STUDIES ON THE HERPES SIMPLEX VIRUS TYPE 1 gB GLYCOPROTEIN

Dedicated to my parents,

Antonio and Gina,

with all my love.

STUDIES ON THE HERPES SIMPLEX VIRUS TYPE 1 gB GLYCOPROTEIN

BY

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

MASTER OF SCIENCE (1993) McMASTER UNIVERSITY
(Biochemistry) Hamilton, Ontario

TITLE: Studies on the herpes simplex virus type 1 gB glycoprotein

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NUMBER OF PAGES: xiii, 181

ABSTRACT

The glycoprotein gB of HSV-1 is involved in viral entry and membrane fusion functions. It is glycosylated, forms homodimers, and is transported to both the inner nuclear membrane and plasma membrane in infected cells. The gB glycoprotein contains a potential membrane anchoring hydrophobic sequence of 69 amino acids, located near the carboxy terminus of the glycoprotein. This sequence is predicted to span the membrane bilayer three times, and can thus be divided into three distinct segments, each of which could span the bilayer. To define both the membrane anchoring sequence and the role of this 69 residue hydrophobic domain, a series of deletion mutants were constructed. These mutants have one, two or all three of the predicted membrane spanning segments deleted, in every combination possible, thereby creating a total of 7 deletion mutants. These mutant constructs were expressed in COS-1 cells using transient expression systems. All the mutant constructs were expressed and glycosylated in a manner similar to the wild type gB glycoprotein. Mutant glycoproteins lacking the third (or most carboxy terminal) predicted spanning segment of this 69 residue hydrophobic domain were found to be secreted from the cells, indicating that this segment may specify the membrane

anchoring domain of the gB glycoprotein. Further, the mutant glycoproteins containing this third segment were localized in the nuclear envelope, while mutants lacking this segment were not. All the deletion mutants, except for one, were however defective in intracellular transport and processing of the N-linked oligosaccharides. The only mutant that showed any intracellular transport and processing had only the third segment deleted, but even this mutant was transported and processed much slower than the wild type glycoprotein. The mutant glycoproteins also failed to complement a gB-null virus. These results suggest that the carboxy terminus hydrophobic domain contains essential structural determinants of the gB glycoprotein.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. H.P. Ghosh, for the opporotunity to complete these studies. My thanks also goes to all the members of the lab for their help, guidance and friendship.

I would also like to extend my appreciation to my supervisory committee, Dr. J. Capone, Dr. S. Bayley and Dr. R. Rachubinski for their helpful suggestions and constructive critisism of this work.

Special thanks goes to my family, especially my wife, who had to contend with long nights in the lab, failed experiments, and the stresses of science without ever really understanding what it was all about. I'll be home in half an hour, honest.

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LIST OF ABREVIATIONS

ATP: adenosine 5'-triphosphate
BHV-1: bovine herpesvirus type 1
BSA: bovine serum albumin
cDNA: DNA complementary to RNA
CIP: calf intestinal phosphatase
CMV: cytomegalovirus
dATP: deoxyadenosine 5'-triphosphate
dCTP: deoxycytidine 5'-triphosphate
ddATP: dideoxyadenosine 5'-triphosphate
ddCTP: dideoxycytidine 5'-triphosphate
ddGTP: dideoxyguanosine 5'-triphosphate
ddTTP: dideoxythymidine 5'-triphosphate
dGTP: deoxyguanosine 5'-triphosphate
dITP: deoxyinosine 5'-triphosphate
DMSO: dimethyl sulfoxide
DME: Dulbecco's modified Eagle's medium
DNA: deoxyribonucleic acid.
dNTP: dATP, dCTP, dGTP and dTTP
DTT: dithiotreitol
dTTP: deoxythymidine 5'-triphosphate
EBV: Epstien-Barr virus
EDTA: ethylene-diaminetetra-acetic acid
EHV-1: equine herpesvirus type 1
EHV-4: equine herpesvirus type 4
Endo H: ß-endoglycosidase H
FBS: fetal bovine serum
g: gram
HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HHV-6: human herpesvirus 6
HSV-1: herpes simplex virus type 1
HSV-2: herpes simplex virus type 2
HVS: herpes virus saimiri
ILTV: infectious laryngotracheitis virus
IPTG: isopropyl ß-D thyogalactopyranoside
kb: kilobase
kDa: kilodalton(s)
mA: milliampere(s)
mCi: millicurie(s)
min: minute(s)
mg: milligram(s)
ml: milliliter(s)
mM: millimole(s)
MDV: Marek's disease virus

MOI: Multiplicity of infection
MOPS: 3-[N-Morpholino] propanesulfonic acid
MW: molecular weight
ng: nanogram(s)
OD: optical density
PPO: 2,5-diphenyloxazole
PRV: pseudorabies virus
RFI: Replicative form I of bacteriophage M13
RPM: rotations per minute
SDS: sodium dodecyl sulfate
sec: second(s)
TEMED: N,N,N',N',-tetramethylethylenediamine
Tris: Tris(hydroxymethyl)aminomethane
μCi: microcurie(s)
μg: microgram(s)
μl: microliter(s)
μM: micromole(s)
vol: volume
VSV: vesicular stomatitis virus
VZV: varicella-zoster virus
w: weight
X-gal: 5-bromo 4-chloro 3-indolyl ß-D-galactoside

1.0 INTRODUCTION

The body of this works deals with the structure and function relationships in the glycoprotein gB of herpes simplex virus type 1 (HSV-1). HSV-1 is a member of the alphaherpesvirus family, and as such has some unique characteristics which are worthy of study. One interesting feature of members of this virus family is that they contain a variety of membrane glycoproteins within the virus envelope. These viral encoded glycoproteins have been implicated in a number of strategic functions in the virus life cycle, such as adsorption, membrane fusion, entry into cells, and egress from infected cells (reviewed in Roizman and Sears, 1990). Other enveloped viruses, such as vesicular stomatitis virus (VSV), influenza virus, Semliki Forest virus (SFV), and Newcastle disease virus, to name but a few, contain only one or two membrane glycoproteins with which to carry out many of these same functions (reviewed in Schlesinger and Schlesinger, 1987). This complexity certainly makes HSV-1 an interesting virus. Further, the HSV-1 glycoprotein gB is unlike most other viral encoded glycoproteins, such as VSV G, which have a bulky extracytoplasmic domain, a single spanning membrane anchoring domain, and a fairly short cytoplasmic domain (reviewed in Schlesinger and Schlesinger, 1987). The HSV-1

glycoprotein gB, on the other hand, has a long cytoplasmic domain and a fairly large hydrophobic domain that anchors it to the lipid bilayer. These features, and others which will be discussed, make the glycoprotein gB a particularly interesting membrane protein.

