Effect of dextran sulfate addition on HSV-1 infection

Expt	Experimental conditions <sup>a</sup>	Infection Efficiency <sup>b</sup>
A	Infect $\xrightarrow{4^{\circ}}$ Rinse $\xrightarrow{4^{\circ}}$ Add DS in DMEM $\xrightarrow{37^{\circ}}$ Rinse $\xrightarrow{37^{\circ}}$ Incubate with media + IgG	$2 \pm 1$
B	Infect $\xrightarrow{4^{\circ}}$ Rinse $\xrightarrow{4^{\circ}}$ Add DS in DMEM $\xrightarrow{37^{\circ}}$ Rinse $\xrightarrow{37^{\circ}}$ Incubate with media + IgG	$1 \pm 0.5$
C	Infect $\xrightarrow{4^{\circ}}$ Rinse $\xrightarrow{4^{\circ}}$ Add DS in DMEM $\xrightarrow{4^{\circ}}$ Rinse $\xrightarrow{4^{\circ}}$ Add DS in DMEM $\xrightarrow{37^{\circ}}$ Rinse $\xrightarrow{37^{\circ}}$ Incubate with media + IgG	$4 \pm 0.5$
D	Add DS in DMEM $\xrightarrow{4^{\circ}}$ Rinse $\xrightarrow{4^{\circ}}$ Infect $\xrightarrow{4^{\circ}}$ Rinse $\xrightarrow{4^{\circ}}$ Add DS in DMEM $\xrightarrow{37^{\circ}}$ Rinse $\xrightarrow{37^{\circ}}$ Incubate with media + IgG	$31 \pm 4$

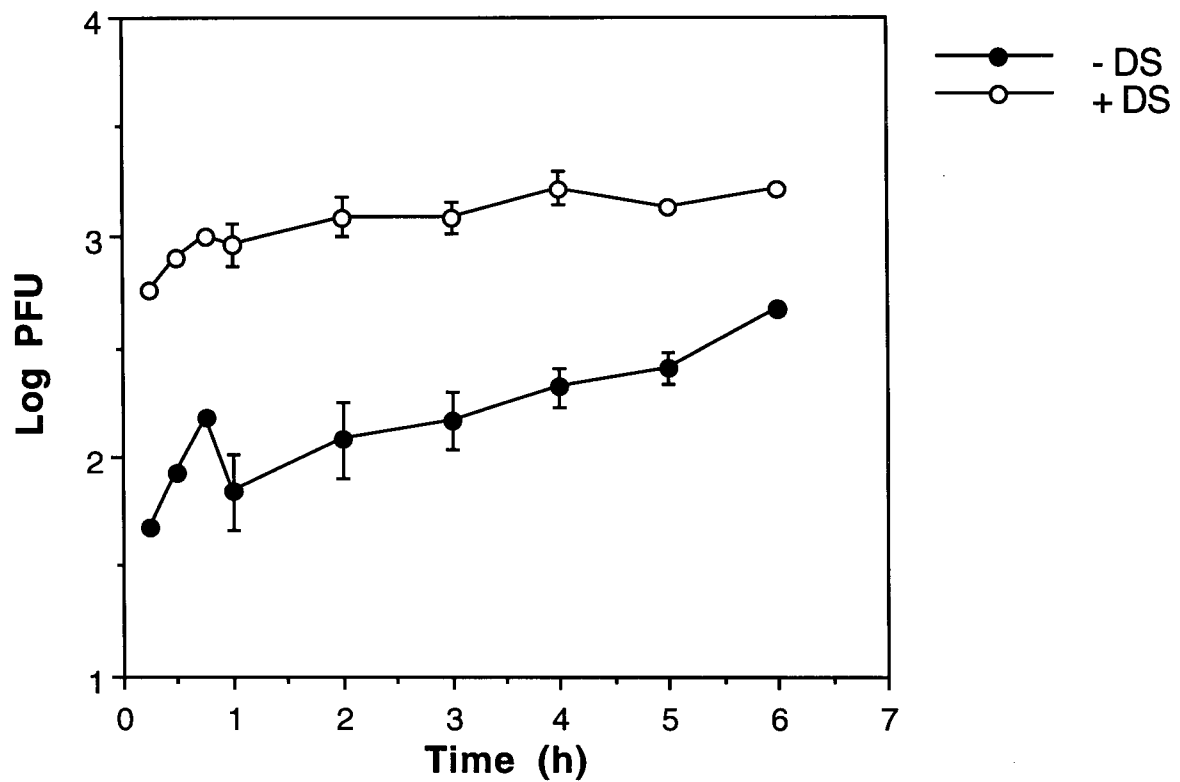
<sup>a</sup> For experiments A, B and C, HSV-1 (F) was allowed to adsorb to cells for 1 h at  $4^{\circ}$ , followed by the indicated treatments. In experiment D, cells were incubated with 100  $\mu\text{g/ml}$  DS for 1 h at  $4^{\circ}$  prior to infection. For all experiments, cells were rinsed once with PBS following removal of virus and 3 times after incubation with DS. All incubations were conducted for 1 h. <sup>b</sup> Results are expressed as the ratio of infection efficiency (plaque-forming units) obtained in the presence of DS compared to that obtained in the absence of DS. Values represent the average  $\pm$  the range from two independent experiments.

These results are consistent with previous findings demonstrating that DS stimulated adsorption of radiolabelled HSV-1 to sog 9 cells at 4°C (Banfield *et al.*, 1995a). Banfield and colleagues (1995a) showed that DS could enhance adsorption to sog9 cells by nearly 5-fold, but exerted little effect on virus adsorption to control L cells and gro2C cells. I wanted to demonstrate definitively that HSV-1 could bind DS. To do this, radiolabelled HSV was incubated with DS immobilized on microtiter wells (Fig.3.9). As a control for adventitious binding, virus was incubated with wells coated with BSA. In these experiments, approximately 20% of input HSV-1 bound to DS, which was substantially more than that observed for the BSA control wells. By contrast, less than 5% of input HSV-2 bound to DS. These data are consistent with a model in which HSV-1 adsorbs more efficiently than HSV-2 to sog9 cells in the presence of DS on the cell surface. This could account in part for the results showing that only HSV-1 infection is stimulated by DS.

If DS was tethering HSV-1 to the cell surface, then it could possibly accelerate the interaction of virus with a secondary, saturable receptor. To test this, sog9 cell monolayers were inoculated with HSV-1 in the presence or absence of DS for up to 6 h (Fig. 3.10). In the absence of DS, virus infection continued to increase during the 6 h incubation, which suggested that saturation of cell surface receptors had not been achieved. To control for degradation and/or dissociation of DS during this incubation, an experiment was performed in which fresh virus mixed with DS was added every 2 h throughout the 6-h time course (data not shown). The results from the two experiments were indistinguishable. An interpretation of these results is that in the absence of the primary cell surface glycosaminoglycan receptor, HSV-1 could not efficiently engage a downstream receptor. In the presence of DS, however, saturation was reached by 1 h of incubation with virus. Moreover, by 6 h, there was only a 3 +/- 0.5-fold difference between DS-stimulated and control infections. These data are consistent with a model in which DS functions to tether HSV-1 to the cell surface, thereby facilitating its interaction with a downstream receptor.



**Figure 3.9.** Binding of HSV-1 (A) and HSV-2 (B) to DS. HSV-1 and HSV-2 were labelled with [<sup>35</sup>S]methionine and purified by centrifugation through a sucrose gradient. Maxisorp 96-well dishes were coated with 5mg/ml DS and incubated with virus for 2 h at room temperature. Unbound material was removed by several washes with PBS. Bound virus was harvested with lysis buffer and transferred to scintillation vials for quantitation of radioactivity by liquid scintillation spectroscopy. Two independent experiments were performed with similar results. The results from a single experiment are shown. Open circles, binding to DS; closed circles, binding to BSA control.



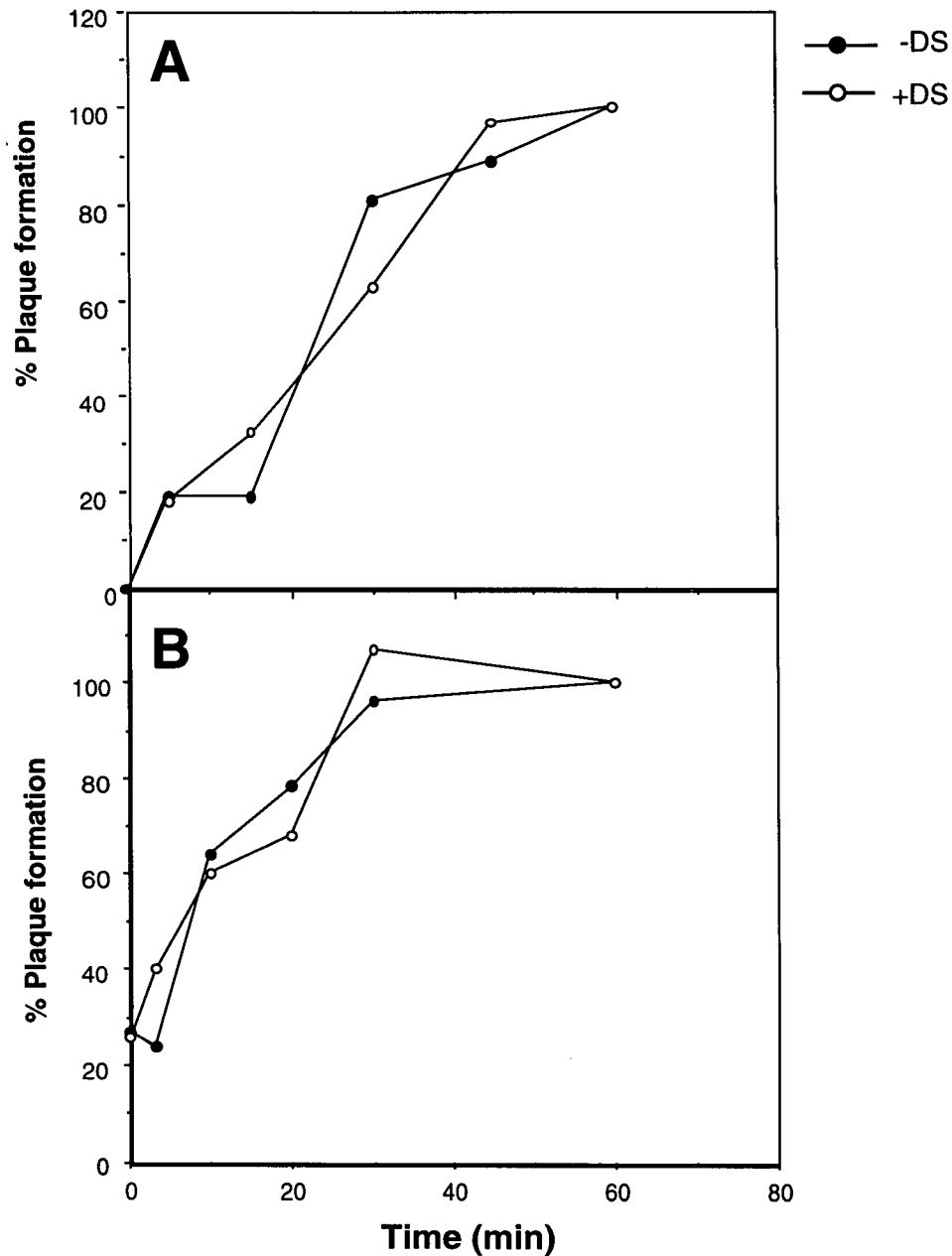
**Figure 3.10.** Time course of HSV-1 infection of sog9 cells in the presence of DS. Sog9 cells were inoculated with HSV-1 previously mixed with 10 $\mu$ g/ml DS and incubated at 37°C. At the indicated times post-infection, the inoculum was removed and DMEM with pooled human IgG was added. Values represent the average  $\pm$  range from two independent experiments.

### 3.1.4 Effect of Dextran Sulfate on Viral Penetration

To investigate whether DS exerted any effect on the rate of viral penetration, which might be suggestive of an alternative entry pathway, several penetration assays were performed. In one experiment, virus was adsorbed to the sog9 cell surface in the presence or absence of 10  $\mu\text{g/ml}$  DS at 37°C for 1 h to allow for virus adsorption and entry (Fig. 3.11 A). At various times, monolayers were washed with citrate buffer (pH 3.0) to inactivate extracellular virions, and the resulting plaques were counted after 3 days. The adsorption-penetration rates for DS-treated and control cells were similar. In a variation on this assay, cells were pretreated with DS for 30 min at 4°C, rinsed, incubated with virus for 30 min at 4°C, and then incubated at 37°C (Fig. 3.11 B). Monolayers were then treated with citrate buffer. Once again, there was no significant difference in virus penetration between DS-treated and untreated controls.

On the basis of these results, it appeared that HSV-1 engaged the normal entry pathway in the presence of DS. Dextran sulfate, however, is capable of adsorbing specifically to several cell surface receptors, including scavenger receptors (Krieger, 1992; Krieger *et al.*, 1993). It has also been shown to stimulate endocytosis in certain cell types (Thiele and Steinbach, 1994) and to block endocytosis by competing with other molecules for the endocytic machinery (Tokuda *et al.*, 1993; Xu *et al.*, 1993; Greenspan and Gutman, 1994). Low temperature blocks the movement of ligands between compartments of the endocytic pathway. To inhibit steps in endocytosis, sog9 cells were treated with 100  $\mu\text{g/ml}$  of DS at 4°C for 30 min. The cells were rinsed and then incubated with HSV-1 at 4°C for 30 min. The incubation temperature was then shifted to either 15°C, to inhibit steps in endocytosis, or 37°C (control) for 30 min. The results indicated that HSV-1 infection was stimulated by 20-fold at both temperatures (data not shown). These results suggest that DS-mediated infection occurs via the normal entry pathway.





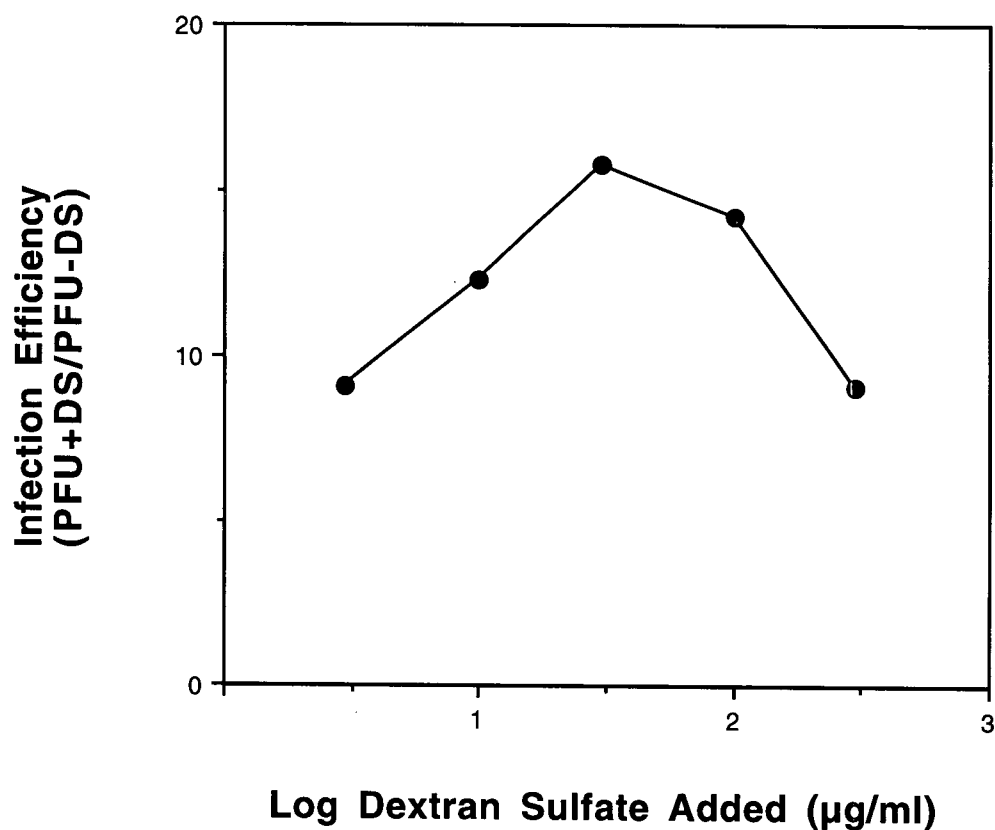
**Figure 3.11.** Rates of HSV-1 penetration into sog9 cells in the presence and in the absence of DS. (A) Confluent monolayers in 35-mm-diameter dishes were inoculated with HSV-1 (F) in the presence of 10 $\mu$ g/ml DS at 37°C for 1 h. The monolayers were washed at various times with citrate wash buffer (pH 3.0), and the resulting plaques were counted. (B) Sog9 cell monolayers were incubated with 100 $\mu$ g/ml DS/ml in DMEM for 30 min at 4°C. The monolayers were rinsed three times with PBS, and then the inoculum was added. Following a 30-min adsorption period at 4°C, the cells were transferred to a 37°C environment. At different times after the temperature shift, the monolayers were treated with citrate wash buffer. Two independent experiments were performed with similar results. Results from single experiments are shown. The results are PFU surviving citrate treatment expressed as a percentage of the number of plaques obtained after 60 min of infection, which for each experiment was taken as 100%.

### 3.1.5 Effect of Dextran Sulfate on HSV Infection of GAG-deficient sog8 Cells

I wanted to test the hypothesis that DS could enhance HSV-1 infection of other cell lines that are deficient in cell surface GAGs. One cell line that was tested was the sog8 cell line (Fig.3.12). Similar to sog9 cells, sog8 cells were isolated from gro2C cells and do not express any cell surface GAGs. Due to additional defects in this cell line (Esford, 1999), HSV-1 is unable to form plaques on sog8 cells. To quantify infection of sog8 cells in the presence of DS, I used a mutant HSV-1, HSV-1 $\Delta$ gC2-3, in which part of gC is deleted and replaced with the *lacZ* gene. HSV-1 glycoprotein C is not required for DS stimulation of sog9 cell infection (see Chapter 5), and thus this particular virus allowed the measurement of sog8 cell infection as a function of the number of lacZ-expressing cells. As shown in Figure 3.12, DS enhanced infection of sog8 cells by up to 16-fold. This result supported a model whereby DS could replace cell surface HS as a matrix for viral attachment.

## 3.2 DISCUSSION

In this study, I investigated a type-specific phenotype in which sog9 cell infection by HSV-1, but not HSV-2, could be partially rescued by the addition of soluble dextran sulfate. The simplest model to account for DS-mediated HSV-1 infection is that DS binds to sog9 cells in a saturable, reversible manner and tethers the virion to the cell surface. These data, showing that cells can be treated with DS prior to infection, that DS stimulates infection at 4°C and that DS interacts with virions, all lend support to this model. Moreover, there was a size requirement for DS. This makes sense, since it is more likely that virus would collide with and subsequently attach to long-chain DS than to low MW DS. This interaction with DS then allows HSV-1, but not HSV-2, to infect



**Figure 3.12** HSV-1 $\Delta$ gC2-3 infection of sog8 cells in the presence of DS. sog8 cell monolayers were incubated with 30 $\mu$ g/ml DS at 37°C for 10 min prior to infection. The DS was removed and the monolayers rinsed three times with PBS. Cells were incubated with virus for 1 h at 37°C, after which the inoculum was removed and medium was added to the monolayers. 24 h post-infection, cells were stained with 0.1% X-gal in X-gal buffer and incubated for 6 h at 37°C. Blue (infected) cells were counted. Two independent experiments were performed with similar results. Results from a single experiment are shown.

cells more efficiently. If this model is correct, DS essentially functions as a substitute for cell surface heparan sulfate, albeit one that confers type-specific infection.