In order to better appreciate and understand the work in this thesis, it is important to review certain aspects of HSV-1, as well as delve into more detail concerning the gB glycoprotein.

1.1 Morphological structure of herpes simplex virus

The herpes simplex virion basically consists of four structural elements: a central core containing the viral DNA; a capsid which encapsulates the core; a lipid envelope that surrounds the entire virion; and a fibrous tegument that separates the lipid envelope from the capsid. These will be briefly examined here, but are covered extensively, along with other aspects of the virus and its life cycle, in several excellent reviews (Roizman and Furlong, 1974; Spear and Roizman, 1981; Roizman, 1990; Roizman and Sears, 1990; Whitley, 1990).

The first of these structural elements, starting from the inside of the virion and working outwards, is a central core. The core contains the viral DNA as a linear, double stranded molecule in the form of a torus (Furlong *et al.*, 1972). This

DNA is 152 kilobase pairs in the case of HSV-1, and ranges from 86 to 170 kilobase pairs within the entire Herpesviridae family. It is interesting, and experimentally important, to note that the viral DNA of HSV-1 is very rich in the bases guanine (G) and cytidine (C). The G and C residues make up approximately 67 percent of the HSV-1 and 69 percent of the HSV-2 genomes, and this composition has made sequencing sections of this genome somewhat tricky (Becker *et al.*, 1968; Kieff *et al.*, 1971; McGeoch *et al.*, 1985; McGeoch *et al.*, 1988).

The second element of the herpes virion is the proteinaceous, symetrical capsid, composed of 162 subunits, or capsomers (Gibson and Roizman, 1972; Cohen *et al.*, 1980). It is about 100 nm in diameter, and encapsulates the central core (Wildy and Watson, 1963). Surrounding all this is the fourth and outermost component of the virion, the outer envelope. This envelope is derived from the cellular membranes of the infected cell (Morgan *et al.*, 1959), and will be discussed further in section 1.2. Separating the outer envelope from the capsid is the tegument, which is asymetrical and can vary in thickness (Morgan *et al.*, 1959; Morgan *et al.*, 1968). The overall size of the virion can range anywhere from 120 nm to 300 nm (Roizman and Furlong, 1974).

The HSV-1 genome has been completely sequenced, and the open reading frames identified to date are capable of encoding

about 70 polypeptides, although more may be possible (McGeoch *et al.*, 1985; McGeoch *et al.*, 1988). Studies on preparations of purified HSV-1 virions have identified some 35 viral encoded polypeptides associated with the virion particle itself (Spear *et al.*, 1970; Heine *et al.*, 1972; Spear and Roizman, 1972; Spear, 1976; Sarmiento and Spear, 1979; Spear, 1984; Gompels and Minson, 1986; Richman *et al.*, 1986; Longnecker *et al.*, 1987; Hutchinson *et al.*, 1992a; Hutchinson *et al.*, 1992b; Baines and Roizman, 1993).

1.2 The life cycle of herpes simplex virus

The life cycle of the herpes simplex virus can be divided into several discrete steps, starting with attachment of the virus to the host cell, followed by infection (which itself involves several steps), and ending with egress of the virus from the cell. Several of the more important steps in this cycle will be examined.

The first step in the virus life cycle is attachment to the host cell. HSV-1 has been shown to bind to heparan sulfate proteoglycans present on the cell surface (WuDunn and Spear, 1989; Lycke *et al.*, 1991; Shieh *et al.*, 1992; Gruenheid *et al.*, 1993). Exactly which viral proteins are involved in recognition and interaction with these receptor molecules are not clearly understood.

Attachment to the host cell is followed by penetration of

the virus into the cell. This can occur by one of two pathways: the first is by fusion of the viral envelope to the plasma membrane; the second is by phagocytosis of the virus. Most evidence to date favours the fusion pathway (Morgan *et al.*, 1968; Ali *et al.*, 1987; Campadelli-Fiume *et al.*, 1988; Butcher *et al.*, 1990; Hutchinson *et al.*, 1992b; Spear, 1993), and identification of the viral proteins, as well as the steps involved in this fusion pathway, is a topic of current investigations.

Following viral penetration, the virion is de-enveloped and the capsid is transported to the nucleus where the viral DNA is subsequently released into the nucleus (Tognon *et al.*, 1981; Batterson *et al.*, 1983). This leads to the subsequent transcription and replication of the viral DNA. These are complex and highly regulated events, requiring a number of viral components, and fall outside the scope of this short review.

The transcribed mRNA is transported into the cytoplasm, where the viral encoded proteins are transcribed. This synthesis occurs on either free or bound polysomes, depending on the signals inherent in the protein being synthesized. These proteins are then processed and transported to their individual destinations within the cell.

The proteins required for constructing the capsid are transported back into the nucleus, where the capsids are

assembled. Packaging of the viral DNA and associated proteins into these capsids takes place by mechanisms not yet fully understood (Ladin *et al.*, 1980; Ladin *et al.*, 1982; Deiss *et al.*, 1986; Deiss and Frenkel, 1986).

Herpes simplex virus acquires its outer envelope by budding through the inner membrane of the nucleus of the infected cell (Darlington and Moss, 1968; Nii *et al.*, 1968). The sites where this envelopment occur appear as thick, invaginated patches. Since no cellular proteins have been detected in the envelope of mature virions, these patches must exclude cellular proteins (Spear and Roizman, 1972; Spear, 1976). Further, the appropriate viral proteins necessary for envelopment must be targeted to these budding sites in the nuclear membrane.

The final stage in the virus life cycle is egress from the infected cell. Stackpole, (1969), has proposed a model whereby capsids are enveloped at the inner nuclear membrane, de-enveloped at the outer nuclear membrane, re-enveloped at the endoplasmic reticulum, and released outside the cell either by envelopment at the plasma membrane or by fusion of vesicles carrying enveloped particles with the plasma membrane. Johnson and Spear, (1982), have proposed a model in which the enveloped virions are secreted via the Golgi apparatus using a pathway similar to that of secreted soluble proteins (Palade, 1975). Any model of egress must explain the

following observations. Firstly, intact enveloped particles are usually seen bounded within membrane structures or vesicles in the cytoplasm of infected cells (Darlington and Moss, 1969; Schwartz and Roizman, 1969). These vesicles may serve as transport vesicles by which the virions transit through the cytoplasm. Secondly, conditions that block glycoprotein maturation cause an accumulation of enveloped particles in the cytoplasm (Pizer *et al.*, 1980; Kousoulas *et al.*, 1983). Thirdly, the nuclear envelope contains the immature form of viral glycoproteins (Compton and Courtney, 1984) while the mature virions contain fully processed, mature glycoproteins (Spear and Roizman, 1972; Spear, 1976; Compton and Courtney, 1984).

1.3 HSV-1 glycoproteins

Herpes simplex virus type-1 codes for at least 11 antigenically distinct glycoproteins (Heine *et al.*, 1974; Spear, 1976; Sarmiento and Spear, 1979; Gompels and Minson, 1986; Richman *et al.*, 1986; Longnecker *et al.*, 1987; Hutchinson *et al.*, 1992a; Hutchinson *et al.*, 1992b; Baines and Roizman, 1993). These glycoproteins have been designated gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL and gM. At least 3 of these glycoproteins, namely gB, gD and gH, are required for virus replication (Sarmiento *et al.*, 1979; Little *et al.*, 1981; Longnecker *et al.*, 1987; Longnecker and Roizman, 1987;

Weber *et al.*, 1987; Desai *et al.*, 1988). These glycoproteins have been implicated in functions such as binding to cells, membrane fusion, and penetration (Manservigi *et al.*, 1977; Sarmiento *et al.*, 1979; Fuller and Spear, 1987; Cai *et al.*, 1988; Campadelli-Fiume *et al.*, 1988; Johnson and Ligas, 1988; Ligas and Johnson, 1988; Fuller and Wee, 1992). It has also been shown that the glycoproteins gI and gE form a complex (Johnson *et al.*, 1988) and also that gL forms a complex with gH (Hutchinson *et al.*, 1992b).

The glycoproteins are also extensively processed. They are synthesized on the rough endoplasmic reticulum, and have both N-linked and O-linked oligosaccharides attached to varying degrees (reviewed in Campadelli-Fiume and Serafini-Cessi, 1984).

As mentioned previously, these glycoproteins are localized to the nuclear envelope, from where the virus buds (see section 1.2), and these proteins are not fully processed (Compton and Courtney, 1984). These glycoproteins are also localized to the plasma membrane in infected cells, and these species are fully processed (Heine *et al.*, 1972; Spear and Roizman, 1972; Spear, 1976; Eberle and Courtney, 1980; Koga *et al.*, 1986). The significance of this plasma membrane localization is not known.

1.4 The HSV-1 glycoprotein gB

1.4.1 Biological role of gB

As indicated in section 1.3, the gB glycoprotein is one of three envelope glycoproteins required for HSV-1 replication. Studies with mutant HSV-1 viruses have implicated the gB glycoprotein as specifically playing an indispensable role in two important events in the viral life cycle: (i) fusion of the viral envelope to the cell membranes and (ii) the penetration, or entry, of the attached viral particle into the cell. The gB glycoprotein, however, is not required for the assembly of virus particles or attachment of the virus particle to the cell (Sarmiento *et al.*, 1979; Little *et al.*, 1981; Cai *et al.*, 1988).

Early studies used temperature sensitive isolates of HSV-1. One temperature sensitive isolate, tsB5, fails to accumulate gB glycoprotein at the non-permissive temperature of 39°C. However, non-infectious virus particles are released at this temperature, indicating that the gB glycoprotein is not required for the assembly of virus particles. These particles can attach to cells, and if the fusogenic agent polyethylene glycol (PEG) is added, productive infection occurs. This strongly suggests that a lack of gB glycoprotein results in failure of the virion envelope to fuse to cellular membranes (Manservigi *et al.*, 1977; Sarmiento and Spear, 1979; Sarmiento *et al.*, 1979). As well, HSV-1 tsB5, grown at the

permissive temperature of 34°C, exhibits a syncytia phenotype and a fast entry phenotype. This means that cells infected at the permissive temperature are extensively fused, and the virus enters these cells much faster than wild type HSV-1 (DeLuca *et al.*, 1982). Both of these phenotypes map to the gB glycoprotein, and sequence analysis of the mutant gB gene revealed two point mutations. Marker rescue experiments mapped the fast entry phenotype to the substitution of Ala for Val at residue 552 of the glycoprotein, and the syncytia phenotype to the substitution of His for Arg at residue 857 of the glycoprotein (Bzik *et al.*, 1984b). Similar results were obtained with another temperature isolate, tsJ12. Like tsB5, tsJ12 failed to accumulate gB glycoprotein at the non-permissive temperature, but non-infectious virus particles were produced. These particles could attach to cells, and in the presence of PEG, these particles were infectious (Little *et al.*, 1981).

One drawback with the temperature sensitive isolates of HSV-1 is that they were obtained by chemical mutagenesis. The result is that one does not know what other lesions may have been produced and what effect these other lesions may have on the experiments. This was resolved by Cai *et al.*, (1987) when they engineered a specific mutation in the gB gene. They took a cloned copy of the gB gene, and introduced a chain termination codon at amino acid 42 of the gB protein by site-

directed mutagenesis. They then introduced this mutant gene into the wild type HSV-1 by transfecting this gene into cells, and then infecting with the wild type HSV-1. The resultant recombinant virus, designated K082, expressed a gB protein truncated after only 42 amino acids. Like the temperature sensitive isolates, this virus produced virus particles that contained no gB glycoprotein. These particles could bind to cells, but were non-infectious. The plating efficiency of these particles could be significantly enhanced by treatment with PEG. This indicates that gB glycoprotein is required at some point after viral attachment to cells but before expression of virus-specific proteins.

A final line of evidence implicating gB glycoprotein in membrane fusion comes from the transient expression of the gB glycoprotein in monkey cells (COS-1). These studies involved the cloning of the gB gene into an eucaryotic expression vector. Transfecting this DNA into COS-1 cells resulted in transient expression of the gB glycoprotein in the absence of other HSV-1 proteins. These gB expressing cells exhibited pH dependent fusion activity, and this fusion can be blocked by pre-treating with antibodies specific to gB glycoprotein (Ali *et al.*, 1987; Butcher *et al.*, 1990). This indicates that the gB glycoprotein can mediate membrane fusion.

1.4.2 Nucleotide sequence analysis of HSV-1 gB

The nucleotide sequence of the gene encoding the glycoprotein gB has been determined for at least four strains of HSV-1, namely HSV-1 strain F (Pellet *et al.*, 1985b), HSV-1 strain KOS (Bzik *et al.*, 1984; corrections to this sequence in Bzik *et al.*, 1986), HSV-1 strain Patton (Stuve *et al.*, 1987), and HSV-1 strain 17 (McGeoch *et al.*, 1988). The DNA sequences amongst these HSV-1 strains share significant homology, which is further reflected in the predicted amino acid composition of the corresponding gB glycoprotein.