Heparan sulfate is a ubiquitous cellular GAG that promotes efficient HSV adsorption to the host cell (WuDunn and Spear, 1989). Heparan sulfate may also serve other functions as well, such as promoting efficient fusion of the virus with the host cell (Shieh and Spear, 1994). Because the structure of DS does not resemble that of the highly complex heparan sulfate molecule, it is unlikely that DS could provide more than a simple tethering function during infection. This may account, in part, for the inability of DS to completely restore wild-type levels of infection in the *sog9* cells. Moreover DS is not covalently linked to the host cell surface, which is likely to severely compromise its ability to stabilize HSV-1 virions that collide with the cell surface.

The finding that DS could actually *stimulate* HSV-1 infection is highly significant. Stimulation of infection requires that specific host-virus interactions be maintained. Indeed, this data indicates that DS mediates HSV-1 infection via the normal entry pathway. Time course assays of HSV infection in the presence of DS suggest that DS is capable of binding to HSV-1 and then “handing-off” the particle to its co-receptor, such that saturation of the second receptor is quickly achieved. This investigation into the mechanism of DS stimulation thus corroborates the paradigm of HSV entry, in which cellular GAGs serve as a matrix for viral binding, concentrating viral particles at the host cell surface and facilitating attachment to a downstream receptor.

It is well established that DS normally inhibits infection of cells by enveloped viruses. DS has been shown to prevent fusion of influenza virus with erythrocytes (Krumbiegel *et al.*, 1992) and the formation of syncytia in human T-cell lymphotropic virus type 1- infected cells (Ida *et al.*, 1994). Infection by human cytomegalovirus (Neyts *et al.*, 1992) and Sendai virus is also inhibited by this polyanion (Ohki *et al.*, 1992). It was therefore surprising that HSV-1 infection of *sog9* cells was markedly enhanced. These results also indicate that this effect was mediated specifically by DS; HSV-1 infection was not affected by soluble heparin. Earlier findings demonstrated that heparan

sulfate and chondroitin sulfate neither enhanced or inhibited HSV-1 infection on sog9 cells (Banfield *et al.*, 1995a). This is interesting, because heparan sulfate is the natural cell surface receptor for the virus. Several cell types, including murine macrophages and endothelial cells, as well as CHO cells, express scavenger receptors capable of binding various polyanionic ligands (Krieger, 1992; Krieger *et al.*, 1993). In particular, scavenger receptors present on the surface of murine macrophages have broad ligand binding specificity and can bind DS and fucoidan but not heparin or chondroitin sulfate. Although the ligands for these receptors are all polyanions, not all polyanions can function as ligands. It is possible, therefore, that sog9 cells possess a receptor with a binding specificity similar to that of macrophage scavenger receptors, and this may account for the observation that cells pretreated with DS retain enhanced susceptibility to HSV-1 even after the soluble DS is removed. If this is true, then presumably sog9-EXT1 and L cells also express this receptor. However, pre-treatment of L cells and sog9-EXT1 cells with DS did not enhance viral infection (plaque formation was inhibited). The stimulatory effects of DS may not be detectable in cells that display heparan sulfate.

To demonstrate definitively that DS could act as an artificial co-receptor to initiate viral infection, DS stimulation assays were performed using another GAG-deficient cell line, sog8. HSV-1 infection of sog8 cells line was enhanced by soluble dextran sulfate. This was not necessarily surprising, since the sog8 and sog9 cell lines were both derived from gro2C cells. During the course of the present investigation, however, another project was initiated in our laboratory which allowed us to test the DS stimulation model in an unrelated GAG-deficient cell type. Mouse muscle fibers exhibit a maturation-dependent loss of susceptibility to infection by HSV-1, such that HSV can infect newborn muscle fibers *in vitro*, but not those from older animals (Acsadi *et al.*, 1994; Feero *et al.*, 1997). Work by Sonia Yeung in our laboratory has demonstrated that heparan sulfate biosynthesis is down regulated during skeletal muscle maturation (Yeung *et al.*, 1999). Newborn muscle fibers express significant amounts of heparan sulfate and chondroitin sulfate. By contrast, GAG expression in mature myofibers is reduced.

Further experiments demonstrated that mature fibers expressed secondary viral receptors but that in the absence of heparan sulfate, HSV-1 was unable to effectively bind to these receptors. In an attempt to overcome this block to infection, DS was added to cultured mature myofibers either prior to or during infection. In both experiments, infection was enhanced by the addition of dextran sulfate. In another experiment, adult mice were injected with HSV-1 in the tibialis anterior muscle, along with dextran sulfate. Remarkably, HSV infectivity was enhanced by DS, consistent with the *in vitro* results. This provides sound support for a model in which DS functions as a surrogate receptor to stabilize the virus at the cell surface, thereby allowing HSV to engage a downstream receptor.

## CHAPTER 4: USE OF NOVEL *EXT1*-EXPRESSING CELL LINES TO CHARACTERIZE HSV-GLYCOSAMINOGLYCAN INTERACTIONS

### 4.0 INTRODUCTION

The initial attachment of HSV to cell surface GAGs is a critical component of the HSV entry pathway; the exact nature of this interaction, however, is poorly understood. Several lines of evidence indicate that HSV gB and gC are able to interact with cell surface GAGs and thus, play an important role in viral adsorption. This interaction would appear to be partly ionic in nature, as demonstrated in this study whereby DS, a polyanion structurally distinct from HS, could act as a surrogate HSV receptor on GAG-deficient cell lines. Nevertheless, recent studies have indicated that certain structural features of cell surface HS may also be significant in mediating attachment (Feyzi *et al.*, 1997; Herold *et al.* 1995, Lycke *et al.*, 1991; Herold *et al.*, 1996). To examine further HSV attachment to GAGs, HSV-1 infection of novel mouse cell lines expressing *EXT1* was investigated.

Sog9-*EXT1* cells were generated by transfecting sog9 cells with a single cDNA clone encoding the gene *EXT1* (McCormick *et al.*, 1998). *EXT1* expression restored wild-type levels of susceptibility to HSV-1 infection in sog9 cells. The rescue of HSV-1 infection conferred by *EXT1* expression is due to the fact that *EXT1* encodes a heparan sulfate polymerase, thereby restoring HS synthesis in sog9 cells. Moreover, the type of HS that is synthesized in *EXT1*-expressing cells appears to be distinct from that produced in wild-type L cells. HPLC analysis demonstrated that L-*EXT1* and sog9-*EXT1* cells synthesize a heparan sulfate GAG that is eluted at lower salt concentration. This suggests that HS produced by *EXT1*-expressing cells is either undersulfated and/or of shorter chain length relative to HS produced in control L cells. One objective of the work presented in this chapter, therefore, was to identify the specific features of HS that are important for viral binding and infection of *EXT1* and non-*EXT1* cell lines. To do this,

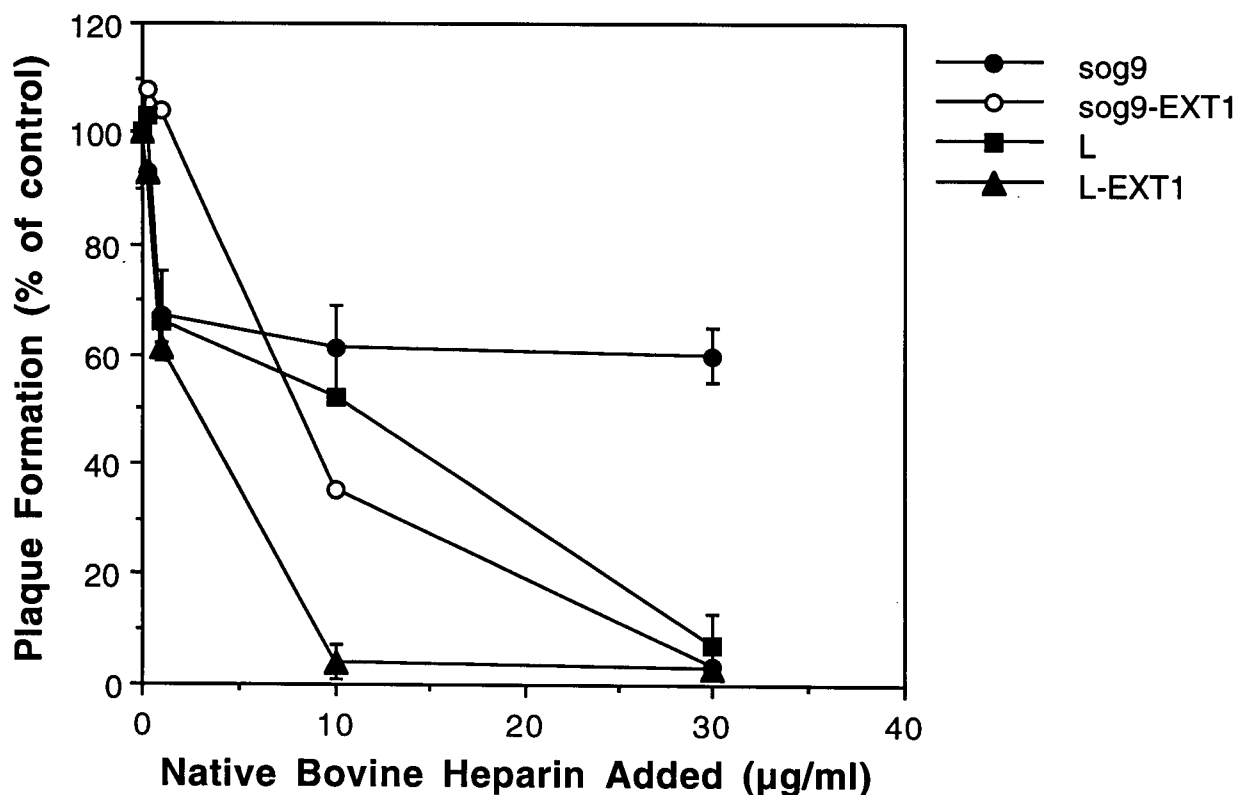
HSV-1 plaque formation on L cells, sog9 cells, L-EXT1 cells and sog9-EXT1 cells was examined in the presence of chemically modified heparin compounds. The hypothesis underlying these experiments was that HSV-1 infection would be inhibited by modified heparin compounds which resembled viral receptors on the cell surface. Modified heparin compounds which were no longer similar to viral GAG receptors would not inhibit HSV-1 infection. It was also desirable to determine if HSV-1 attachment to these novel *EXT-1* expressing cell lines employed the heparin-binding glycoproteins gB and gC. For this, a panel of HSV mutants bearing deletions in the heparin-binding domains of gB and gC was tested. By doing so, the ability of these glycoproteins to functionally recognize a unique HS moiety on *EXT1* cells could be assessed. The results obtained suggest that HSV recognizes similar HS features in *EXT-1* and non-*EXT1* cell lines and that in both instances, this recognition is primarily mediated by the heparin-binding domain of gC.

## 4.1 RESULTS

### 4.1.1 Effect of Sulfated Polyanions on HSV-1 Infection of Mouse *EXT-1* Cells

In a previous study, *EXT-1* expression was shown to restore HSV-1 infection of sog9 cells to wild-type levels (McCormick *et al.*, 1998). We hypothesized that this was due to the ability of the virus to attach to HS GAGs on sog9-EXT1 cells. To test this, HSV-1 infection assays were performed on sog9-EXT1 cells in the presence of soluble heparin (Fig. 4.1). HSV-1 infection of L cells, L-EXT1 cells and sog9-EXT1 cells was effectively inhibited, indicating that heparin could act as a competitor for HSV attachment to cellular GAGs. To substantiate these results, HSV-1 infection assays were performed once again, only this time using soluble DS (Fig. 4.2). Under these conditions, sog9-EXT1 cells behaved like control L cells and L-EXT1 cells in that DS inhibited HSV-1 infection. As observed previously, DS stimulated HSV-1 infection of sog9 cells. Taken together, these results indicate that sog9-EXT1 cells resemble control L cells with regard

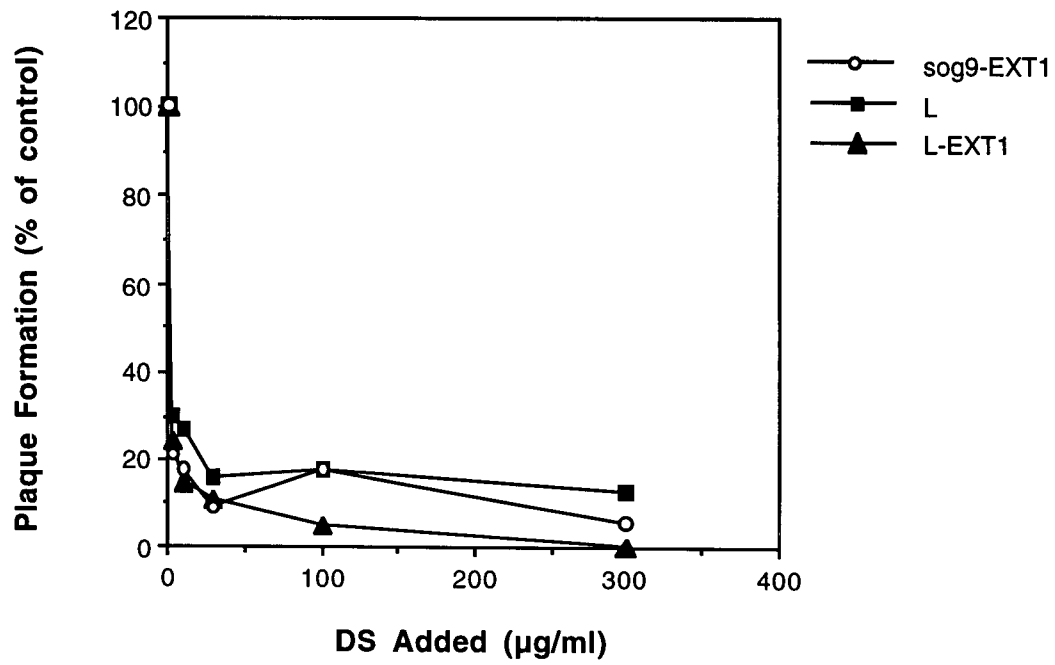




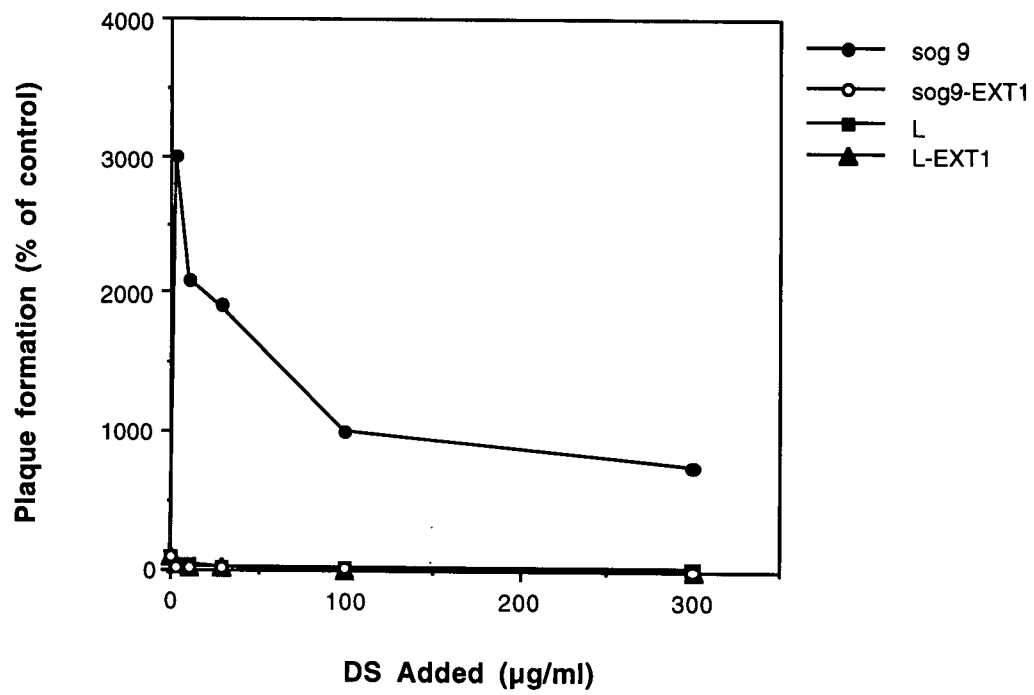
**Figure 4.1.** Effect of soluble native bovine heparin on HSV-1(F) infection of sog9, sog9-EXT-1, L and L-EXT1 cells. Cell monolayers were inoculated with HSV-1 diluted in media containing various concentrations of unmodified heparin. After a 1 h adsorption period at 37°C, virus was removed and media containing 0.1% pooled human IgG was added to the cells. Plaques were counted after three days. Results are the number of plaques formed in the presence of heparin expressed as percentages of plaque formation in the absence of heparin. The value shown is the average  $\pm$  range from two independent experiments.

**Figure 4.2.** Effect of DS on HSV-1 infection of mouse EXT-1 cell lines. Graphs A and B show experiment plotted two different ways in order to illustrate both the stimulatory (B) and inhibitory (A) effects of DS on HSV-1 infection. Cell monolayers were inoculated with HSV-1 (F) in the presence of various concentrations of soluble DS. Following a 1-h adsorption period at 37°C, virus was removed and DMEM containing 0.1% pooled human IgG was added. Plaques were counted after three days. Two individual experiments were performed with similar results. Data shown are from single experiments and are expressed as percentages of the infection that occurs in the absence of dextran sulfate. In a variation of this experiment, cell monolayers were pretreated with DS prior to infection. The data obtained were similar to the results shown.

**A**



**B**



to HSV-1 infection. Moreover, it would appear that *EXT1* expression radically alters the cell surface architecture of *sog9* cells, such that *sog9-EXT1* cells express cell surface GAGs to which HSV-1 can functionally attach.

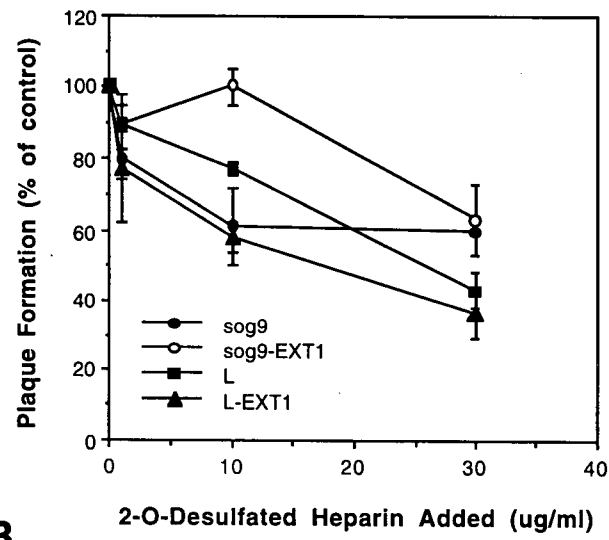
#### 4.1.2 Analysis of HSV-1 Infection of Mouse *EXT1* Cells in the Presence of Modified Heparin Compounds

Both heparin and heparan sulfate display a number of chemical groups that may potentially contribute to viral attachment. Several lines of evidence indicate that both N- and O- sulfate groups are key determinants of viral binding to heparan sulfate (Lycke *et al.*, 1991; Sheih *et al.*, 1992; and Herold *et al.*, 1994). In an attempt to define the importance of specific HS sulfate groups synthesized by control L cells and *EXT1*-expressing cell lines, HSV infection assays were performed on *sog9*, *sog9-EXT1*, L-*EXT1* and L cells in the presence of chemically desulfated heparins.

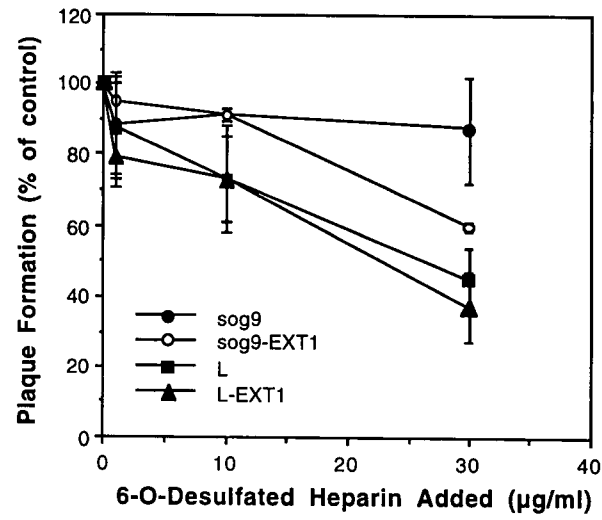
To determine the relative contributions of 6-O and 2-O sulfations to viral infection, the effects of 6-O desulfated, 2-O desulfated, and 2, 6-O desulfated heparin on the inhibition of viral plaque formation were compared (Fig. 4.3). None of the aforementioned modified heparin compounds had any significant effect on infection of *sog9* cells. However, both 6-O-desulfated and 2-O desulfated heparin inhibited HSV-1(F) infection on L, L-*EXT1* and *sog9-EXT1* cells, albeit less efficiently than unmodified heparin (Fig. 4.3 A and B). At the highest dose tested, plaque formation on *sog9-EXT1* cells in the presence either 6-O or 2-O desulfated heparin was reduced by approximately 30%. Infection of L and L-*EXT1* cells was somewhat more sensitive to the antiviral effects of these compounds, as plaque formation was inhibited by up to 50% and 60%, respectively. Removal of both 2-O and 6-O sulfate groups, however, abrogated the ability of heparin to act as a competitor for viral attachment, since 2,6-O desulfated heparin had little inhibitory effect on HSV-1 infection of either L, L *EXT1* or *sog9-EXT1* cells (Fig. 4.3 C). Taken together, these results suggest that the HS chains expressed on

**Figure 4.3.** Effects of O-desulfated heparin on HSV-1 infection of sog9, sog9-EXT1, L and L-EXT1 cells. Cell monolayers were infected with HSV-1 diluted in various concentrations of either (A) 2-O-desulfated heparin, (B) 6-O-desulfated heparin or (C) 2,6-O-desulfated heparin. Cell monolayers were inoculated with HSV-1 diluted in media containing various concentrations of the O-desulfated heparin compounds. After a 1-h adsorption period at 37°C, the inoculum was removed and media containing 0.1% pooled human IgG was added. Plaques were counted after 3 days. Results are plaques formed in the presence of O-desulfated heparin expressed as percentages of plaque formation in the absence of O-desulfated heparin. Values represent the average  $\pm$  range from two independent experiments.

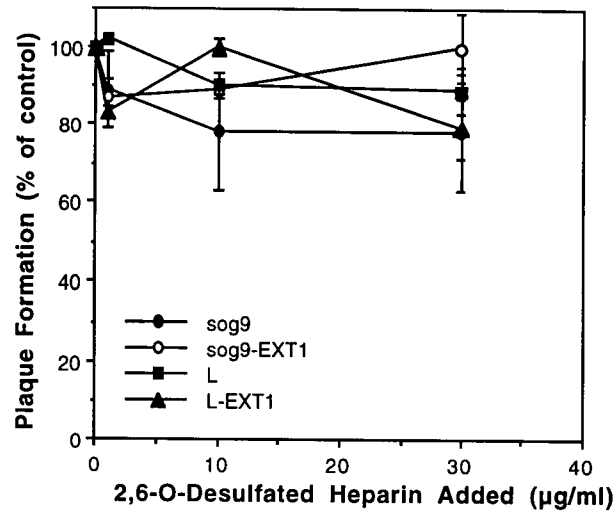
**A**



**B**



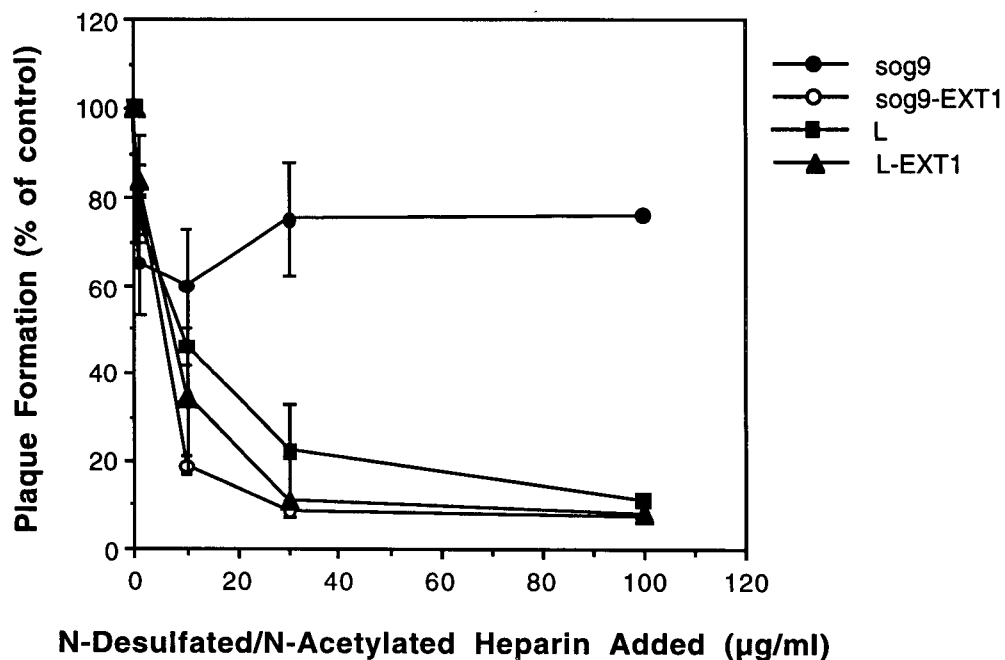
**C**



L, L-EXT1 and sog9-EXT1 are rich in O-sulfate groups and that these moieties are important for virus-cell interactions.

I next wanted to test the importance of N-sulfation in mediating HSV adsorption and infection. For this, a modified heparin compound in which all N-sulfate groups were replaced by the charge neutral acetyl group (N-desulfated/N-acetylated heparin) was used. Interestingly, HSV-1 infection on sog9-EXT1, L and L-EXT1 cells was inhibited as effectively by N-desulfated/N-acetylated heparin as it was by unmodified heparin (Fig. 4.4). This could imply that N-sulfate groups are not important for HS recognition by HSV-1. These results, however, are difficult to interpret, since previous studies have observed that reacetylation of heparin at N-desulfated sites partially restores antiviral activity to the compound (Herold *et al* , 1995; Feyzi *et al.* 1997). I was, therefore, unable to establish the role of N-sulfate groups in HSV-1 attachment using this particular heparin preparation.

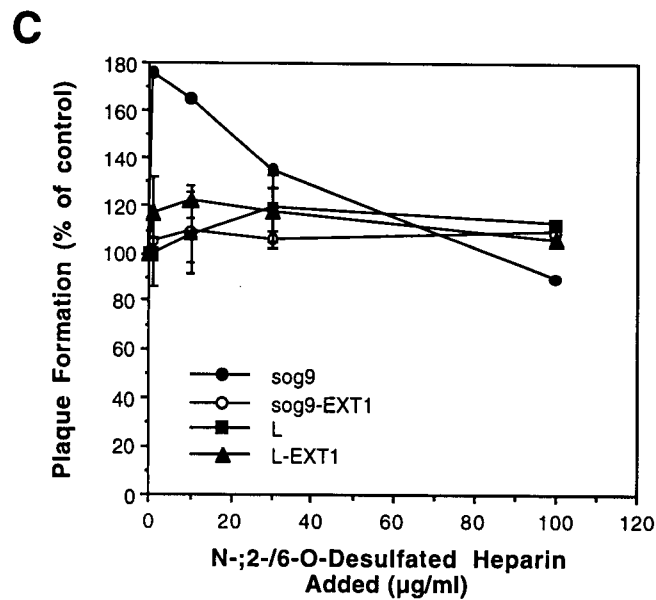
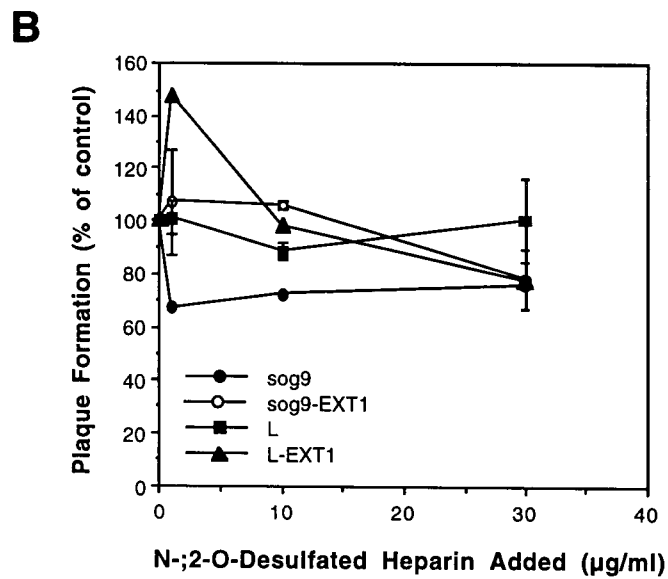
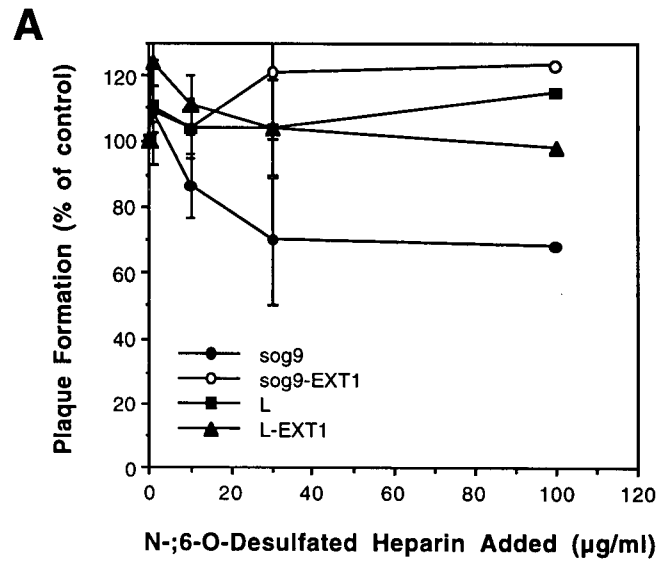
To examine the combined contributions of N- and O- sulfated regions in virus-cell interactions, N-; 6-O-desulfated, N-;2-O-desulfated and N-; 2-/6-O-desulfated heparin compounds were tested in HSV-1 infection assays (Fig 4.5). None of the three N-;O-desulfated heparin compounds inhibited HSV-1 infection of L, L-EXT1 cells or sog9-EXT1 cells. Moreover, N;6-O-desulfated and N;2-O-desulfated heparin were less effective than their 6-O-desulfated and 2-O desulfated counterparts at inhibiting viral infection (Fig.4.3). This supports the notion that N-sulfate groups are involved in viral-cell interactions. Taken together, these results demonstrate that both O- and N-sulfated regions of HS contribute to viral attachment.



**Figure 4.4.** Effect of N-desulfated/N-acetylated heparin on HSV-1(F) infection of sog9, sog9-EXT1, L and L-EXT1 cells. Cell monolayers were inoculated with HSV-1 diluted in media containing various concentrations of N-desulfated /N-acetylated heparin. After a 1h adsorption period at 37°C, the inoculum was removed and media containing 0.1% pooled human IgG was added. Plaques were counted after three days. Results are plaques formed in the presence of N-desulfated/N-acetylated heparin expressed as percentages of plaque formation in the absence of the modified heparin compound. Values are the averages  $\pm$  the range from two independent experiments.



**Figure 4.5.** Effects of N-/O-desulfated compounds on HSV-1(F) plaque formation on sog9, sog9-EXT1, L and L-EXT1 cells. Cell monolayers were inoculated with HSV-1 diluted in media containing various concentrations of either (A) N-;6-O-desulfated heparin, (B) N-;2-O-desulfated heparin, or (C)N-;2-/6-O-desulfated heparin. After a 1h adsorption period at 37°C, the inoculum was removed and media containing 0.1% pooled human IgG was added. Plaques were counted after 3 days. Results are plaques formed in the presence of N-/O-desulfated heparin expressed as percentages of plaque formation in the absence of N-/O-desulfated heparin. Values represent the average  $\pm$  the range from two independent experiments.



### 4.1.3 Relative Contributions of HSV gB and gC to Infection of Host Cells

From these results, it appeared that HSV-1 interacted with similar structural features of HS on both *EXT1* cells and control L cells. It was reasoned that these HS moieties were likely being recognized by HSV gB and /or gC, two glycoproteins previously shown to bind heparin. To test this hypothesis, plaque formation on these cell lines was compared using a panel of HSV gB and gC mutants that have reduced heparin-binding capabilities. Results from these experiments are shown in Table 5. To discern any differences between infection of *EXT1* and non-*EXT1* cells with the HSV mutants, plaque formation on each cell line was calculated relative to infectivity observed on sog9 cells. As a control, wild-type HSV-1 (KOS) was used, since all the mutants were originally derived from this strain. In agreement with an earlier study on HSV-1 infection of sog9-EXT1 cells (McCormick *et al.*, 1998), infection of sog9-EXT1 cells by wild-type HSV-1 was at a level similar to that observed for control L cells. To determine if gC played a role in the infection of these cell lines, the gC-deficient virus HSV-1(KOS) $\Delta$ gC2-3, was tested. The relative infectivity of HSV-1(KOS) $\Delta$ gC2-3 on sog9-EXT1, L, and L-EXT1 cells was substantially less than that of wild-type HSV-1(KOS). This was similar to the results obtained using another gC-deficient HSV-1, KCZ. The relative infectivity of KCZ on sog9-EXT1 cell lines was lower than that observed for HSV-1(KOS) $\Delta$ gC2-3. The reason for this is not clear. Collectively, however, these observations demonstrate that gC facilitates HSV-1 infection of both control L cell and *EXT1*-expressing cell lines.

The HSV-1 mutant KgBpK<sup>-</sup>, in which a putative HS binding lysine-rich (pK) domain of gB is deleted (Laquerre *et al.*, 1998b), was tested next. Significantly, the relative infectivity of KgBpK<sup>-</sup> on sog9-EXT1 and L cells was comparable to that of wild-type HSV-1. This implied that the heparin-binding domain of gB was not essential for mediating HSV-1 infection. To extend this observation further, infection of the cell lines

**Table 5.** Relative infectivity of HSV-1 heparin-binding mutants on EXT1 cell lines.

<sup>a</sup> Data shown is HSV plaque formation on sog9-EXT1, L and L-EXT1 cells relative to that observed on sog9 cells ( $\equiv 1.0$ ) for each strain tested. For each virus strain, cell lines were inoculated with equivalent amounts of virus for 1h at 37°C. Virus was removed and media containing IgG was added to facilitate plaque formation. Plaques were counted after three days. The results shown are from a single determination, which were similar to data obtained from two other experiments. (NA) Not available.

<sup>b</sup> See Table3 (Chapter 2) for information regarding virus strains used in this experiment.

Table 5. Relative Infectivity<sup>a</sup> of HSV Heparin-Binding Mutants on EXT1 Cell Lines

Cell Line		Virus Strain <sup>b</sup>						
		HSV-1 (KOS)	HSV-1 ΔgC2-3	KCZ	KgBpK <sup>-</sup>	KgBpK <sup>-</sup> gC <sup>-</sup>	KgBpK <sup>R</sup> gC <sup>-</sup>	KgBpK <sup>-</sup> gC <sup>R</sup>
sog9	1	1	1	1	1	1	1	1
sog9-EXT1	307	43	3	451	4	3.4	208	
L	923	398	40	857	32	28	676	
L-EXT1	659	122	13	NA	8.2	28	1081	

using the double-mutant virus KgBpK<sup>-</sup>gC<sup>-</sup> and the two repaired viruses, KgBpK<sup>R</sup>gC<sup>-</sup> and KgBpK<sup>-</sup>gC<sup>R</sup>, was compared. The relative infectivity KgBpK<sup>-</sup>gC<sup>R</sup> was comparable to that of wild-type HSV-1(KOS). By contrast, the relative infectivities of KgBpK<sup>-</sup>gC<sup>-</sup> and KgBpK<sup>R</sup>gC<sup>-</sup> on the various cell lines were similar to that observed with KCZ. Taken together, these results support a model whereby HSV-1 gC is the primary mediator of attachment to HS GAGs presented on *EXT1* cells and control L cells. Moreover, the pK sequence of gB is not essential for infection of these cell lines.

## 4.2 DISCUSSION

Heparan sulfate recognition by HSV is an important determinant of cell and tissue tropism. The interaction of HSV-1 with HS was studied by examining the ability of modified heparin compounds to inhibit viral infection of novel *EXT1*-expressing cell lines. Previous analysis in our laboratory indicated that the HS moiety expressed on *EXT1* cell lines is distinct from that presented on control L cells (McCormick *et al.*, 1998). An advantage of using these cell lines, therefore, was that they allowed the comparison of the ability of virus to use different HS structures as attachment receptors and to assess which features of HS were important for viral binding.

*EXT1* expression rescues HSV-1 infection of sog9 cells to wild-type levels (McCormick *et al.*, 1998). We hypothesized that the rescue of HSV-1 infection conferred by *EXT1* expression was the result of the enhanced expression of HS GAGs in sog9-*EXT1* cells. In this chapter, it was shown that HSV-1 infection of sog9-*EXT1* cells is inhibited by the addition of soluble heparin and dextran sulfate. The ability of these polyanions to inhibit HSV-1 infection is a clear indicator that HSV-1 can engage the cell surface HS presented on sog9-*EXT1* cells and thereby gain entry into host cells.

Having determined that HSV-1 could use HS on sog9-*EXT1* cells to mediate infection, I wanted to identify structural features of the molecule that were important for viral recognition. Previous studies have shown that in particular, sulfate groups are

important for viral attachment. For example, cells expressing undersulfated heparan sulfate due to genetic mutation bind HSV poorly (Shieh *et al.*, 1992). To clarify which sulfate groups were present on the HS GAGs on *EXT1* and non-*EXT1* cell lines, and whether or not they were important for viral infection, the effects of various desulfated heparin compounds on HSV-1 infection of the *EXT1* cell lines was tested. 2-O and 6-O desulfation of heparin reduced the antiviral activity of the compound on sog9-*EXT1*, L-*EXT1* and control L cells. Interestingly, however, these compounds were less effective at inhibiting viral infection of sog9-*EXT1* cells than they were on L and L-*EXT1* cells. One interpretation of this result is that sog9-*EXT1* cells express HS that has relatively more 2-O and 6-O sulfate groups than L and L-*EXT1* cells. Infection of all the cell lines was, however, insensitive to the addition of 2,6-O desulfated heparin. It can be concluded, therefore, that the HS GAGs on L, sog9-*EXT1* and L-*EXT1* possess both 2-O- and 6-O sulfate groups and that these are important for viral infection. This is in agreement with results from another study which demonstrated that 6-O-desulfation of heparin markedly reduced the antiviral activity for HSV-1 on Vero cells (Herold *et al.*, 1995). Work by Feyzi *et al.* (1997) further demonstrated that the minimal requirements for HSV-1 binding to HS consisted of 10-12 monosaccharide units containing at least one 2-O and one 6-O sulfate group.