The predicted protein sequence generated from these DNA sequences delineates a glycoprotein of 904 amino acid residues for HSV-1 strains KOS, 17 and Patton, while the HSV-1 strain F glycoprotein is 903 residues in length. The glycoprotein gB has a molecular weight of 94 kDa when non-glycosylated, whereas the mature glycoprotein has a molecular weight of around 120 kDa (Sarmiento and Spear, 1979; Pizer *et al.*, 1980; Wenske *et al.*, 1982; Johnson and Spear, 1983; Claesson-Welsh and Spear, 1987). These proteins share 98 to 99 percent homology, with about half of the amino acid differences localized to the amino terminal 78 residues (Bzik *et al.*, 1986; Stuve *et al.*, 1987). Further, the gB glycoprotein is conserved amongst all herpesviruses examined to date, including bovine herpesvirus (Misra *et al.*, 1988; Hammerschmidt *et al.*, 1988), cytomegalovirus (Cranage *et al.*,

1986), Epstein-Barr virus (Baer *et al.*, 1984; Gong *et al.*, 1987; Pellet *et al.*, 1985), equine herpesvirus (Whalley *et al.*, 1989; Riggio *et al.*, 1989), herpes simplex virus type 2 (HSV-2) (Bzik *et al.*, 1986; Stuve *et al.*, 1987), herpesvirus saimiri (Albrecht and Fleckenstein, 1990), infectious laryngotracheitis virus (Griffin, 1991), Marek's disease virus (Ross *et al.*, 1989), pseudorabies virus (Robbins *et al.*, 1987) and varicella-zoster virus (Davison and Scott, 1986). Comparison of the glycoprotein gB amino acid sequence of HSV-1 strains KOS and Patton to the sequence of the gB homolog in HSV-2 strains 333 and HG52 reveals that the gB homologs from these two viruses share from 85 to 86% homology (Bzik *et al.*, 1986; Stuve *et al.*, 1987). These three factors, namely conservation across HSV-1 strains, conservation between HSV-1 and HSV-2, and conservation amongst all herpesviruses, suggest that the glycoprotein gB serves an essential and common function in the herpesvirus life cycle.

The gene for HSV-1 gB has been cloned into several eukaryotic expression vectors, and has been expressed transiently in mammalian cells (Ali *et al.*, 1987; Cai *et al.*, 1987; Pachl *et al.*, 1987). This transient expression, along with a number of mutations generated in these cloned genes, has led to a considerable amount of data on the structure and function of the gB glycoprotein (Ali *et al.*, 1987; Pachl *et al.*, 1987; Cai *et al.*, 1988b; Highlander *et al.*, 1989; Ali,

1990; Butcher *et al.*, 1990; Raviprakash *et al.*, 1990; Highlander *et al.*, 1991; Navarro *et al.*, 1991; Qadri *et al.*, 1991; Navarro *et al.*, 1992; Navarro *et al.*, 1993). Some of these findings will be discussed below.

1.4.3 Structure and topology of the HSV-1 glycoprotein gB

Analysis of the primary amino acid sequence of the HSV-1 glycoprotein gB leads to several interesting observations. The first of these is the presence of a typical amino terminal signal sequence. This sequence directs the protein to be synthesized on membrane bound ribosomes, with the concomitant insertion of the newly synthesized protein into the endoplasmic reticulum (ER) (Blobel and Sabatini, 1971; Blobel and Dobberstein, 1975; Walter and Blobel, 1981). Present within this signal sequence is a common signal peptidase cleavage-recognition site, Ala_{-3}-Ser_{-2}-Ala_{-1} (Perlman and Halvorson, 1983). This cleavage site occurs at residues 28 to 30 for HSV-1 strains 17, KOS and Patton, resulting in cleavage of the first 30 residues in these strains. For HSV-1 strain F, the cleavage signal occurs at residues 27 to 29, resulting in cleavage of the first 29 residues, and since the overall length of the gB protein for HSV-1 strain F is 1 residue less than for the other strains (section 1.4.2), this results in all four strains having a post-cleavage protein of 874 residues. These cleavage sites have been confirmed by

sequencing the amino terminus of the mature gB glycoprotein (Claesson-Welsh and Spear, 1987; Pachl *et al.*, 1987).

A second observation from the amino acid sequence analysis is the number of potential N-linked glycosylation sites, which are signalled by the linear amino acid sequences Asn-X-Ser or Asn-X-Thr, where X can be any amino acid except possibly Pro or Asp (Marshall, 1972; Struck and Lennarz, 1980; Hubbard and Ivatt, 1981; Bause, 1983). There are 6 potential N-linked glycosylation sites in HSV-1 glycoprotein gB (Bzik *et al.*, 1984; Pellet *et al.*, 1985b; Stuve *et al.*, 1987; McGeoch *et al.*, 1988). The extent of glycosylation (both N-linked and O-linked), and subsequent processing, is discussed further in section 1.4.5.

The bulk of the work delineating the theoretical secondary structure of the HSV-1 glycoprotein gB was done by Pellet *et al.*, (1985b) and Bzik *et al.*, (1986) using the gB glycoprotein sequence of HSV-1 strains F and KOS. This modelling was done using a combination of hydropathy analysis (Kyte and Doolittle, 1982), which is shown in Figure 1.4.3.1, empirically based secondary structure predictive algorithms (Chou and Fasman, 1978; Garnier *et al.*, 1978), and helical wheel models (Schiffer and Edmundson, 1967). The resultant secondary structure model was generated, which is shown in Figure 1.4.3.2.

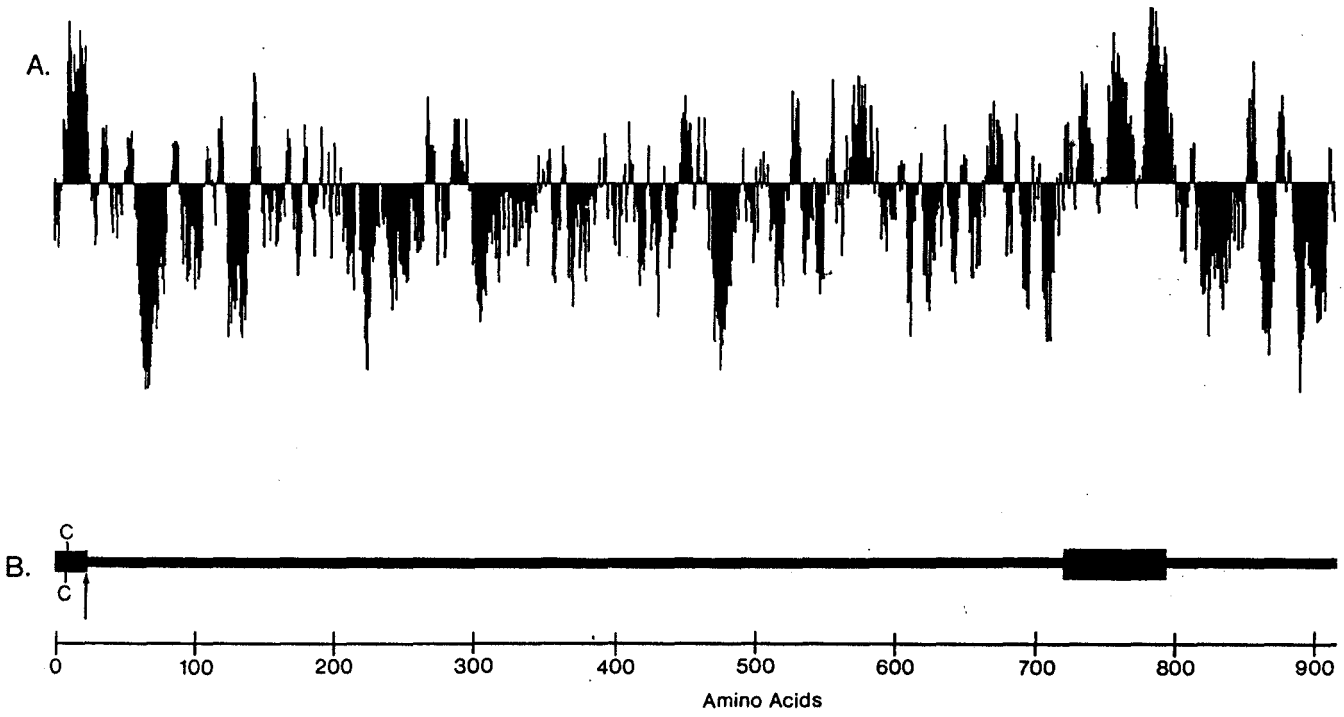


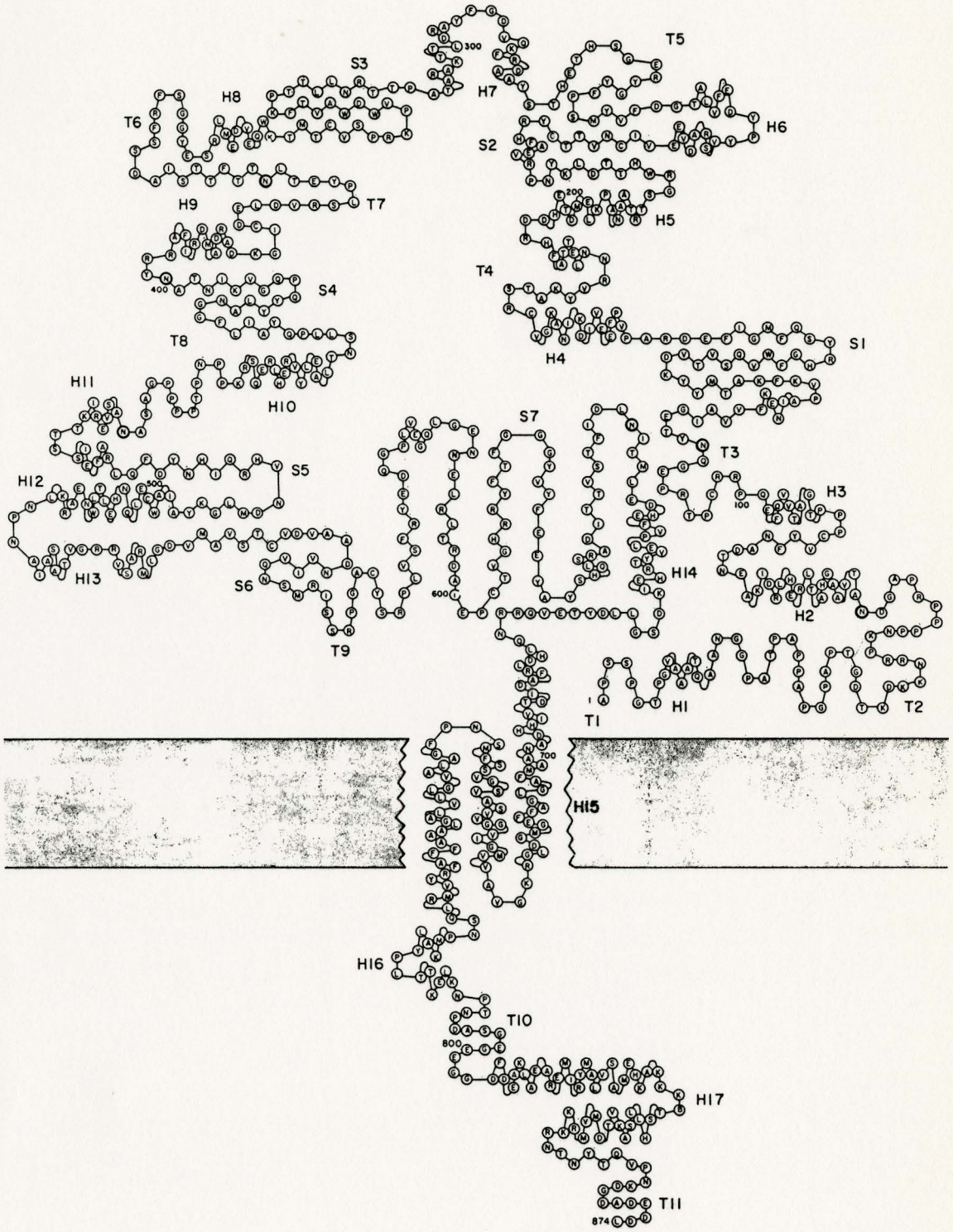
Figure 1.4.3.2. Hydropathic analysis of HSV-1 gB.

A. The relative hydropathy was determined for each amino acid position by using a moving window of seven amino acids. Points above the line indicate hydrophobicity values, while those below indicate hydrophilicity values.