In addition to O-sulfate groups, N-sulfate groups have also been shown to be important for binding of HSV-1 to HS (Feyzi *et al.*, 1997). Cell lines mutant in N-sulfotransferase display a reduced susceptibility to HSV infection (Shieh *et al.*, 1992). Moreover, N-desulfation of heparin has been shown to abolish its antiviral activity for HSV-1 (KOS) (Herold *et al.*, 1995). The contribution of N-sulfate groups to viral binding was examined by testing N-desulfated/N-acetylated heparin in the HSV infectivity assay. HSV infection of all *EXT1* and L cells was inhibited as effectively by N-desulfated/N-acetylated heparin as it was by unmodified heparin. This is contrary to a report by Feyzi *et al.* (1997), who showed that N-acetylation of N-desulfated sites reduced the inhibitory effect of heparin in a virus infectivity assay by 10-fold. It is difficult to explain this

discrepancy. It should be noted, however, that Herold and colleagues (1995) observed that re-acetylation of heparin at N-desulfated sites partially restored the antiviral activity of heparin. N-desulfation yields a positive charge ( $\text{NH}^+_3$ ) in the molecule, whereas, N-desulfation, N-reacetylation produces a neutral charge. As proposed by Herold *et al.* (1995), it may be that it is the absence of a positive charge at that site, rather than the presence of a negatively charged sulfate group, that is important for antiviral activity. Nonetheless, the observation that N-desulfated/N-acetylated heparin inhibits HSV-1 infection of the mouse cell lines is significant. The presence of a neutral group clearly allows the modified compound to retain enough structural similarity to compete with cell surface HS for viral binding. This demonstrates that not only are the charged sulfate groups of HS determinants of viral binding, but importantly, so is the actual backbone of the molecule. This is in agreement with Lycke and colleagues (1991) who demonstrated that HSV-1 could bind to a heparan sulfate preparation which contained 1.5 sulfate groups/disaccharide unit but not with a dermatan sulfate preparation which had a similar negative charge density (1.3 sulfate groups/disaccharide unit).

The ability of HSV to recognize GAGs is a function of the two heparin-binding glycoproteins, gC and gB. Until recently, the role of gB in the attachment of virus to HS GAGs has been difficult to establish. Laquerre and colleagues (1997) constructed a mutant HSV-1 particle deleted in the heparin-binding region of gB ( $\text{KgBpK}^-$ ) and demonstrated that binding of this virus to cells was reduced 20% compared to wild-type virus. The binding capacity of a gC-deficient HSV-1 (KCZ) was reduced by 65% and that of a double gB/gC mutant ( $\text{KgBpK}^- \text{gC}^-$ ) by 80%. These data led the authors to conclude that the heparin-binding region of gB contributes less to virus binding to HS than gC. The infectivity of these heparin-binding mutants was tested using our *EXT1*-expressing cell lines. These results support those of Laquerre and colleagues (1997), in that the relative infectivity of HSV-1 $\Delta\text{gC}2-3$ , KCZ and  $\text{KgBpK}^- \text{gC}^-$  on L, L-EXT1 and sog9-EXT1 cells was reduced compared to that of wild-type HSV-1. The relative infectivity of  $\text{KgBpK}^-$ , on the otherhand, was similar to that of wild-type virus. Thus, HSV-1 gC is likely the



principle virion component mediating HSV-1 attachment to HS GAGs on *EXT1* cells. Finally, it was interesting that the two gC-deficient viruses used in this study, KCZ and HSV-1ΔgC2-3, had different relative infectivities on the cell lines tested; the relative infectivity of KCZ was noticeably lower than that of HSV-1ΔgC2-3. It is difficult to account for this result. One possibility is that one of these viruses carries a secondary mutation in another viral glycoprotein and that this affects a step in viral infection. Work is underway in our laboratory to investigate this possibility.

The initial aim of this study was to compare HSV-1 infection of novel *EXT1*-expressing cells with control L cells. These results indicate that HSV-1 infection of sog9-*EXT1* cells resembles that of normal L cells. Infection of both *EXT-1* and non-*EXT1* cells was facilitated by HSV-1 gC. Moreover, HSV-1 appears to recognize similar HS features in both cell types. This does not imply, however, that the HS moieties on *EXT1* and control cells are identical, since HPLC data clearly suggests otherwise (McCormick *et al.*, 1998). Rather, HSV-1 has likely evolved the ability to recognize particular polysaccharide sequences which may be present in different types of HS GAGs. Given that HS expressed on the surface of cells may differ with respect to chain length, fine structure and amount produced, it would be of benefit for HSV-1 to be somewhat versatile in its ability to bind HS.

How could the virus adapt to different HS types? Trybala and colleagues (1998) recently addressed this question by analyzing the interaction of PrV gC heparin-binding domain mutants with modified heparin preparations. Their results indicated that different regions of the gC heparin-binding domain may promote PrV binding to different structural features of HS. It remains to be determined whether the same holds true for the heparin-binding region of HSV-1 gC. It does appear, however, that HSV-1 gC and HSV-2 gC have evolved to recognize different HS features. Herold and colleagues (1996) demonstrated that HSV-1 and HSV-2 differed in their susceptibility to modified heparin compounds. O-sulfate groups were determinants primarily for HSV-1 infection. These differences in susceptibility to modified heparin compounds mapped to gC. This suggests that

differences in the interactions of HSV-1 and HSV-2 with cell surface HS may influence tissue tropism. The studies reported here confirm that the specific interaction of HSV-1 with HS is important for an efficient infection of cells by the virus.

## CHAPTER 5: GLYCOPROTEIN B MEDIATES DEXTRAN SULFATE STIMULATION OF HSV-1 INFECTION

### 5.0 INTRODUCTION

Several lines of evidence show that for both HSV-1 and HSV-2, the initial interaction with cells is binding of virus to cell surface HS. This, however, would appear to contradict observations that the two serotypes display differences in epidemiology, cell tropism and susceptibility to inhibitors of viral binding. These results, however, can be rationalized by the observations that i) the relative contributions of viral glycoproteins to viral binding are different (Herold *et al.*, 1994), ii) the glycoproteins of HSV-1 and HSV-2 recognize different structural features of heparan sulfate (Gerber *et al.*, 1995) and iii) HSV-1 and HSV-2 may use distinct secondary receptors to gain entry into the host cell (Geraghty *et al.* 1998).

At the time this work was initiated very little was understood about the different entry pathways used by HSV-1 and HSV-2. Work presented earlier in this thesis on HSV infection of sog9 cells suggested that a particular component of the HSV-1 particle could interact with the GAG analogue dextran sulfate, since HSV-1, but not HSV-2, infection of sog9 cells could be enhanced by the addition of the polyanion. Differences in the ability of HSV-1 and HSV-2 to interact with DS could reflect differences in the adsorption of these viruses to GAG moieties displayed on the host cell surface. The aim of the work presented in this chapter, therefore, was to identify the viral component(s) that could mediate an interaction of HSV-1 with dextran sulfate.

## 5.1 RESULTS

### 5.1.1 Mapping the DS Activation Site on the Virus

In an attempt to map the viral components that facilitate DS stimulation, sog9 cells were treated with DS either before or during inoculation with a panel of intertypic recombinants and deletion mutants (Table 6). Sog9 cell infection with several of the glycoprotein mutants, including, UL10<sup>-</sup> (HSV-1 gM<sup>-</sup>), HSV-1 macroplaque (gC<sup>-</sup>), HSV-1 ANG (gB *syn*) and HSV-1 ANG-path (gB *syn*; base change at amino acid 84 in gD), was enhanced 5- to 14-fold in the presence of DS, a level of stimulation that is less than that of HSV-1 (F), but greater than that observed for HSV-2 (G). These results suggest that: i) gM-1 and gC-1 are not essential for DS stimulation and ii) the HSV-1 particle can functionally interact with DS regardless of *syn* mutations in gB and a base change in gD, the latter alteration conferring neuroinvasive properties to ANG-path. It cannot be concluded, however, that these glycoproteins do not contribute to DS-mediated infection since stimulation by DS is greater when these glycoproteins are intact.

The most significant finding in this series of experiments was that DS was unable to stimulate (less than two-fold) infection of the HSV-1 intertypic recombinant RH1G13, in which gB-1 is replaced by gB-2 (Table 6, Figure 5.1). This indicated that DS stimulation is mediated, at least in part, by gB-1. By contrast, infection with RS1G25, an HSV-1 strain in which gC-1 is replaced by gC-2, was stimulated by DS, although by only five- to sevenfold, significantly lower than the level observed using HSV-1 (F). This implied a role for gC-1 in DS-mediated infection, which was somewhat surprising because HSV-1(KOS) $\Delta$ gC2-3, a gC-deficient HSV-1 strain was stimulated by up to 18-fold (Figure 5.1, Table 6). This is similar to the stimulation observed with wild-type HSV-1(F). One way to account for these results was the possibility that gC-2 is an inhibitor of DS stimulation. I tested this, using a gC-2-deficient strain, HSV-2(G)gC2<sup>-</sup>, and found that it was stimulated by up to eight-fold when sog9 cells were pre-treated with DS. This

<sup>a</sup> (-), stimulation of the prototype HSV-2 strain G, which varied from zero- to fourfold above that of controls in different experiments; (++) fold stimulation of the prototype HSV-1 strain F, which varied from 15- to 35-fold above that of controls in different experiments; (+), stimulation which lies intermediate to those of the two prototype strains.

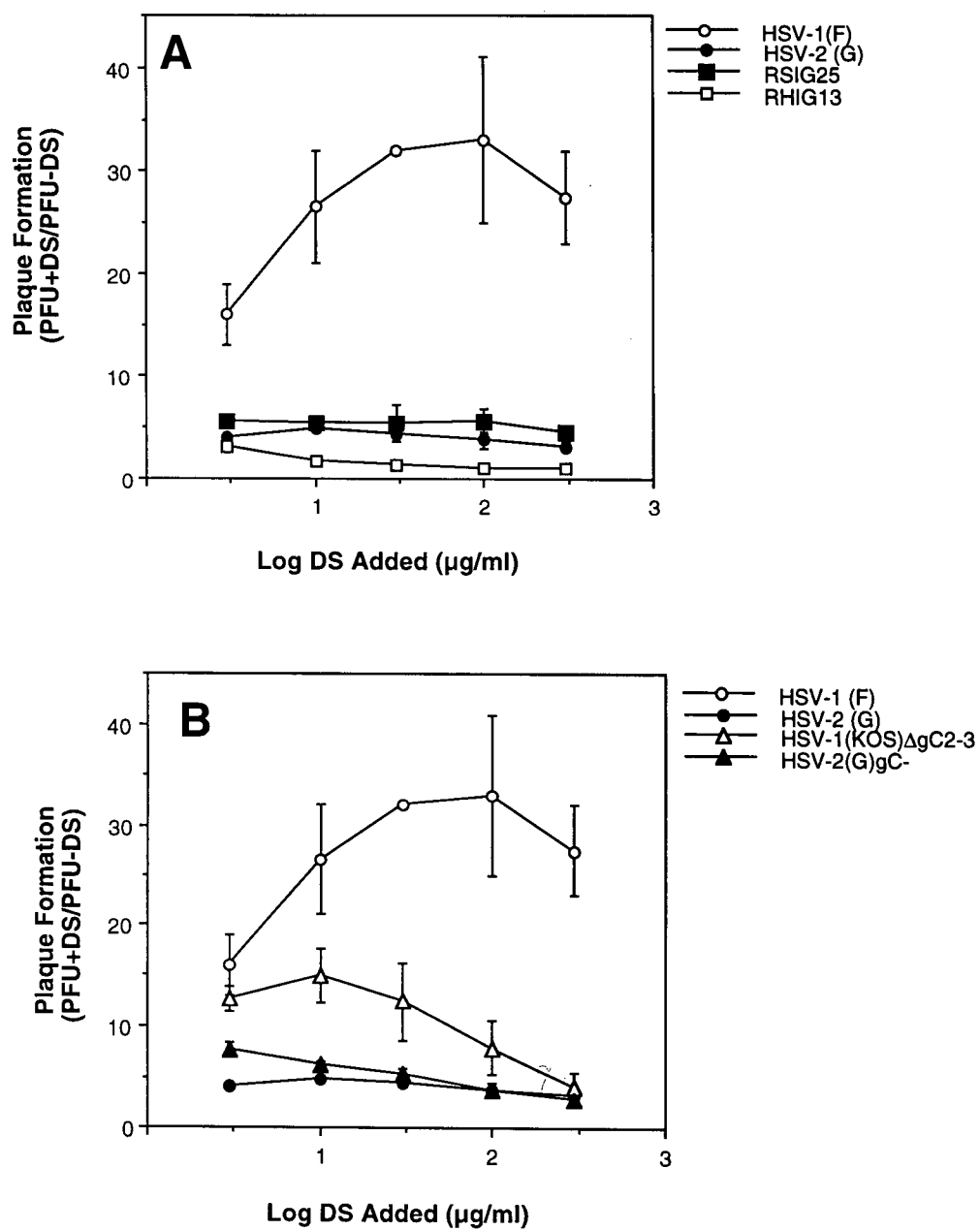
<sup>b</sup> HSV-1 background containing gC-2 in place of gC-1. Other strains of virus are designated in a similar manner.

<sup>c</sup> Data published by Banfield *et al.* 1995, J. Virol 69:3290-3298

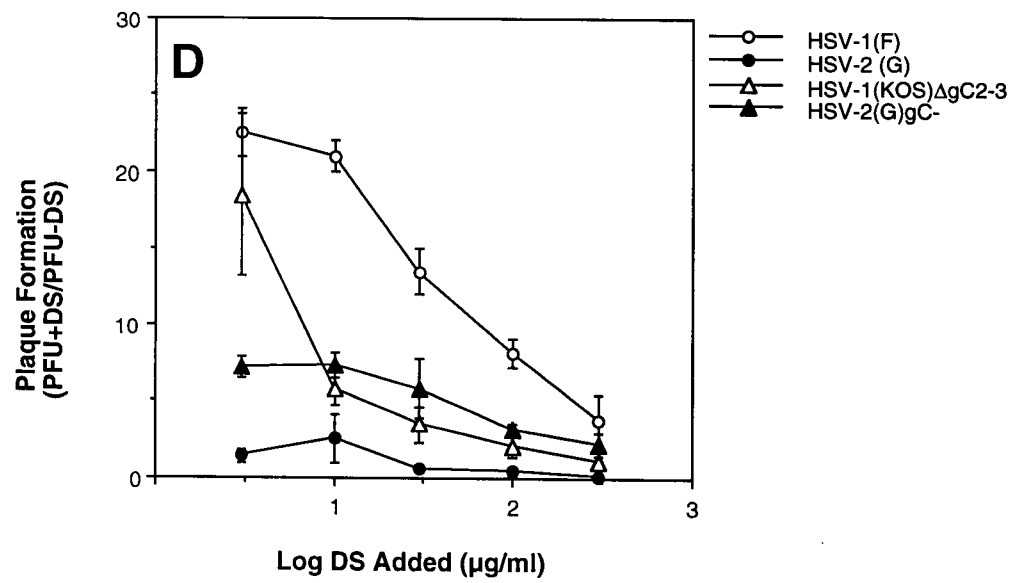
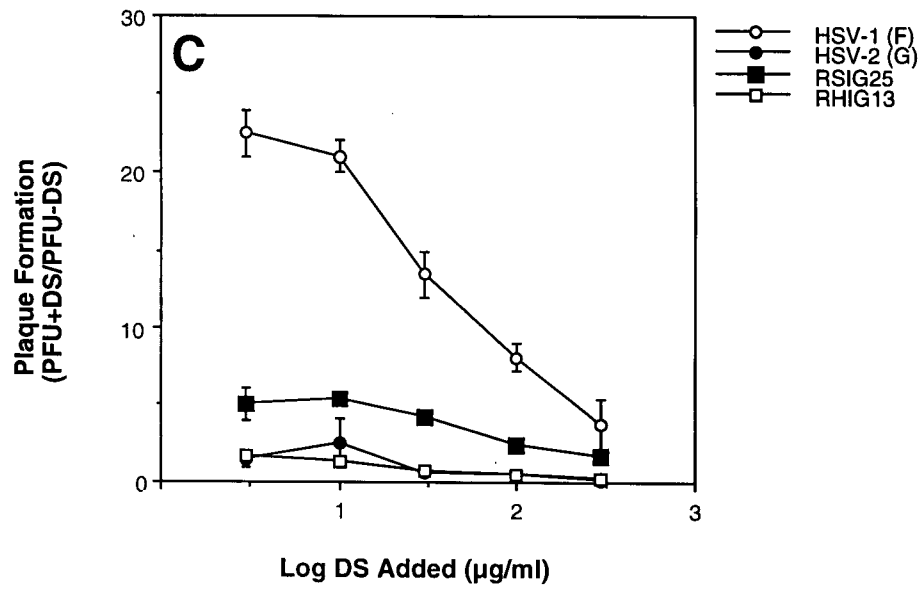
**Table 6.** Effect of dextran sulfate on HSV infection

Virus Strain	Phenotype	Stimulation by DS <sup>a</sup>
HSV-1 (F)	wild type	++
HSV-1 (KOS) <sup>c</sup>	wild type	++
HSV-2 (G)	wild type	-
RSIG25	HSV-1; gC-2 <sup>b</sup>	+
RHIG13	HSV-1; gB-2	-
HSV-1(KOS)ΔgC2-3	HSV-1; gC-1-deficient	++
HSV-2(G)gC2-	HSV-2; gC-2-deficient	+
KCZ	HSV-1(KOS); gC-deficient	++
KgBpK <sup>-</sup>	HSV-1(KOS); gB HBD mutant	++
KgBpK <sup>-</sup> gC <sup>-</sup>	HSV-1(KOS); gB HBD mutant; gC-deficient	++
R7015	HSV-1; gD-2, gE-2, gG-2	++
UL10-	HSV-1; gM-deficient	+
HSV-1(MP)	HSV-1; gC-deficient syncytial strain	+
HSV-1 (ANG)	wild type; gB <i>syn</i> mutation; gB fast rate of entry determinant ( <i>roe</i> )	+
HSV-1 (ANG-path)	HSV-1 ANG derivative; gB <i>syn</i> and <i>roe</i> mutations; neuroinvasive determinant at residue 84 in gD	+

**Figure 5.1.** Effect of soluble DS on herpes simplex virus infection. (A and B) Fold stimulation of HSV plaque formation observed when sog9 cell monolayers were treated with DS prior to infection. Sog9 cells grown in 6-well dishes were incubated at 37°C for 10 min with DS diluted in DMEM. The wells were then rinsed three times with PBS, and the viral inoculum was added. After a 1-h adsorption period at 37°C, the virus was removed and medium containing 0.1% pooled human IgG was added to facilitate plaque formation. (C and D) Fold stimulation of HSV plaque formation when sog9 cell monolayers were inoculated with virus in the presence of various concentrations of DS. Virus was incubated with cells for 1-h at 37° C, after which the inoculum was removed. Medium containing IgG was added to facilitate plaque formation. Data are fold stimulation of plaque formation that occurs in the presence of DS. Values represent the average  $\pm$  the range of two independent experiments.







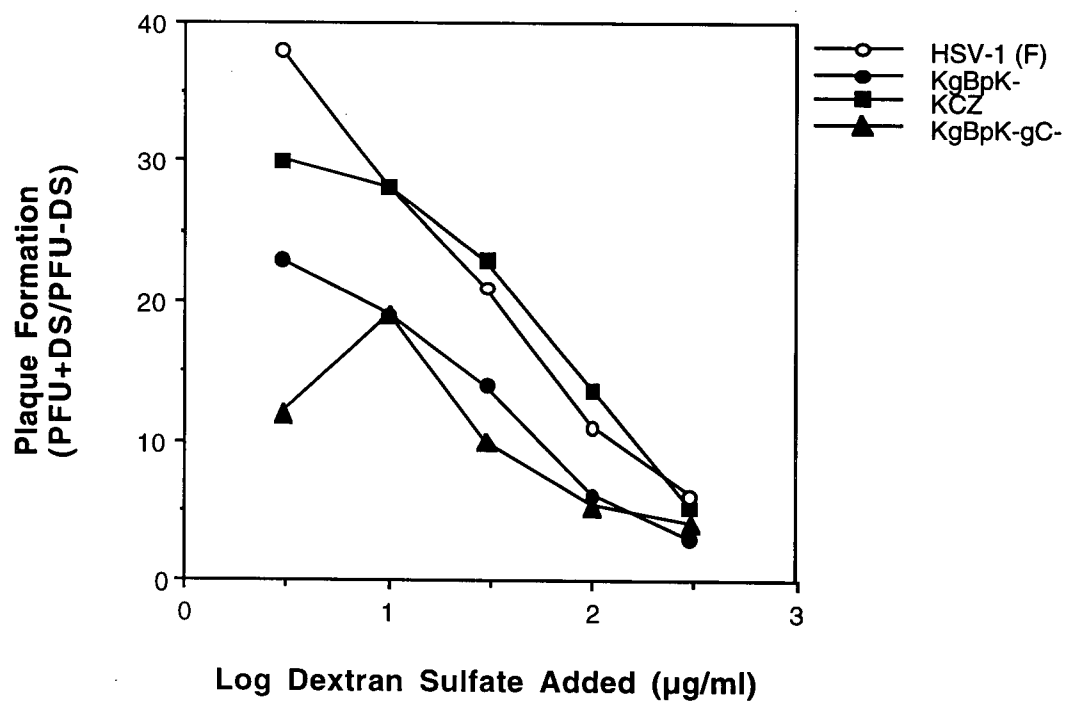
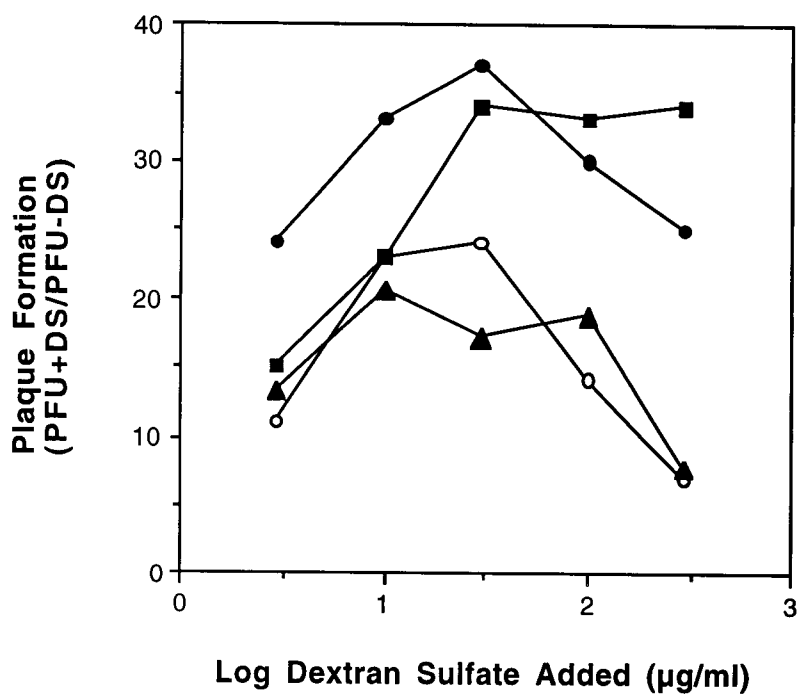
level of infection was substantially better than that for HSV-2. On the basis of these results, it can be concluded that gB-1, in part, facilitates DS stimulation and that gC-2 may be a weak inhibitor of DS-mediated stimulation of infection. This property might account, in part, for the poor DS stimulation observed with RS1G25 and control HSV-2.

### **5.1.2 Contributions of gB-1 and gC-1 Heparin-Binding Domains to the Interaction of HSV-1 with Dextran Sulfate**

The above findings suggest that the interactions of gB-1 and gB-2 with DS are quite distinct from one another. I therefore wanted to identify the domains of gB-1 that mediate DS stimulation of HSV-1 infection, since these regions could be important for interactions of HSV-1 and HSV-2 with host cell GAGs. One candidate domain was the heparan sulfate (HS) binding domain in gB-1 (amino acids 68 to 76) since i) DS could possibly interact with the positively charged lysine residues found in this domain and ii) the HS-binding domain lies within the most divergent region between gB-1 and gB-2. To determine whether or not the HS binding domain of gB-1 was involved in DS-mediated HSV-1 infection, the DS stimulation assay was performed using the virus KgBpK<sup>-</sup>, an HSV-1 virus in which only the gB HS binding domain (pK) has been removed. Laquerre and colleagues (1998b) demonstrated that the pK sequence of gB-1 was solely responsible for the HS binding function of gB. As shown in Figure 5.2, infection of sog9 cells by KgBpK<sup>-</sup> was stimulated by as much as 37-fold in the presence of DS. This clearly indicates that the HS-binding domain of gB is not required for DS-mediated stimulation of infection.

One possibility to account for this observation is that in the absence of the gB HS-binding domain, HSV-1 uses gC to interact with DS. This hypothesis was tested directly using the double HSV-1 mutant, HSV-1gBpK<sup>-</sup>gC<sup>-</sup> in which both the HS binding domain of gB and the entire coding region of gC have been deleted. As a control for the gC deletion, the gC-deficient HSV-1 virus KCZ was also tested. Infection of sog9 cells by

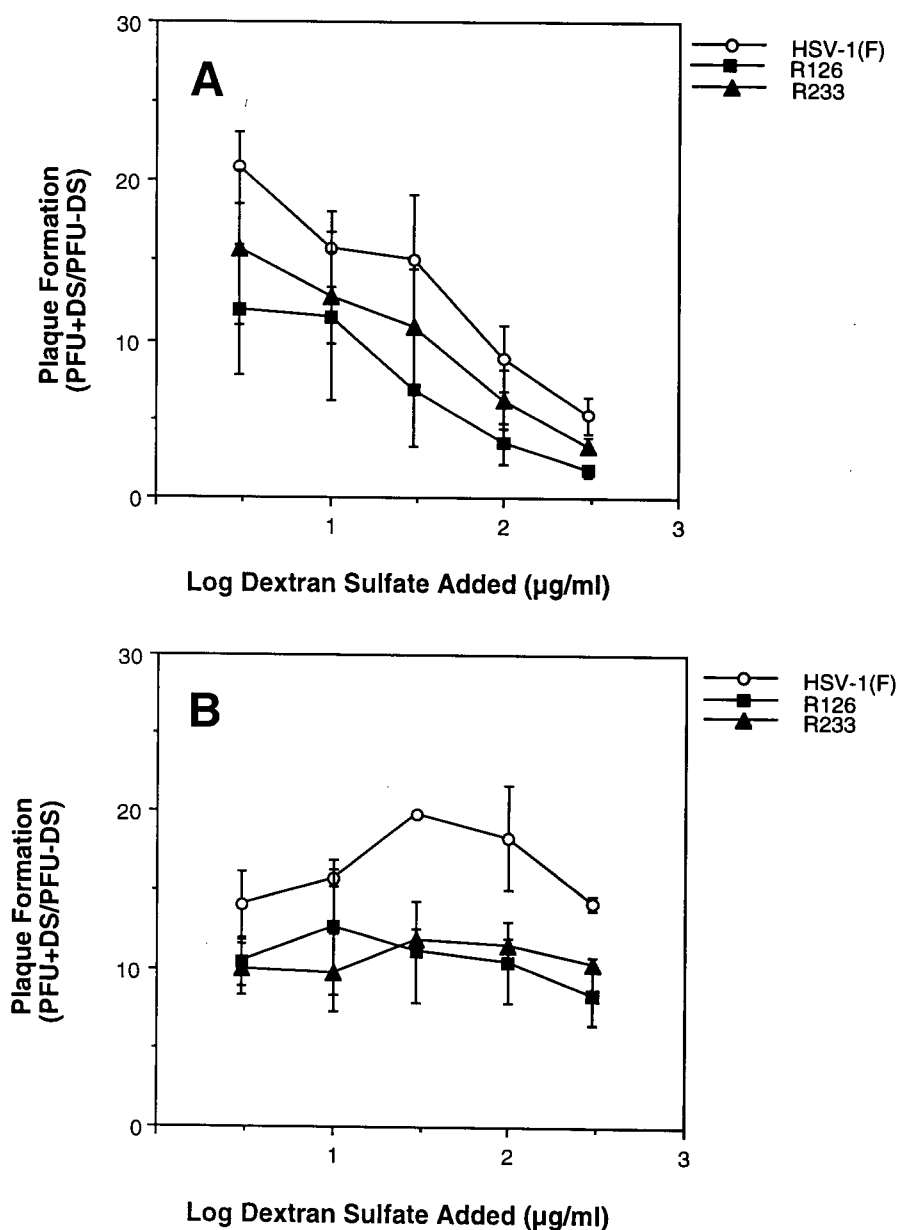
**Figure 5.2** Effect of DS on infection of sog9 cells by HSV-1 heparin-binding mutants. (A) sog9 cell monolayers were inoculated with virus in the presence of various concentrations of DS. (B) sog9 cell monolayers were incubated with DS in DMEM for 10 min at 37°C and washed three times with PBS prior to inoculation with virus. Equivalent concentrations of virus were used for both experiments. After a 1-h adsorption period at 37°C, the inoculum was removed and medium containing 0.1% pooled human IgG was added to the monolayers. Plaques were counted after three days. The results are the ratios of plaques formed on DS-treated monolayers to plaques formed on untreated controls. Two independent experiments were performed with similar results. The results from single experiments are shown.

**A****B**

KCZ and KgBpK<sup>-</sup>gC<sup>-</sup> was stimulated by 35-fold and 20-fold, respectively. Taken together these results provide evidence that gC is not essential for viral interactions with DS.

### 5.1.3 Analysis of DS-mediated Infection Using HSV-1 gB Antigenic Variants

The interaction of gB-1 with DS might be determined by not only the primary sequence of the molecule, but also by its secondary and tertiary structure. To test whether or not DS-mediated HSV-1 infection was sensitive to changes in the secondary structure of gB-1, two HSV-1 gB monoclonal antibody resistant (*mar*) mutants, R126 and R233 were studied. These HSV variants were isolated on the basis of their ability to escape neutralization by various gB-specific monoclonal antibodies (Pellet *et al.*, 1985). Mutant R126 contains three substitutions, i.e., Thr for Ser+283, and Gln for Arg+305 and Asn for Ser+443. Mutant R233 contains a single substitution of Thr+285. These substitutions alter the secondary structure of the protein at both epitopic sites, which are in exposed domains of the gB-1 molecule. The R126 and R233 viruses were either added to cells pre-treated with DS or diluted in DMEM containing DS and then used to inoculate sog9 cells. As shown in Figure 5.3, R126 infection was stimulated by DS although less than that of HSV-1(F) in both experiments. One interpretation of these results could be that the site comprising these residues is involved at some level in recognizing DS. By contrast, DS stimulation of R233 infection was near wild-type levels when virus and DS were added to cells simultaneously. However, R233 infection was somewhat lower than that of HSV-1(F) when sog9 cells were pre-treated with DS. Likewise to R126, the mutation in gB of R233 did not block the ability of DS to stimulate HSV-1 infection. However, because a slightly lower infection efficiency was observed using R233 in one of the experiments, the possibility that this region of gB (comprising residue 285) is involved in mediating interactions with DS cannot be ruled out.



**Figure 5.3.** Effect of DS on infection of sog9 cells by HSV-1 gB *mar* mutants. (A) sog9 cell monolayers were inoculated with virus in the presence of various concentrations of DS. (B) sog9 cell monolayers were incubated with DS in DMEM for 10 min at 37°C and washed three times with PBS prior to inoculation with virus. Equivalent concentrations of virus were used for both experiments. After a 1 h adsorption period at 37°C, the inoculum was removed and medium containing 0.1% pooled human IgG was added to the monolayers. Plaques were counted after three days. The results are the ratios of plaques formed on DS-treated monolayers to plaques formed on untreated controls. Values represent the average  $\pm$  range from two independent experiments.

## 5.2 DISCUSSION

It has been reported recently that HSV-1 and HSV-2 display type-specific differences in their interactions with host cells. These differences include preference for binding to various cell types (Vahlne *et al.*, 1979; Vahlne *et al.*, 1980), binding to sulfated glycosaminoglycans (Herold *et al.*, 1996) and interactions with specific cellular components, such as the C3B receptor and Prr1 (Eisenberg *et al.*, 1987; Friedman *et al.*, 1984; Friedman *et al.*, 1986; Fries *et al.*, 1986; Geraghty *et al.*, 1998). In this study, an additional type-specific phenotype was investigated which involves the interaction of virions with host cell surfaces devoid of GAGs. Infection of sog9 cells by HSV-1, but not HSV-2, could be rescued by the addition of dextran sulfate.

What could account for the failure of DS to stimulate HSV-2 infection? There are several possibilities which must be considered. It is clear that DS stimulation of HSV-1 is mediated, at least in part, by gB-1 and that gB-2 is inactive in this capacity when it is present in an otherwise unperturbed HSV-1 virion (RH1G13; Table 6). This observation is extended by the work of others in our laboratory, who have recently shown that infection of GAG-deficient adult mouse muscle fibers by RH1G13 is unresponsive to the addition of DS (Yeung *et al.*, 1999). The role of gB-1 could be to interact with a distinct cellular receptor accessible to the virion at all times. This interaction is promoted when the virion is held in place even transiently by cell surface DS. If RH1G13, which contains gB-2 in an HSV-1 virion, can still interact with DS, then it is likely that access to the gB-2 receptor is not enhanced in this instance.

gB-1 and gB-2 are highly conserved, with 86% amino acid similarity (Stuve *et al.*, 1987). For the most part, regions that have been shown to have functional significance are conserved, including cysteine residues and predicted glycosylation sites in the external domain (Norton *et al.*, 1998; Stuve *et al.*, 1987). Despite the overall similarity, however, there exist clustered regions of marked divergence between the two proteins. Could the structural differences between gB-1 and gB-2 alter the ability of virus to interact with DS?

Interestingly, and perhaps most relevant, are clustered amino acid substitutions within the N-terminal 85 amino acids of the mature protein and a second region that includes amino acids 451 to 495. The N-terminal divergence includes domains of high positive charge density that could interact with GAGs, including the gB-1 HBD (Laquerre *et al.*, 1998b). Studies of GAG-binding proteins such as gC of HSV-1, BHV-1 and PrV, show that sulfated polyanions are recognized by protein domains that are rich in basic residues, particularly lysine (Flynn and Ryan, 1995; Langeland *et al.*, 1988; Liang *et al.*, 1993; Tal-Singer *et al.*, 1995; Trybala *et al.*, 1994). Moreover, the inhibitory effects of DS on HIV-1 infection appear to be mediated by specific binding of the polyanion to positively charged amino acids concentrated in the V3 loop of the envelope glycoprotein gp120 (Callahan, 1991). I was curious as to whether or not the HBD of gB was involved in mediating DS stimulation. The results show that infection by KgBpK<sup>-</sup>, an HSV-1 particle deleted for the gB HBD, was stimulated as efficiently by DS as wild-type infection. It can therefore be concluded that the HBD of gB-1 is not required for DS-mediated infection. It could be that the binding of DS to proteins is dependent upon specific peptide domains containing suitably positioned cationic residues and in this respect, some site in gB, other than the HBD, may be able to mediate binding to DS.

Using another approach to identify a possible domain of gB that could interact with DS, the effect of DS on sog9 cell infection by two gB-1 *mar* mutants, R126 and R233, was tested. HSV-1 *mar* mutants have been successfully used in past studies to help localize heparan sulfate binding sites in gC (Trybala *et al.*, 1994) and to identify domains of gB that function in viral penetration (Highlander, 1989). Despite significant changes in the secondary structure of gB between N-terminal amino acids 283 to 305, DS was still able to stimulate infection of both R126 and R233, implying that this region of the molecule may not be critical for mediating an interaction of the virus with DS. Nevertheless, the level of DS stimulation of R126, and in one experiment with R233, was lower than that of wild-type HSV-1. Therefore, one cannot exclude the possibility that this region assists in mediating the interaction. Perhaps this region is only one of several



peptide domains on gB that help stabilize the interaction with DS. It is interesting that for several other GAG-binding proteins, such as gC, the consensus sequence elements are not close together in the primary sequence and only by folding of the protein structure, do these regions come together to form a crevice into which the GAG binds (Cardin and Weintraub, 1989; Trybala *et al.*, 1994).

The molecular recognition of GAGs by proteins is clearly a complex process. Therefore, other approaches will need to be used to elucidate the role of gB-1 in DS-mediated infection. Future experiments could include an assessment of the ability of soluble gB as well as truncated forms of the protein to bind to DS. The construction and characterization of viruses bearing gB-1/gB-2 chimaeric proteins could also help identify key sites of the protein. Nevertheless, the results presented here support the concept that gB-1 and gB-2 may differ in their ability to adsorb to sulfated polyanions such as DS or to recognize a cellular receptor. Differences in gB-1 and gB-2 can also be inferred from studies showing that gC-deficient HSV-2 exhibits no loss in specific binding activity, specific infectivity, or rate of viral penetration (Gerber *et al.*, 1995). This is very different from studies on gC-deficient HSV-1, which showed a serious impairment in virus adsorption. It may be that gC-1 and gB-2 predominate in their respective viruses to regulate the early interactions that lead to a productive infection.

It was also interesting to discover that gC-2 reduces DS-mediated infection. In this instance the presence of gC-2 may interfere with the binding of the virion to DS at the cell surface. Alternatively, gC-2 may directly impede the interaction of gB-2 with DS. These experiments using a gC<sup>-</sup> virus support the possibility that gC-2 is responsible for at least part of the unresponsiveness of the HSV-2 virion to DS stimulation. It is important to consider that the HSV-2 virion does show some level of DS stimulation in the absence of gC-2, thereby indicating that HSV-2 can engage the DS-mediated pathway. There is no evidence, however, that gB-2 mediates this process in HSV-2.