B. The top line diagram represents the linear gB molecule, with the linear amino acid number mapping shown below. The cleaved amino terminal signal sequence and 69 residue hydrophobic domain are shown as solid boxes. Adapted from Stuve *et al.*, (1987).

Figure 1.4.3.2. Predicted secondary structure and membrane orientation of HSV-1 glycoprotein gB.

The theoretical secondary structure and membrane orientation of HSV-1 glycoprotein gB was determined as described in the text (section 1.4.3). The α-helical regions are drawn as helices, ß-sheets as zig-zags, and chain direction changes drawn at predicted ß-turns. Adapted from Pellet *et al.*, (1985b).


T5
S3
H8
H7
T6
H6
S2
H9
T7
H5
S4
T4
T8
H4
S1
H11
H10
S7
T3
S5
H12
H3
H14
H13
S6
H2
T9
H1
T2
T1
H15
H16
T10
H17
T11

An examination of Figure 1.4.3.2 reveals several interesting points. The most visible aspect of the model is the delineation of the mature HSV-1 gB molecule into 3 distinct domains: a membrane spanning domain of 69 amino acids separating a carboxyl terminus cytoplasmic domain of 109 amino acids from a large amino terminus extracytoplasmic domain, or ectodomain of 726 amino acids (which is trimmed down to 696 amino acids after cleavage of the signal sequence).

The hydropathy analysis (Figure 1.4.3.1) reveals a 69 residue hydrophobic domain located near the carboxyl terminus of the glycoprotein (from residues 727 to 795). This can serve as a membrane spanning domain, and is shown as such in Figure 1.4.3.2. In fact, this domain is predicted to span the lipid bilayer three times, and can thus be divided into 3 distinct membrane spanning segments: segment 1 being closest to the amino terminus and segment 3 closest to the carboxyl terminus. The hydrophobicity value of segment 1 is 0.8, of segment 2 is 1.7 and of segment 3 is 2.4. Kyte and Doolittle (1982) define a membrane spanning domain as a contiguous sequence of at least 19 residues with a hydrophobicity value of 1.65 or greater. Using this criterion alone, only the two most carboxyl spans of the lipid bilayer should be possible, since the hydrophobicity value of the span closest to the amino terminus (segment 1) is below the Kyte and Doolittle hydrophobicity minimum of 1.65. This predicted membrane span

contains three charged residues (Glu, Asp and Arg) that decrease its hydrophobicity value to 0.8. However, this traverse of the membrane is postulated based on the fact that thermodynamic analyses of Engelman and Steitz (1981) predict that the thermodynamic stress generated by the presence of these charged residues (which are located on the same side of the α-helix and hence could form a charge-neutralizing salt bridge) is not prohibitive enough to preclude their presence in the lipid bilayer.

The topology of HSV-1 gB has been studied by Claesson-Welsh and Spear (1987). Their experiments yielded several important findings. Firstly, amino acid sequencing of the mature glycoprotein gB from infected cells confirmed cleavage of the 29 residue signal sequence from HSV-1 strain F and the 30 residue signal sequence from HSV-1 strain KOS. Secondly, protection experiments confirmed both the orientation of the glycoprotein and the approximate size of its cytoplasmic domain. In these experiments, trypsin-chymotrypsin was used to digest gB glycoprotein synthesized *in vivo* or *in vitro* in the presence of membranes. The size of the protected fragment was 93 kDa, compared to a translation product of 108 kDa when protease treatment was omitted. This suggests a 15 kDa domain exposed to the cytoplasm. This also confirms that the gB glycoprotein has a type I membrane orientation (amino terminus in the lumen of the endoplasmic reticulum, carboxy terminus in

the cytoplasm), as predicted. It should be noted, however, that these experiments do not necessarily confirm the size nor the number of spanning domains in the 69 residue hydrophobic membrane anchoring domain.

Some indication of the membrane anchoring region of glycoprotein gB has come from analysis of chain termination mutants (Ali *et al.*, 1987; Cai *et al.*, 1988; Ali, 1990; Qadri *et al.*, 1991; Navarro *et al.*, 1993). In these studies, truncation of the gB protein at or upstream of residue 737 resulted in secretion of the protein, indicating that the molecule is not stably anchored to the membrane. This places the membrane anchoring domain downstream of residue 737, which is in agreement with the predicted model (the membrane anchoring domain is predicted to be from residues 727-795). There are not sufficient mutants currently available, however, to definitively map the membrane anchoring domain, nor to confirm the exact number of membrane spans in this domain.

1.4.4 Oligomerization of the HSV-1 glycoprotein gB

The glycoprotein gB of HSV-1 has been shown to exist as homodimers in both HSV-1 infected cells and in cells transiently expressing the glycoprotein gB gene (Sarmiento and Spear, 1979; Claesson-Welsh and Spear, 1986; Zhu and Courtney, 1988; Ali, 1990; Highlander *et al.*, 1991). The ability of transiently expressed glycoprotein gB to form homodimers

indicates that other HSV-1 proteins are not required for the oligomerization of glycoprotein gB.

Further characterization of these gB dimers reveals several interesting points. Firstly, these homodimers cannot be dissociated by 2-mercaptoethanol, and they are also resistant to dissociation by a mixture of 2-mercaptoethanol and sodium dodecyl sulfate (SDS), except at elevated temperatures (Sarmiento and Spear, 1979). The dimers are readily dissociated by heating in the presence of SDS. Secondly, this dimerization takes place in the endoplasmic reticulum (ER) shortly after synthesis, as shown by the fact that gB dimers are observed following a pulse labelling with [^{35}S]methionine of only 7 minutes. These pulse labelled homodimers are sensitive to endoglycosidase H digestion, indicating that they are still located in the ER and have not yet moved to the Golgi apparatus (Claesson-Welsh and Spear, 1986). Thirdly, N-linked glycosylation of the glycoprotein is not required for dimerization. This was determined by expression of the gB protein in the presence of tunicamycin, an inhibitor of N-linked glycosylation (Takatsuki *et al.*, 1971; Takatsuki *et al.*, 1975; Tkacz and Lampen, 1975; Struck and Lennarz, 1980). These experiments showed that gB dimers were formed in the presence of tunicamycin, even though transport of the protein from the ER to the Golgi apparatus was abolished (Claesson-Welsh and Spear, 1986; Highlander *et*

al., 1991). This result confirms that dimerization of the glycoprotein gB occurs in the ER.