## CHAPTER 6: SUMMARY

The HSV entry pathway normally involves viral attachment to cell surface HS and CS GAGs (Sheih *et al.*, 1992; Banfield *et al.*, 1995a). This interaction is mediated by the heparin-binding proteins gB and gC in the virion envelope (Herold *et al.* 1991, Laquerre *et al.*, 1998). These glycoproteins contain heparin-binding regions that recognize and bind to particular structural features of GAG moieties (Herold *et al.*, 1995; Tal-Singer *et al.*, 1995; Feyzi *et al.*, 1997; Laquerre *et al.*, 1998). The binding of virus to GAG receptors is a critical component of the HSV entry pathway as it i) concentrates the virus at the cell surface and ii) brings the virus in proximity with its co-receptor. HSV co-receptors have recently been identified as HveA, a member of the TNF receptor family, and HveB, HveC and HlgR, members of the Ig superfamily (Montgomery *et al.*, 1996; Cocchi *et al.*, 1998b; Geraghty *et al.*, 1998). Significantly, different HSV-1 and HSV-2 strains vary in their ability to use these co-receptors. This likely influences the spread of virus in the human host. Attachment of the HSV to its co-receptor primes the virus for penetration into the cell, which involves the fusion of the virion envelope with the cell plasma membrane and results in the release of the viral capsid into the cell cytoplasm. The process is not yet fully understood, although appears to be regulated by the combined activities of gB, gD, gH and gL (Handler *et al.* 1996b).

In this study, I uncovered a functional difference between HSV-1 and HSV-2 in their ability to use dextran sulfate as a surrogate receptor on glycosaminoglycan-deficient sog9 cells. The absence of glycosaminoglycans on the sog9 cell surface reduces the adsorption of HSV in a manner that allows for a more sensitive readout of the virus-host interactions that ensue during the infection process. The significance of this study is that DS stimulates HSV-1 infection of sog9 cells, thereby acting as an artificial receptor to initiate a productive infection. By contrast with previous experiments in which HSV-1 and HSV-2 were differentiated by their susceptibility to inhibitors, I have identified an

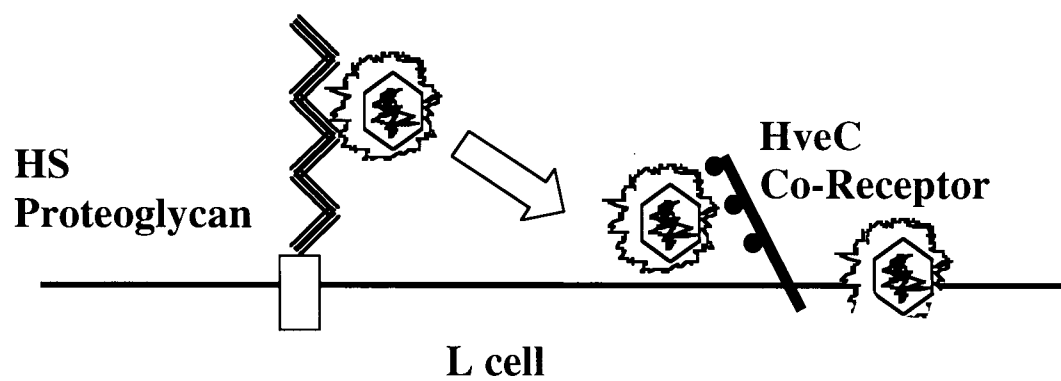
interaction that promotes infection. This distinction is significant; whereas inhibition of infection could be caused by relatively non-specific blocking, stimulation of infection requires that specific host-virus interactions be maintained.

A model for DS stimulation of HSV-1 infection of sog9 cells is illustrated in Figure 6.1. In Chapter 3, it was demonstrated that sog9 cells can be treated with DS prior to infection, that DS interacts with virions and that DS stimulates infection at 4°C. This supports a model in which DS binds to sog9 cells and tethers the virion at the cell surface. Possible receptors for DS on sog9 cells include scavenger receptors, which are capable of binding a variety of polyanions (Krieger *et al.* 1993). Using intertypic recombinants, it was demonstrated that HSV-1 interacted with DS, in part, via glycoprotein B. This interaction of virus with DS likely serves to concentrate HSV-1 virions at the cell surface, much the same way as the virus' natural receptor, heparan sulfate, does. In this manner, HSV-1 is brought into proximity with a second, downstream cell surface receptor, such as HveC. Once the virus engages this receptor, viral penetration can ensue. In the absence of DS, however, the probability of HSV-1 engaging its co-receptor is reduced, which in turn decreases productive infection.

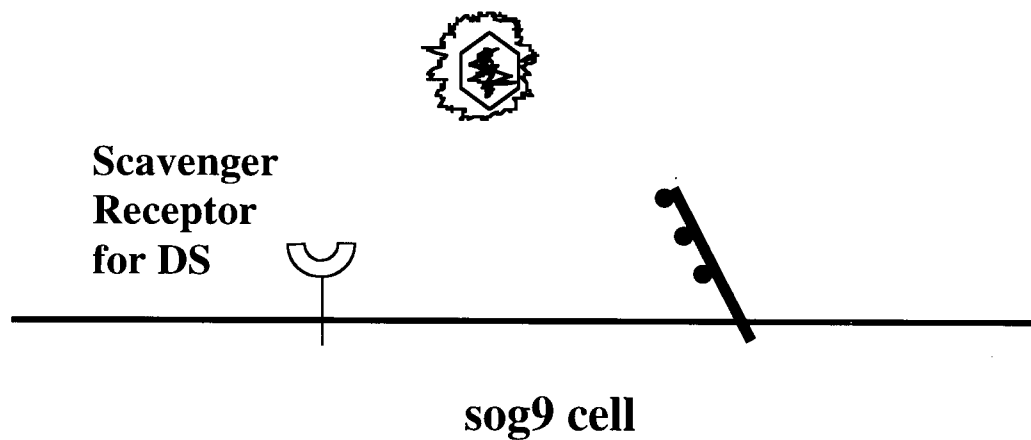
The striking differences in the behaviour of HSV-1 and HSV-2 in the DS assay are most likely defined by differences in the propensity of principally gB to interact with sulfated polyanions, as well as perhaps additional cell surface receptors. It will be interesting to take advantage of the differences in susceptibility conferred by DS to identify and characterize the domains of gB that mediate this effect. These domains are likely to be important in conferring type-specific properties on the respective virions. Results presented in this thesis show that the heparin-binding region of gB-1 is not required for mediating an interaction of HSV-1 with DS. The interactions of proteins with glycosaminoglycans are defined by several parameters and are not simply electrostatic in nature. It seems likely then that there may be another domain of gB-1 comprising a specific sequence and spatial arrangement of amino acid residues with which DS can interact.

**Figure 6.1** Proposed model for dextran sulfate stimulation of HSV-1 infection of sog9 cells. (A) Normal infection (e.g. L cell infection) involves the binding of virus to cell surface HS and/or CS proteoglycans. This facilitates a downstream interaction with a co-receptor, such as HveC. Stable attachment of HSV with its co-receptor promotes penetration of the virus into cells. (B) HSV infection of sog9 cells. In the absence of cell surface GAGs, the ability of HSV to engage its co-receptor is reduced, resulting in decreased infection efficiency. (C) HSV-1 infection of sog9 cells in the presence of soluble dextran sulfate. DS binds to the sog9 cells surface, possibly to scavenger receptors. DS functions to tether the virus at the cell surface. This interaction is mediated, in part, by HSV-1 gB HSV-1 and facilitates attachment of the virus with its co-receptor, resulting in a productive infection.

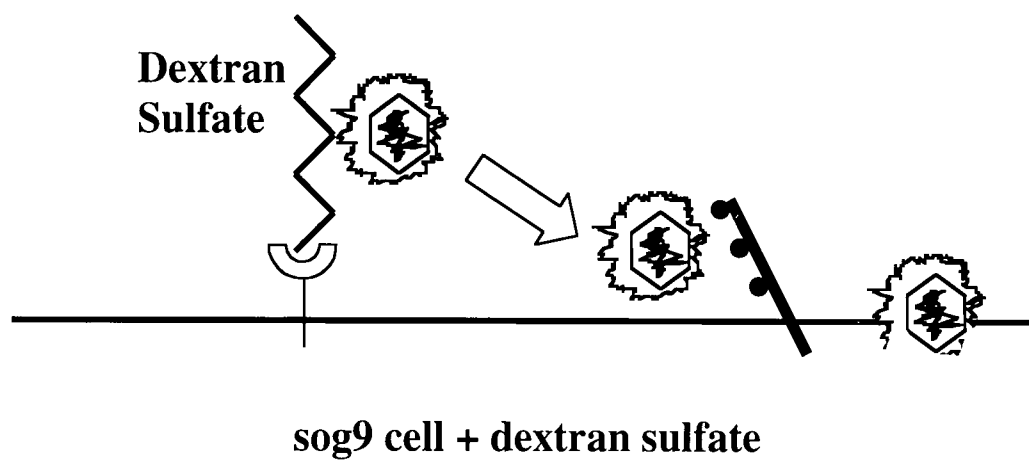
**A**



**B**



**C**



By contrast to DS-mediated infection, HSV-1 infection of cell lines expressing heparan sulfate, including the novel *EXT1*-expressing cell lines, is mediated principally by gC-1. Moreover, the HBD of gB-1 appears to play a minimal role in binding of virus to heparan sulfate GAGs. Using chemically modified heparin compounds, I determined that the virion recognizes particular structural features of HS, including, 2-O-, 6-O- and N-sulfate groups. These findings further suggest that the cell and tissue tropism of HSV may, in part, be due to the structural variations of HS.

The recent advances in identifying factors governing HSV-GAG interactions, such as those described herein, have made a substantial contribution to our understanding of the HSV attachment process. This in turn has had a significant impact on other areas of HSV research. For example, Laquerre and colleagues (1998a) altered the tropism of HSV-1 by replacing the HS binding domain of gC-1 with erythropoietin (EPO). This engineered virus, but not wild-type virus, could bind to cells expressing the EPO receptor, and as such is the first evidence that HSV-1 attachment can be targeted to non-HSV cell surface receptors. The ability to alter host range of the virus can be exploited for HSV-based gene therapy purposes. This is underscored by work in our laboratory demonstrating that *in vivo*, DS can mediate HSV-1 infection of adult mouse myofibres, cells which are normally refractory to infection due to their lack of HS GAGs (Yeung *et al.*, 1999). Skeletal muscle is an ideal seeding site for the treatment of a variety of disorders, including muscular dystrophy. Therefore, DS-mediated infection represents an approach for expression of HSV vectors in muscle fibers.

On a final note, this study highlights the complex, multi-faceted nature of the interaction of HSV with host cell glycosaminoglycans. Clearly, both viral and host cell components have their own unique parts to play in this exchange. By continuing to identify the finer aspects of this interaction, the role of HSV attachment in determining HSV epidemiology can be fully defined.

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## APPENDIX I: COSMID AND BAC TECHNOLOGY FOR GENERATING HSV RECOMBINANTS

### 7.0 INTRODUCTION

Much of our understanding of the biology and molecular biology of the two HSV serotypes has come largely from studies of HSV-1. It is becoming increasingly evident, however, that HSV-1 and HSV-2 are quite different from one another and that more research on HSV-2, in particular, is required. Studies on HSV-2 are somewhat hindered, though, because of a general scarcity of HSV-2 mutants and unfortunately, the process of constructing HSV recombinants is complex and often lengthy. The large viral genome of 152 kb is just one factor that complicates the manipulation of viral DNA. Conventional methods for construction of viral recombinants have involved co-transfection of cells with intact viral DNA and a plasmid which has been modified by insertion of a marker. Mutants arise by recombination and are isolated from wild-type virus by virtue of expression of the marker. Two new technologies have been developed which may expedite and simplify the construction of HSV-1 mutants. The first is a cosmid-based system, which is centered around a set of cosmids whose inserts overlap and represent the entire HSV-1 genome. When co-transfected into cells, viral plaques are produced via recombination between overlapping DNA fragments (Cunningham and Davison, 1993). Mutants are constructed by replacing a wild-type cosmid with a specifically mutated derivative. The second method relies on bacterial artificial chromosome (BAC) technology to clone the entire HSV-1 genome as a single molecule (Messerle *et al.*, 1997; Horsburgh *et al.*, 1999). Viral recombinants are generated by using bacterial genetics to mutate the BAC-cloned virus genome.

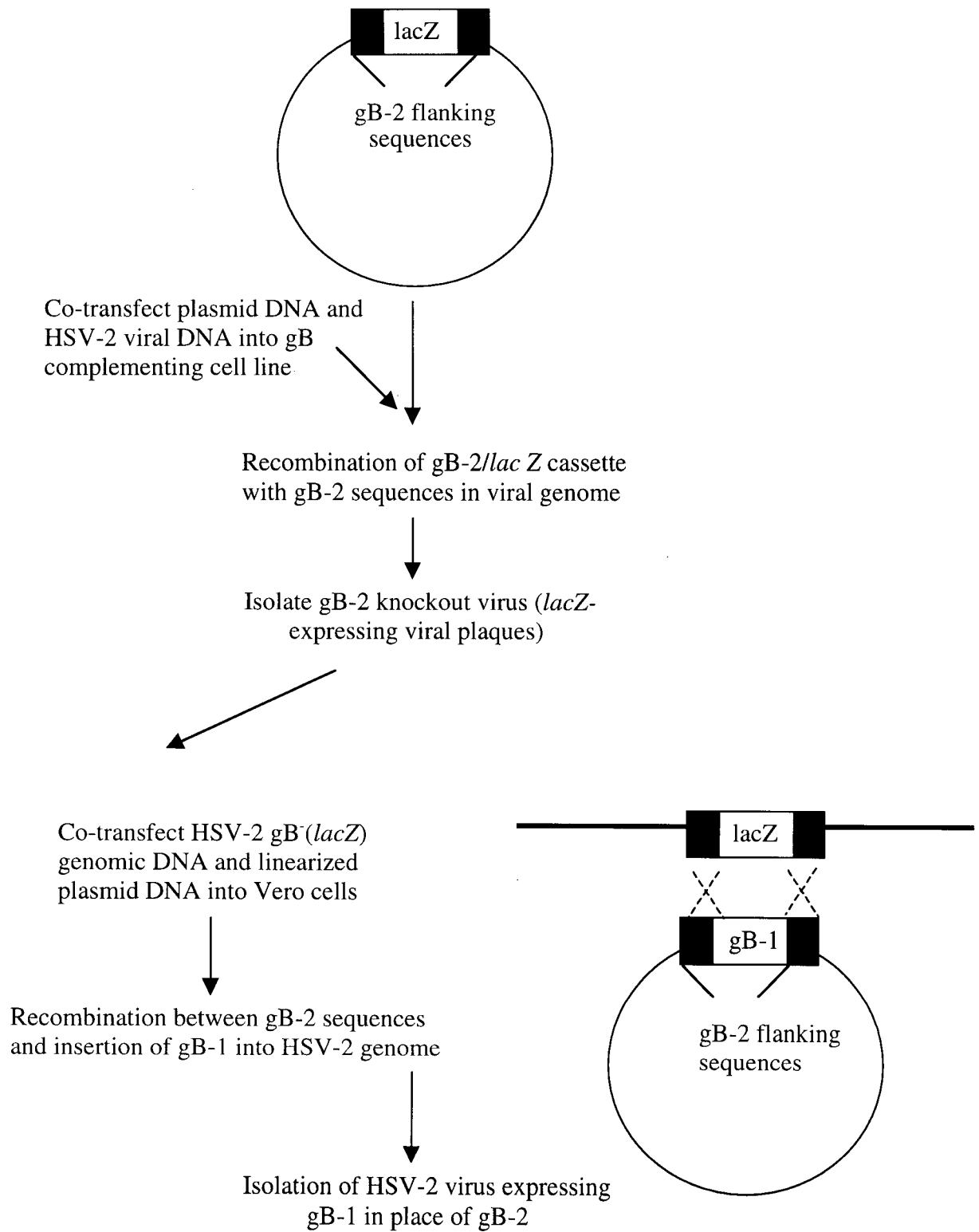


Given that the heparin-binding domain of gB was not required for DS stimulation, I wanted to determine which other domains of gB could be important for mediating this effect. This would require the generation of a variety of HSV gB mutants. One recombinant that I wanted to generate was an HSV-2 particle expressing gB-1 in place of gB-2. This intertypic virus could then be used to test whether or not expression of gB-1 could restore DS stimulation of HSV-2. As evident from the work presented in this chapter, it is often difficult to construct HSV gB mutants because gB is essential for viral replication. In light of this, we wanted to explore the possibility of developing a reagent, such as an HSV-2 cosmid set or an HSV-2 BAC, that would make the HSV-2 genome more accessible to mutagenesis. For various reasons, construction of both the HSV-2 cosmid set and BAC proved troublesome. The work described herein addresses particular problems that can arise when manipulating the HSV genome. A discussion of some of the advantages and disadvantages of the various systems used to construct HSV recombinants is included.

## 7.1 RESULTS

### 7.1.1 Construction of HSV-2 gB2(*lacZ*) virus

To confirm further the role of gB-1 in mediating DS stimulation, I wanted to construct and later characterize an HSV-2 intertypic recombinant expressing gB-1 in place of gB-2. Figure 7.1 illustrates the plasmid-based strategy that was used for the construction of this mutant. The plasmid pHS208-*lacZ*, which encodes the *E. coli lacZ* gene flanked by gB-2 sequences, was linearized and co-transfected into gB-1 complementing D6 cells with HSV-2 genomic DNA. Recombination between homologous sequences in pHS208-*lacZ* and in the viral DNA would generate virus deficient in gB-2 and expressing *lacZ*. To select for recombinant virus, infected monolayers were stained with buffer containing X-gal. The appearance of blue-staining plaques indicated that the desired HSV-2 gB knockout virus had likely been constructed.



**Figure 7.1.** Strategy for construction of an HSV-2 intertypic recombinant expressing gB-1 in place of gB-2 using a traditional plasmid-based strategy.

This was also supported by the observation that *lacZ*-expressing plaques were not observed when non-complementing cell lines were infected with virus. The next step was to plaque purify the HSV-2 gB<sup>-</sup> (*lacZ*) virus. Several attempts to do so, however, failed, simply because the size of the plaques produced by the mutant were too small to pick, i.e. after 3 days post-infection, the number of blue-staining infected cells in a given plaque ranged from 5-20. By comparison, wild-type HSV can be expected to produce plaques containing approximately 100 - 200 infected cells. When plaques formed by the HSV-2 gB<sup>-</sup> (*lacZ*) virus were picked, wild-type HSV-2 virus was always isolated instead. In an attempt to produce larger HSV-2 gB<sup>-</sup> (*lacZ*) plaques, infection was allowed to proceed beyond the standard three days. This was to little avail, however, since wild-type HSV-2 infection proceeded at a faster rate than the mutant, resulting in any uninfected cells being infected by wild-type HSV-2.

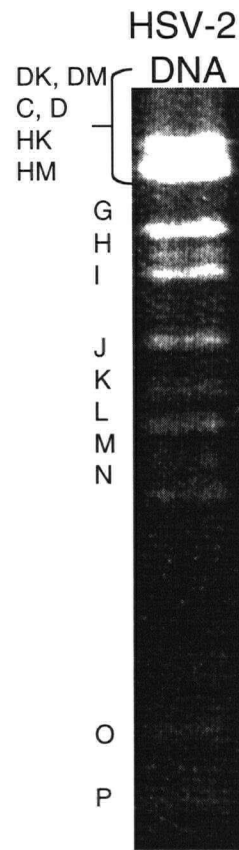
I was unable to plaque purify the HSV-2 gB<sup>-</sup> (*lacZ*) virus and could not, therefore, generate the HSV-2/gB-1 intertypic virus according to the strategy originally proposed. The difficulties encountered using a plasmid-based method for constructing the HSV-2 mutant underscored the need to develop a system that would facilitate the manipulation of the HSV-2 genome. For this reason, attempts were made to develop an infectious HSV-2 cosmid set.

### **7.1.2 Cosmid Technology for Generating HSV Mutants: Construction and Characterization of an HSV-2 Cosmid Library**

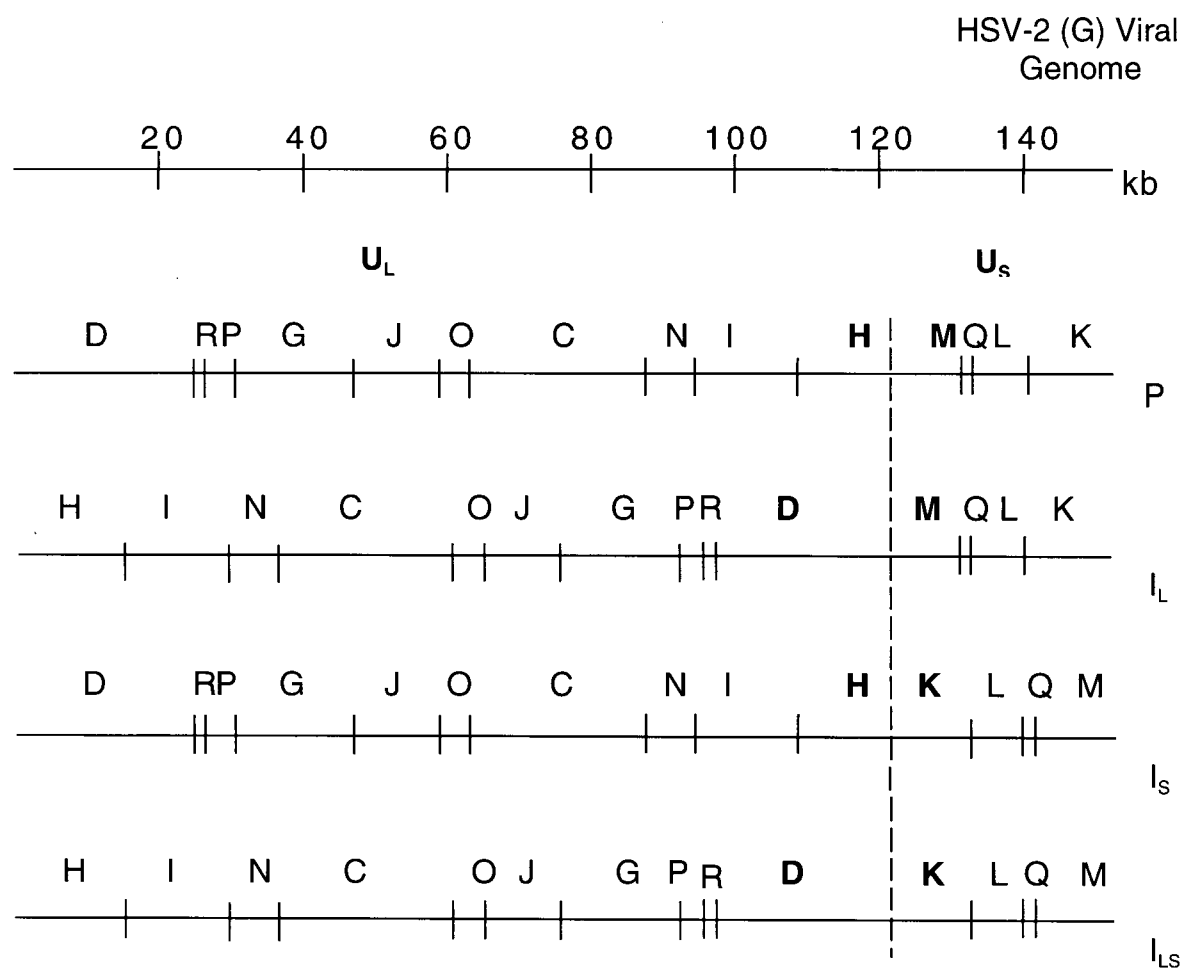
To develop a cosmid-based system for generating HSV-2 mutants, an HSV-2 cosmid library was first constructed. In characterizing the cosmid clones in the library, Bgl II restriction analysis was used to approximate the locations of viral cosmid inserts with respect to the HSV-2 genome. Bgl II was chosen for restriction analysis because compared to other enzymes, Bgl II digestion of wild-type HSV-2 DNA yielded a banding pattern that was the most effective for mapping purposes. The Bgl II restriction profile

for HSV-2(G) is shown in Figure 7.2. The Bgl II H, K, and M fragments are of lower intensity than the other fragments in the Bgl II HSV-2 restriction profile due to recombination in the repeat regions of the HSV-2 genome. This recombination generates the four isomeric forms of the HSV-2 genome, the Bgl II restriction maps of which are shown in Figure 7.3. Approximately 200 cosmid clones were analyzed and as one might expect, viral fragments from the different isomeric forms of the genome were found. For simplicity, I studied cosmid clones in which viral inserts were of the prototype form. The results from Bgl II restriction analysis are shown in Figure 7.4. The generation of a functional cosmid set required cosmids to contain viral DNA that was all of the prototype form, with the exception of one cosmid which contained the Bgl II DK fragment found in the  $I_L I_S$  form of the genome. The latter cosmid was required in order to provide sequences that could overlap with DNA at the ends of the genome.

To confirm that viral inserts overlapped with one another, the ends of selected cosmids were sequenced. The results from sequencing analysis are presented in Figure 7.5. These results are, however, obviously incomplete, in that for the majority of cosmids studied, only one end of the viral insert was sequenced. The reason for this is that by this point in the study, I was aware that there were inherent problems with the HSV-2 cosmid library and the method by which I was characterizing it. First, it was extremely difficult by Bgl II restriction analysis to distinguish between the sequences found in the repeat regions of the HSV-2 genome and, moreover, to determine from what isomeric form of the genome they were derived. As shown in Figure 7.3, the HM (23 kb) and HK (24 kb) fragments, found in the prototype and  $I_S$  (inverted short) isomers respectively, are of similar size. Likewise, it was difficult to distinguish between the DM (32 kb) and DK (33 kb) fragments found in the  $I_L$  (inverted long) and  $I_L I_S$  isomeric forms, respectively. Thus, it was only when the ends of cosmids were later sequenced, that the viral inserts could be accurately mapped. Indeed, sequence analysis of cosmids pF2-35 and pF-98 (Fig. 7.5), demonstrated that the viral inserts did not contain the DK fragment, as

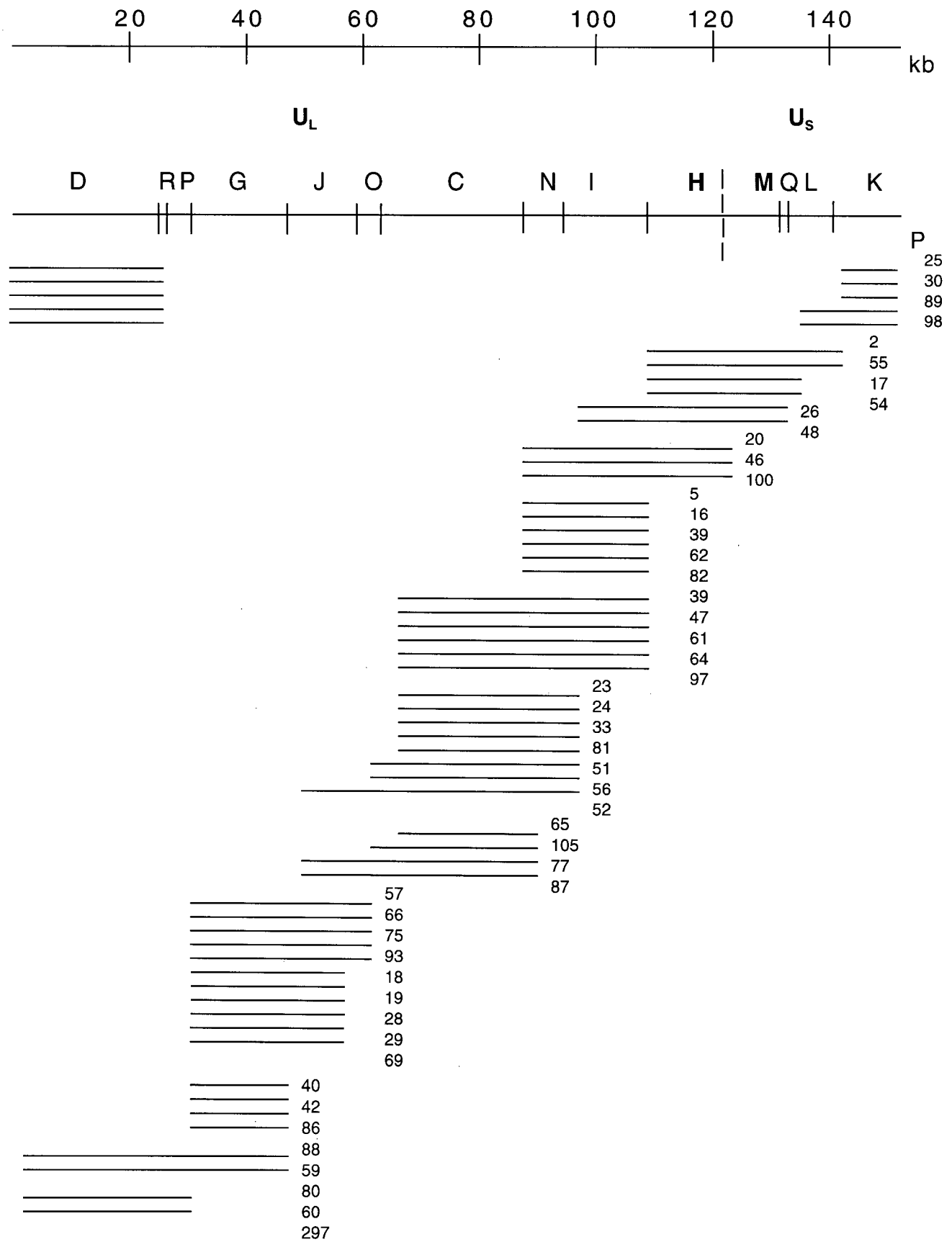


**Figure 7.2.** Bgl II restriction profile of HSV-2 genomic DNA. HSV-2 genomic DNA was digested with Bgl II restriction enzyme overnight and the products were analyzed on a 0.5% agarose gel stained with ethidium bromide. Each viral DNA fragment is designated by a letter. For the corresponding HSV-2 Bgl II restriction maps, see Figure 6.3.



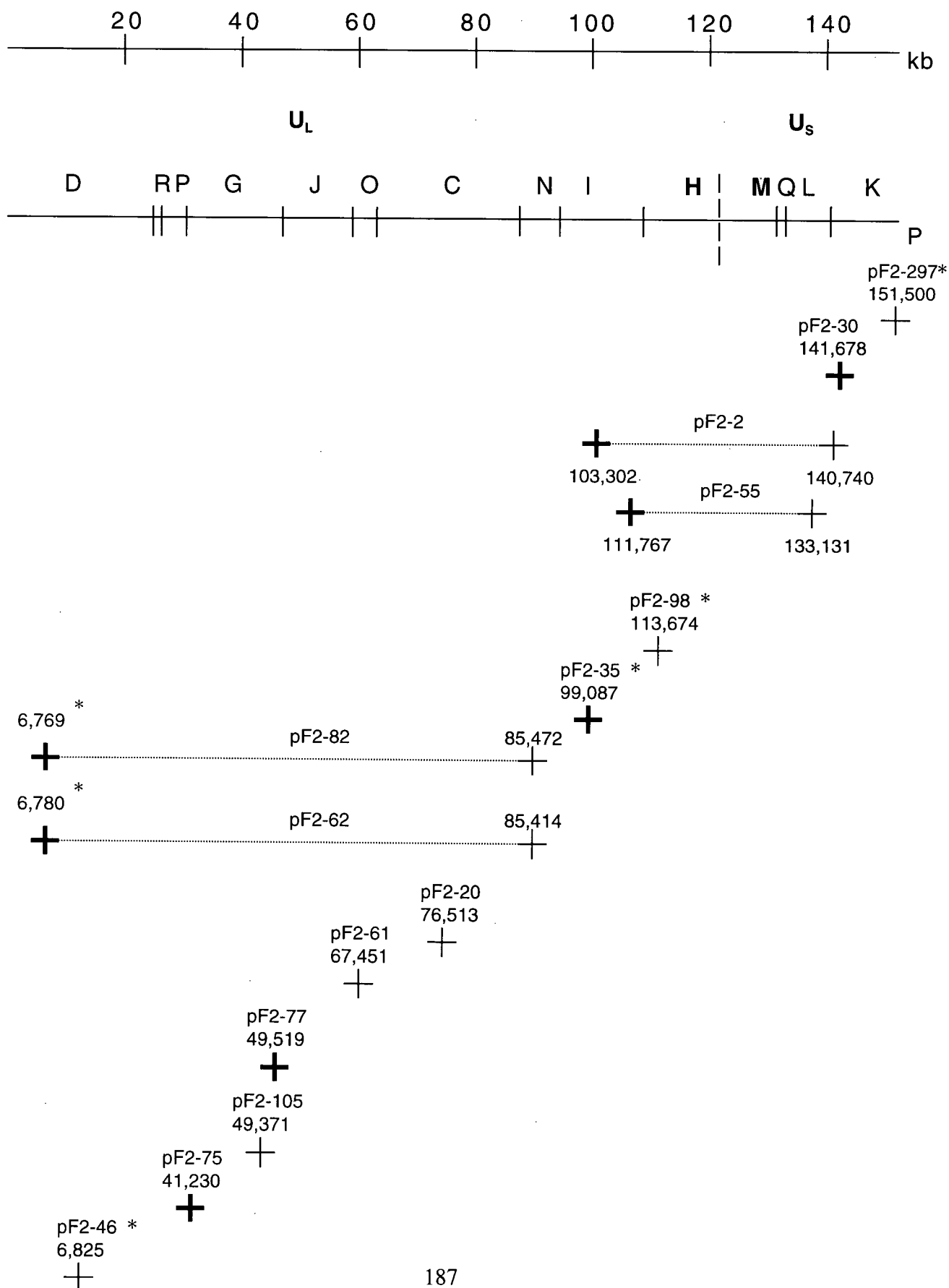
**Figure 7.3** Bgl II restriction maps for all four isomeric forms of HSV-2 (G) genomic DNA. The dashed line indicates the point of genomic inversion. (U<sub>L</sub>) unique long sequence, (U<sub>S</sub>) unique short sequence, (P) prototype form of the genome, (I<sub>L</sub>) inverted long sequence, (I<sub>S</sub>) inverted short sequence, (I<sub>LS</sub>) inverted long and short sequences.

**Figure 7.4** Bgl II restriction analysis of HSV-2(G) cosmid clones. HSV-2 cosmids were digested with Bgl II restriction enzyme and the digestion products were analyzed by agarose gel electrophoresis and mapped accordingly. Viral cosmid inserts mapping to the prototype position of the viral genome are shown here. The different cosmid clones are designated by a number.





**Figure 7.5** Mapping the HSV-2 cosmid by sequence analysis. Each cosmid is designated by pF-2, followed by a number. Only the ends of viral inserts were sequenced. Bold crosses indicate that the sequence was obtained using the forward sequencing primer; regular crosses indicate that the sequence was obtained using the reverse primer. Asterisks indicate a discrepancy between the sequencing results and data obtained from Bgl II restriction analysis. See text for details.



was suggested by restriction analysis (Fig.7.4). This particular problem was compounded by the fact that cosmids encoding the DK fragment would be under-represented in the library to begin with, simply due to the recombinogenic nature of the viral genome. Restriction analysis also proved inefficient for identifying cosmids encoding the Bgl II D fragment (24 kb), since this fragment was also of similar size to fragments encoding the aforementioned repeat regions (HM, HK, DM and DK). Thus, cosmid pF2-297, which by restriction analysis contained the Bgl II D fragment (Fig. 7.4 ), was by sequence analysis, shown to have one end of its viral insert localized to the Bgl K fragment (Fig. 7.5) In this instance, the Bgl II D fragment may have been mistaken for either the Bgl II HK or DK fragment during restriction analysis.

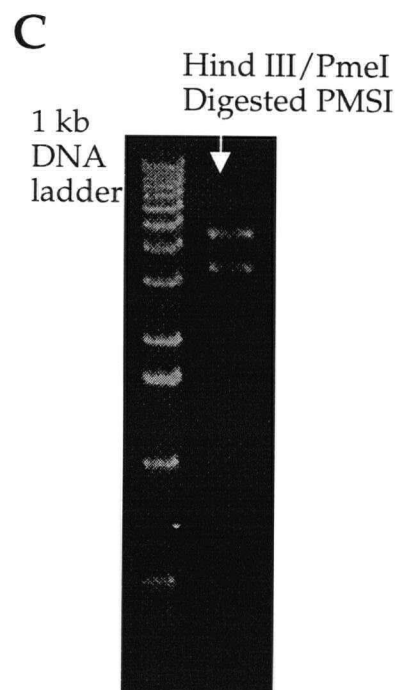
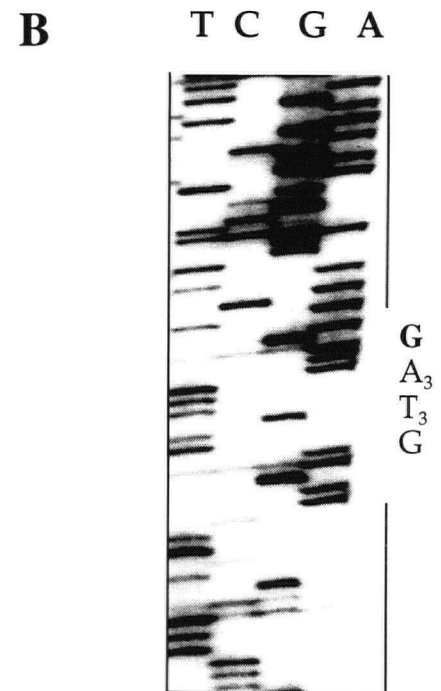
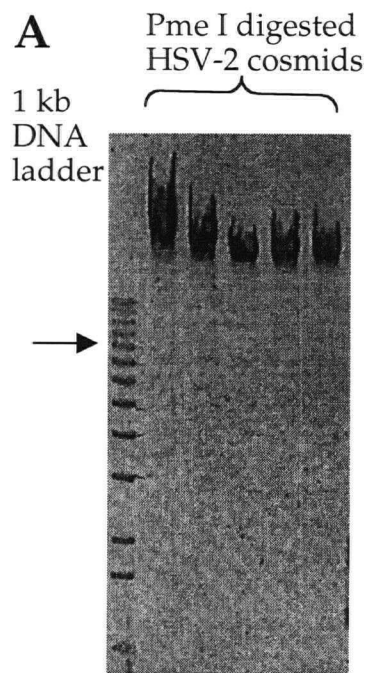
In addition to problems in identifying cosmids containing the Bgl II D and DK fragments, difficulties were also encountered with cosmids mapping to Bgl II fragments N, I and H. For example, restriction analysis clearly indicated that the cosmids pF2-46, pF2-62 and pF2-82, contained viral DNA mapping to Bgl II fragments N, I and/or H. (Fig.7.4) Sequencing the ends of these cosmids, however, suggested that these clones contained DNA mapping between, and partly including, the Bgl II D and N fragments (Fig.7.5). This is a confusing result since the Bgl II G, J, O and C, fragments were clearly not observed when these cosmids are analyzed by restriction digestion. Moreover, this observation implied that these cosmids possessed viral DNA inserts of up to 70kb! Viral fragments of only 40 kb were inserted into the cosmid vector to begin with, and this is the upper limit of what lambda phage can package (C. van Sant, personal communication.) One way to account for this result is if viral sequences in this region (Bgl fragments N, I, and H) are particularly recombinogenic. In this manner, the cosmids may have acquired part of the Bgl II D fragment. This would likely not be detected by restriction analysis but would be detected when the cosmid ends were sequenced.