Several groups have attempted to map the region(s) of the gB protein involved in dimerization. Most of these studies involved generating mutations in the gB protein, and analyzing the effect these mutations had on dimerization. Cai *et al.*, (1988b), using linker insertion mutagenesis, created a large number of addition, deletion, and chain termination mutants in glycoprotein gB. Their analysis was done using a gB null virus (K082) propagated through a gB producing cell line (D6), and they mapped regions required for dimerization by testing for complementation inhibition. Complementation analysis was done by transfecting Vero cells with the mutant constructs, then infecting with K082 virus. The resultant virus from this infection would have the mutant gB protein incorporated. By titering this recombinant virus on both Vero and D6 cells, mutants that could complement the gB null defect were identified. Co-transfecting the wild type gB gene and mutant genes that were complementation defective allowed identification of mutations that inhibited complementation by the wild type gB gene. These particular mutant constructs must be forming dimers with the wild type gB protein, and this hybrid dimer was not biologically functional, hence complementation is inhibited. The mutants that inhibit complementation must therefore contain the sequences necessary

for dimerization. It was found that all the mutants that inhibited complementation contained residues 463 to 791 of glycoprotein gB. This region includes almost all of the 69 residue hydrophobic membrane anchoring domain (residues 727 to 795).

This interaction region between residues 463 to 791 was further confirmed by Ali (1990) using a series of truncation mutants. In these experiments it was found that truncating the gB glycoprotein after residue 802 (that is, removing the last 102 cytoplasmic domain residues), did not affect dimerization, but truncation mutants at residues 507 or 616 (which are upstream of the membrane anchoring domain) resulted in the formation of monomer gB molecules of 507 or 616 residues in length that were secreted by the cells.

Finer mapping of the interaction region was done using more linker insertion mutants (Qadri *et al.*, 1991; Navarro *et al.*, 1993). It was shown that a mutant gB glycoprotein that had residues 720 to 879 deleted, which includes the entire 69 residue hydrophobic anchoring domain (residues 727 to 795) as well as the first 67 residues of the cytoplasmic tail, could form dimers. As well, gB molecules truncated at residue 720 (just upstream of the membrane anchoring domain), were secreted from cells and formed dimers, suggesting that membrane anchoring is not required for dimer formation. Truncation of the gB protein at residue 630 did not result in

dimer formation. Hence, a region essential for the dimerization of glycoprotein gB was mapped between residues 630 and 720.

Highlander *et al.* (1991), mapped the dimerization domain to two separate regions on glycoprotein gB. They mapped these two regions using a co-precipitation assay. Basically, they had a mutant gB molecule with residues 43 to 234 deleted, and they also had a monoclonal antibody whose binding epitope mapped within this deleted region. This meant that gB proteins with these residues present were recognized by this monoclonal antibody, while the deletion mutant lacking these residues was not. Co-transfecting the wild type gB gene along with this deletion mutant gene into cells, and subsequently immunoprecipitating with the monoclonal antibody, pulled down both the wild type and mutant protein, indicating that dimers were formed between the mutant and wild type gB glycoproteins. By replacing the wild type gB with various mutant gB genes in this co-precipitation assay they were able to map out two non-contiguous regions essential for oligomerization: an upstream site between residues 93 and 282; and a downstream site between residues 596 and 711. Glycoprotein gB dimer formation can result from interactions between two upstream sites, two downstream sites or one upstream and one downstream site. This result is somewhat contradictory to those of Ali (1990) and Navarro *et al.* (1993), whose truncation mutants at

residues 507, 616, and 630 did not form dimers, even though they contained the upstream site. This would indicate that perhaps more than just primary sequence information is needed for oligomerization, and that perhaps some minimal folding information may also be required.

1.4.5 Processing and transport of HSV-1 glycoprotein gB

The gB glycoprotein of HSV-1 is synthesized on membrane bound ribosomes and translocated across the ER (see section 1.4.3). The first processing reaction is the removal of the signal sequence by the signal peptidase (see section 1.4.3). The next processing reaction is the co-translational addition of N-linked high mannose oligosaccharides (Honess and Roizman, 1975; Spear, 1976; Eberle and Courtney, 1980b; Pizer *et al.*, 1980). There are 6 N-linked glycosylation sites in the gB protein, but it appears that only 5 of these sites are actually glycosylated (Cai *et al.*, 1988b). This was established using chain termination mutants of gB glycoprotein that contained only 2, 3, 4, 5 or all 6 glycosylation sites, and analyzing the glycosylation patterns before and after partial digestion of the pulse labelled constructs with endoglycosidase H. These patterns suggest that only 5 of the 6 sites are N-linked glycosylated, and the most carboxy terminal site does not appear to be glycosylated.

These high mannose oligosaccharides are eventually

processed to complex oligosaccharide moieties in the Golgi apparatus (Serafini-Cessi and Campedalli-Fiume, 1981; Person *et al.*, 1982; Wenske *et al.*, 1982). This is seen by the fact that the oligosaccharides added to the gB glycoprotein can be cleaved with endoglycosidase H early on in infection, and as the infection progresses, this susceptibility is almost completely abolished. This is a clear indication of the processing of the high mannose type moieties to their complex forms (Tarentino and Maley, 1974; Kornfeld and Kornfeld, 1985).

There is also evidence that the gB glycoprotein contains O-linked oligosaccharides (Olofson *et al.*, 1981; Johnson and Spear, 1983). These oligosaccharides are acquired in the Golgi apparatus (Hanover *et al.*, 1980; Johnson and Spear, 1983). This O-linked oligosaccharide addition appears to be the final processing step, as no other post-translational modifications of the gB glycoprotein have been observed (Spear, 1984).

Each step in this processing pathway results in a change in the molecular weight of the gB glycoprotein. As the glycoprotein matures, its apparent molecular weight increases from a weight of 94 kDa for the non-glycosylated precursor until its mature, fully processed, size of about 120 kDa is reached (Sarmiento and Spear, 1979; Pizer *et al.*, 1980; Wenske *et al.*, 1982; Johnson and Spear, 1983; Claesson-Welsh and

Spear, 1987).