In the cosmid system, a virus genome is reconstructed via homologous recombination between the overlapping sequences in the different clones. It is worthy to note, however, that the cosmid vector backbones are excised prior to co-transfection of

the clones into cells. The presence of the cosmid backbone could interfere with recombination between overlapping viral sequences. For this reason, the cosmid cloning vector used in this study, PMSI, contained two Pme I sites which when cleaved, would release the viral DNA from the cosmid vector (Fig. 2.1). When HSV-2 cosmids from the library were digested with PMSI, I expected to observe two bands by gel electrophoresis: a high molecular weight band representing the viral DNA insert and an 8 kb band representing the cosmid vector. The smaller band representing the cosmid backbone was not observed (Fig. 7.6A). One possibility to account for this was if either one or both of the Pme I sites was not functioning. Sequence analysis using the 5' sequencing primer demonstrated that for one of the sites, the sequence was 5'-GTTTAAAG-3', instead of having the correct Pme I recognition sequence of 5'-GTTTAAAC-3' (Fig. 7.6B). I could show that the other Pme I site in the vector was functional by double digesting PMSI with Hind III (which cut at only one site) and Pme I. This yielded the two expected fragments of sizes 3.3 and 4.5 kb (Fig 7.6C). Since one Pme I site was functional, attempts were made to identify a second restriction site in the PMSI vector that could be used to release viral inserts from the vector. Unfortunately, there were no restriction enzyme sites in the vector which would also not digest the viral DNA insert (data not shown). Thus, viral inserts could not be released from the cosmid vector.

For the reasons addressed above, I was unable to generate a set of HSV-2 cosmids which, when co-transfected into cells, would yield infectious virus. This was a disappointing outcome because the development of such a system would have tremendous utility for generating mutant HSV-2 viruses. At this phase of the work, however, research by other investigators had demonstrated the feasibility to mutate the HSV-1 genome when maintained as a BAC in *E. coli* (Messerle *et al.*, 1997; Horsburgh *et al.*, 1999). This prompted the construction of an HSV2-BAC.

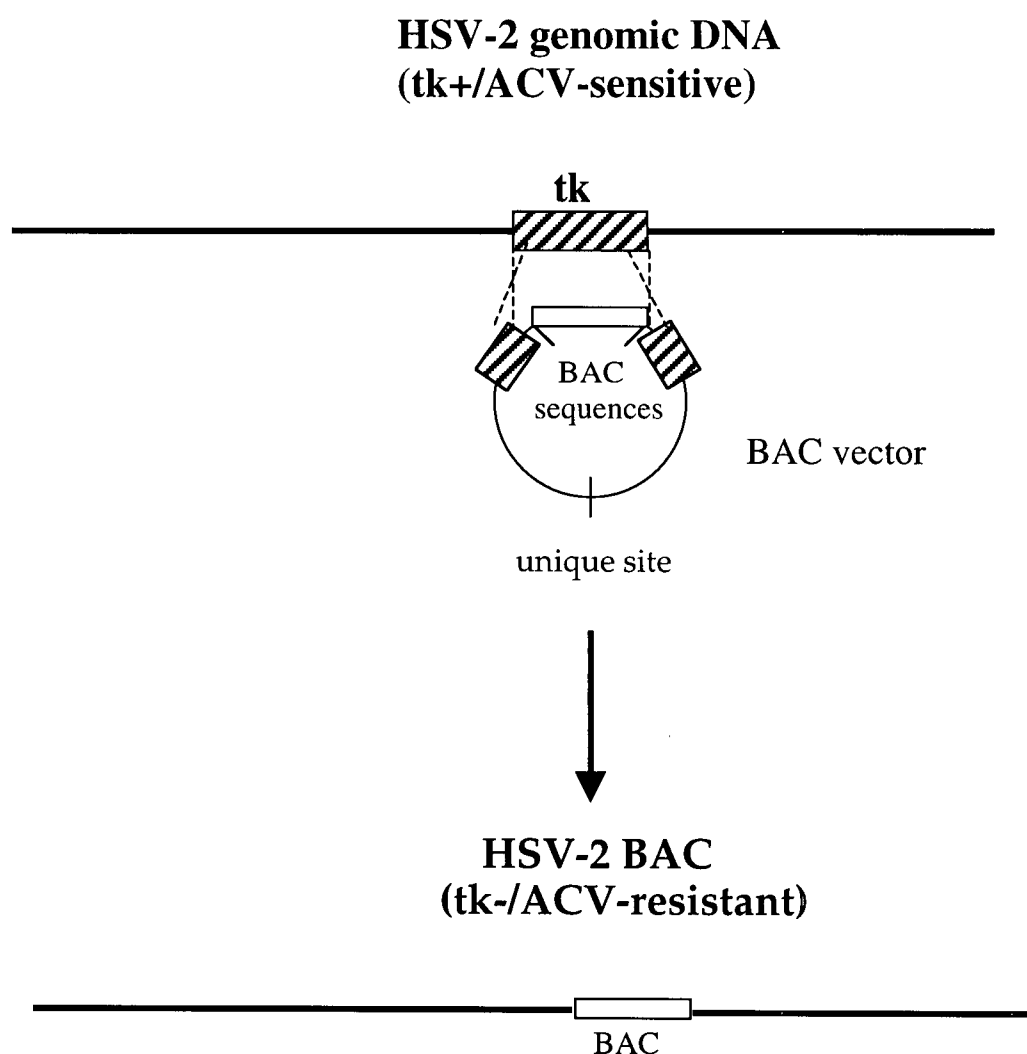
**Figure 7.6.** Impaired release of HSV-2 viral inserts from the cosmid backbone. (A) HSV-2 cosmid clones were digested with PmeI and analyzed by gel electrophoresis. The 8kb DNA fragment representing the PMSI cosmid vector is absent (indicated by the arrow). (B) Sequence analysis of an HSV-2 cosmid clone using the forward sequencing primer. One of the Pme I sites in the PMSI cosmid vector is dysfunctional because of a single bp change, resulting in the sequence 5'-GTTTAAAG-3' instead of the correct sequence of 5'-GTTTAAAC-3'. (C) The second PmeI site in the PMSI cosmid vector is functional. PMSI was double digested with PmeI and Hind III and the products examined by gel electrophoresis. This yielded two expected fragments of 3.3 kb and 4.5 kb in length.



### 7.1.3 Construction of HSV2-BAC

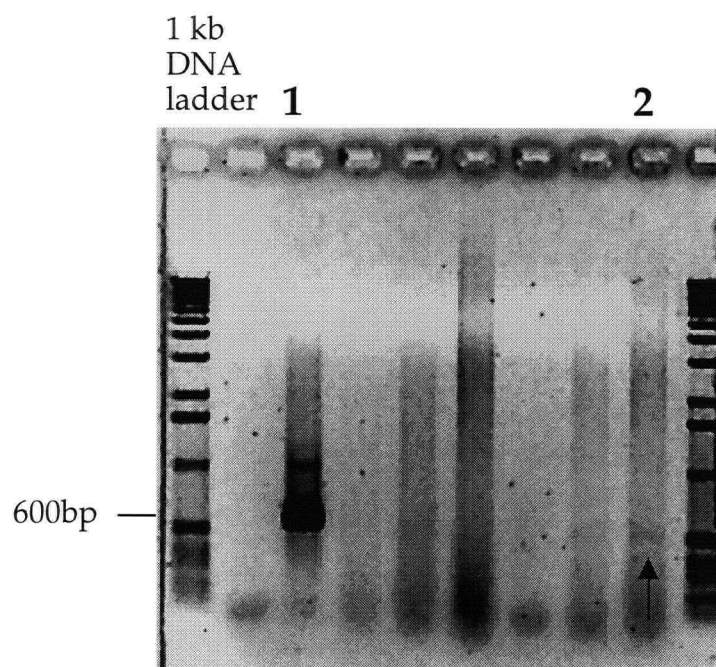
The strategy for construction of the HSV2-BAC is outlined in Figure 7.7. The vector pBAC-TK was used to insert BAC sequences into the HSV-2 genome at the thymidine kinase (tk) locus. The tk locus allows for identification of integration events by screening for acyclovir resistant plaques. It is worthy to note that the tk sequences in pBAC-TK are derived from HSV-1 (F). The HSV-1 and HSV-2 tk genes are quite similar, varying by only 19% with regard to nucleotide sequence (Swain and Galloway, 1983). It was postulated that this degree of DNA sequence similarity was sufficient for recombination to occur between the HSV-1 tk sequences in pBAC-TK and the tk locus in HSV-2. pBAC-TK was linearized by digestion at the unique Hind III site and co-transfected with infectious HSV-2 (G) DNA into Vero cells. Recombinant virus which was resistant to 50  $\mu$ M ACV was harvested. The presence of BAC sequences in the viral genome was verified by PCR using primers which amplified chloramphenicol sequences in the BAC vector (Fig.7.8). A small amount of 600 bp product was produced, which was similar in size to that obtained when PCR was performed on the control pBAC-TK vector. In addition to the correct 600 bp product, PCR of control pBAC-tk also produced a smaller amount of 1kb contaminating product. This 1 kb product was not observed in all PCR trials and its nature is not clear.

To plaque purify a recombinant BAC virus, approximately 50 ACV-resistant plaques were picked and analyzed by PCR (data not shown). For some viral isolates, no PCR products were detected. For others, PCR of chloramphenicol sequences generated multiple products, instead of the expected 600 bp product. Nonetheless, a small amount of 600 bp product was observed for four isolates, in addition to unexpected larger molecular weight products. Due to the fact that non-specific sequences were being amplified in the PCR assay, I could not be certain that this 600bp band represented BAC sequences. To test directly if these viral isolates encoded BAC sequences, circular DNA



**Figure 7.7** Construction of HSV-2 BAC. A BAC vector containing BAC sequences flanked by HSV-1 tk sequences is linearized at a unique restriction site and co-transfected into cells with HSV-2 genomic DNA. BAC sequences are integrated by homologous recombination into the tk locus in the viral genome. HSV-2 BAC virus is selected on the basis of resistance to ACV. The BAC sequences also encode a chloramphenicol marker which is used for selection of HSV-2 BAC.





**Figure 7.8.** Identification of BAC sequences in HSV-2 BAC. The presence of the BAC vector in the HSV-2 genome was established using PCR primers which amplified chloramphenicol sequences. This figure shows the PCR products obtained from an ACV-resistant HSV-2 viral stock produced from co-transfection of HSV-2 genomic DNA and pBAC-tk. Lane 1 shows the PCR products when pBAC-tk is used as template. The 600 bp band represents cm sequences found in the BAC vector. Lane 2 shows results from PCR when DNA from the ACV-resistant HSV-2 BAC viral stock was used. A faint 600 bp band is visible, indicated by the arrow.

from each of the four isolates was electroporated into *E. coli* and cells were plated onto agar plates containing chloramphenicol. Although this procedure was repeated several times, I was unable to isolate recombinant colonies resistant to chloramphenicol and, therefore, unable to isolate HSV2-BAC.

## 7.2 DISCUSSION

During the course of my investigation into the mechanism of DS-mediated HSV infection, it became desirable to construct an HSV-2 intertypic virus in which gB-2 was replaced with gB-1. Initial attempts to construct this mutant were made using a plasmid-based method. This strategy relied on the generation of an HSV-2 gB knockout virus as an intermediate. By co-transfecting HSV-2 DNA and a plasmid bearing the gB-2 gene modified by insertion of a *lacZ* marker, a virus that formed small, *lacZ*-expressing plaques on a gB-1 complementing cell line was generated. These plaques were likely formed by the HSV-2 gB-(*lacZ*) virus, although due to the small plaque size, the virus could not be purified. There are several possibilities to account for the difficulties I encountered when isolating the virus. The first, and most obvious, is that the high background of parental HSV-2 plaques may have obscured the recombinant plaques, particularly since the mutant was disabled in its native gB and relied on gB-1 supplied *in trans* for infection. This in itself raises the question as to whether or not larger plaques would have been produced had the infection been carried out using a gB-2 complementing cell line, rather than the gB-1 complementing D6 cells. The observation that plaques expressing *lacZ* were only observed on complementing cells, and not on control Vero cells, does, however, suggest that gB-1 can complement the gB-2 defect. Nonetheless, there have been no studies to date which have examined the ability of gB-1 to functionally substitute for gB-2 and, therefore, it is not certain whether or not gB-1 can fully complement for gB-2 in this assay.

Given the complexity of the viral genome, one must be wary when modifying viral sequences. The construction of plasmid pHS208-*lacZ* used in this investigation, involved the deletion of gB-2 sequences and then insertion of the *lacZ* cassette. By doing so, the 2.7 kb open-reading frame (ORF) that runs antisense to the gB-2 ORF was also disrupted. At the time of this investigation, it was not known that this ORF, now called U<sub>L</sub>27.5, encodes a protein of 985 amino acids (575 amino acids for HSV-1) which accumulates in the cytoplasm of infected cells (Chang *et al.*, 1998). The gB/U<sub>L</sub>27.5 genes are the third set of genes in the HSV genome located antisense to one another. Although the function of U<sub>L</sub>27.5 is not known, it is worthwhile to consider that the loss of this protein may have contributed to the small plaque phenotype of HSV-2 gB<sup>-</sup> (*lacZ*) virus.

The difficulties encountered in constructing HSV-2 mutants using a plasmid-based method made the development of a cosmid-based system for HSV-2 a particularly desirable objective. Cosmid libraries of VSV, HSV-1, and EBV have proven useful tools for the production of recombinant virus (Cohen and Seidel, 1993; Tompkinson *et al.*, 1993; Kemble *et al.*, 1996). The major advantage of the cosmid system is that it permits the isolation of mutant virus in the absence of wild-type virus, eliminating the need for multiple rounds of plaque purification. For several reasons, I was unable to generate an HSV-2 cosmid set. First, and importantly, viral inserts could not be removed from the PMSI cosmid vector. This would obviously affect the reconstitution of an intact HSV-2 genome in that during recombination between cosmids, the cosmid vector would also be incorporated. Had the PMSI vector contained the necessary restriction sites for excision of the viral insert, the generation of an HSV-2 cosmid set would still have been hindered by the instability of particular viral sequences (Bgl II fragments N, I and H) in the cosmids. Other research groups have noted that some HSV cosmids maintained in *E. coli* are prone to deletion, recombination and rearrangement (Horsburgh *et al.*, 1999). Particularly problematic is that the genome contains palindromic sequences that are often unstable in bacteria. The HSV-1 origin of replication, ori<sub>L</sub>, for example, consists of a large palindrome which is readily deleted when cloned in bacteria (Gray and Kaerner, 1984;

Quinn and McGeoch, 1985; Weller *et al.*, 1985). Hence, this region is unstable in the HSV-1 cosmid and the restriction pattern of serially passaged cosmid clones can alter significantly (Cunningham and Davison, 1993; Stravopoulos and Strathdee, 1998; Horsburgh *et al.*, 1999)

The heterogeneity of cosmid clones is a problem inherent to the cosmid system. Moreover, the reconstitution of viral genomes using cosmids relies on several recombination events in mammalian cells which are difficult to control. These properties are undesirable for the production of reproducible infectious viral DNA. By contrast, herpesvirus sequences maintained as a BAC appear to be stable in bacteria (Messerle *et al.*, 1997; Horsburgh *et al.*, 1999). Horsburgh and colleagues (1999) propose that this may be due to the copy number of BACs in bacteria: approximately one copy per cell. Another advantage of the BAC-system is that the starting material is homogeneous in nature, implying that the properties of HSV virion preparations that are produced from BAC DNA are consistent in their biological properties.

In this study, attempts were made to construct an HSV-2 BAC following the protocol of Horsburgh and colleagues (1999). A viral stock containing ACV-resistant virus which also encoded chloramphenicol sequences (derived from the BAC vector) was generated. This indicated that the HSV-2 BAC virus had likely been produced. However, several attempts to plaque purify the recombinant BAC virus were unsuccessful, suggesting that perhaps the concentration of HSV-2 BAC virus in the viral stock was rather low. A reduced concentration of HSV-2 BAC virus could also explain why PCR of chloramphenicol sequences produced a minimal amount of product (Fig. 7.8A). These difficulties in producing HSV-2 BAC virus may be due to the fact that the incorporation of BAC elements into HSV-2 relied on recombination of HSV-1 tk sequences in the pBAC-TK plasmid with tk sequences in the HSV-2 genome. Although the tk genes in HSV-1 and HSV-2 have significant sequence similarity, the 19% nucleotide variation between them may be sufficient to reduce the efficiency of recombination of BAC sequences into the tk locus of the HSV-2 genome. Another explanation for these results is

that not all ACV-resistant ( $tk^-$ ) virus contained BAC sequences. It is possible that during drug treatment, spontaneous ACV-resistant,  $tk^-$  variants were generated. In the future, this problem could be avoided by integration of the BAC vector and a marker, such as *lacZ*, into another non-essential gene. Because the gene is not essential for replication *in vitro*, the insertion of BAC sequences does not interfere with analyses performed with recombinant viruses.

In light of the success other labs have had using BAC technology to construct herpesvirus mutants, work is ongoing in our laboratory to develop an HSV-2 BAC. This reagent will be useful for the rapid generation of various HSV-2 gB mutants which can then be used to help identify domains of gB involved in mediating DS stimulation of HSV-1 infection. The generation of viral recombinants requires that a vector containing the desired mutation be transformed into HSV-BAC containing bacteria. Following selection, the BAC DNA can be harvested from bacteria and transfected into mammalian cells. Recombinant virus can be harvested 2 days later. In this manner, Horsburgh and colleagues (1999) constructed an HSV-1 virus deleted for the viral packaging/cleavage sites within seven days. By contrast, construction of herpesvirus mutants by the plasmid or cosmid-based methods can be quite challenging. In the cosmid system, fragments of the cosmid are subcloned until a manageable sized DNA fragment is obtained. At this point, the appropriate mutation can be made and, the cosmid, in a series of cloning steps, can be reconstructed to contain the desired mutation.

The cosmid and BAC systems represent cutting-edge technologies for the manipulation of HSV and other herpesviruses. These reagents clearly have advantages over the conventional plasmid-based method for constructing recombinants. The difficulties experienced in this study in constructing both an HSV-2 cosmid set and an HSV-2 BAC, however, demonstrate that these methodologies still have several shortcomings. Thus, it is perhaps best to view the different plasmid, cosmid and BAC systems as being complementary to one another, since each has different merits. For the construction of any given viral recombinant, the benefits and drawbacks of using each of

these systems should be carefully considered. Choosing the most suitable method will certainly facilitate the generation of the desired recombinant.