This maturation of the glycoprotein gB allows its transport within the cell to be monitored easily. By pulse-labelling HSV-1 infected cells, or cells transfected with the gB gene, and subsequent analysis of the labelled gB protein, the rate of transport, and consequently rate of processing, for the gB glycoprotein was determined. Two interesting observation was made in these analyses: (i) glycoprotein gB, and other HSV-1 glycoproteins, were processed and transported faster early on in HSV-1 infections, and this rate of transport slowed later on in infection (Campadelli-Fiume *et al.*, 1988b; Sommer and Courtney, 1991); and (ii) glycoprotein gB, and other HSV-1 glycoproteins, were processed and transported faster in transfected cells (where the proteins were expressed in the absence of other HSV-1 proteins) than when they were in infected cells (Johnson and Smiley, 1985; Campadelli-Fiume *et al.*, 1988b). These differences in transport rates could be due to the following two possibilities: firstly, in HSV-1 infections, the infected cells are healthier early in infection, hence normal transport rates, but as the infection progresses, the cells begin to break down, and perhaps the cellular processing machinery also starts to collapse, resulting in less efficient transport and processing; secondly, in infected cells, the glycoproteins may be sequestered for viral assembly, while in transfected cells

they are not, since there are no other viral proteins. To further elaborate this second point, this protein sequestering would be more pronounced later on in infection, when the amount of viral peptides is greater than in early infection, hence faster transport in early infection versus later infection times (Johnson and Smiley, 1985; Campadelli-Fiume *et al.*, 1988b; Sommer and Courtney, 1991).

The structural requirements of the gB glycoprotein needed for proper transport and processing have been examined by several researchers (Cai *et al.*, 1988; Huff *et al.*, 1988; Raviprakash *et al.*, 1990; Navarro *et al.*, 1991; Qadri *et al.*, 1991; Navarro *et al.*, 1993). A number of linker-insertion, chain termination, and deletion mutants were constructed and tested for their transport and processing. The results can be generalized as follows. Mutations introduced into the ectodomain, that still allowed the gB protein to remain anchored in the membrane, generally resulted in drastically affecting, or abolishing transport and processing. Chain termination mutations upstream of the membrane anchoring domain, resulted in secretion of the protein, and generally did not affect transport and processing as drastically. Mutations in the cytoplasmic domain generally had the least affect on transport and processing, which is consistant with the fact that the last 41 residues of the 109 amino acid long cytoplasmic tail are dispensable for biologically active gB

glycoprotein (Huff *et al.*, 1988). Further, it was found that in the case of some truncation mutants of the gB protein that are not anchored to the membranes, and thus secreted, oligomerization of the gB protein was not necessary for transport and processing (Ali, 1990; Navarro *et al.*, 1993). Dimerization of the gB glycoprotein is not necessarily sufficient for proper transport and processing as shown by the fact that insertion of 4 amino acids in the ectodomain of gB results in a protein that forms stable dimers, but is not transported out of the ER (Navarro *et al.*, 1991; Qadri *et al.*, 1991).

1.4.6 Localization of the HSV-1 glycoprotein gB

The HSV-1 glycoprotein gB, along with other HSV-1 envelope glycoproteins, have been found localized in the lipid envelope of purified virions, as well as in purified plasma membrane preparations of infected cells (Heine *et al.*, 1972; Spear and Roizman, 1972; Spear, 1976). Indirect immunofluorescence studies using antisera specific for the gB glycoprotein have also demonstrated the presence of the gB glycoprotein in the plasma membrane of infected cells (Eberle and Courtney, 1980; Koga *et al.*, 1986) as well as in the plasma membrane of cells transfected with the gB gene and transiently expressing the gB glycoprotein in the absence of other viral proteins (Ali *et al.*, 1987; Pachl *et al.*, 1987;

Cai *et al.*, 1988).

The presence of gB glycoprotein in the nuclear membranes of infected and transfected cells was also established using indirect immunofluorescence (Koga *et al.*, 1986; Ali *et al.*, 1987; Pachl *et al.*, 1987; Cai *et al.*, 1988), as well as by cellular fractionation (Compton and Courtney, 1984; Raviprakash *et al.*, 1990). The gB glycoprotein associated with the nuclear fraction of fractionated cells was shown to be sensitive to digestion with endoglycosidase H (Compton and Courtney, 1984).

The glycoprotein gB, as well as glycoprotein gD, have been further localized using immunoelectron microscopy (Torrisi *et al.*, 1992; Gilbert and Ghosh, 1993). These glycoproteins were localized specifically to both the inner and outer nuclear membranes, in viral particles within the perinuclear cisternae, in virion in the cytoplasm, and in the plasma membrane.

Raviprakash *et al.*, (1990), created a series of chain termination mutants in the cytoplasmic domain of glycoprotein gB. These constructs contained cytoplasmic tails of only 43, 22, 6, 3, or 0 residues, compared to the wild type, which has a cytoplasmic tail of 109 residues. All these mutants were transiently expressed in the absence of other HSV-1 proteins, and were shown to be localized in the nuclear envelope by indirect immunofluorescence and cellular fractionation. These

results suggest that the cytoplasmic domain of glycoprotein gB is not required for nuclear envelope localization, and that the nuclear localization signals must lie within the membrane anchoring or ectodomain of the gB glycoprotein.

1.5 Purpose of the present investigation

The purpose of the present investigation is to study the structure and function relationships in the HSV-1 glycoprotein gB. The function(s) of the 69 residue hydrophobic region are of particular interest.

In order to probe these structure and function relationships in the hydrophobic domain of HSV-1 glycoprotein gB, site directed mutagenesis was used to engineer specific mutations in this region. Since this region can be divided into 3 distinct segments, deletion of one of these segments, either alone or in combination with another segment, will allow the delineation of the functional role(s) of these segments. These mutants of the HSV-1 glycoprotein gB were tested in transient expression systems in order to determine their functionality. By determining the transport and processing, subcellular localization, oligomeric state and the complementation ability of each mutant generated, some insight can be obtained concerning the role(s) of the 69 residue hydrophobic domain of HSV-1 glycoprotein gB.

2.0 Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

All chemicals and reagents were of the highest grade available and used according to the manufacturers' directions unless otherwise specified.

acrylamide	Gibco/BRL
agar	Difco
agarose	Gibco/BRL, Pharmacia
ammonium sulfate	Baker Chemical
ampicillin	Sigma
aprotinin (10000 units/ml)	Miles Canada Inc
ATP	Pharmacia
Bactotryptone	Difco
bis (N,N'-Methylene bisacrylamide)	Gibco/BRL
Bovine serum albumin (BSA)	Sigma
5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal)	Gibco/BRL
Bromophenol blue	Sigma
calf serum	Gibco/BRL
cesium chloride	Terrochem Lab.
citric acid (Monohydrate)	Baker Chemical
crystal violet	Sigma
dideoxynucleotides (dNTP)	Pharmacia
dithiotreitol (DTT)	Calbiochem
ethidium bromide	Sigma
fetal bovine serum (FBS)	Gibco/BRL
N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)	Boehringer Mannheim
8-Hydroxyquinoline	Sigma
Isopropyl ß-D-thyogalactopyranoside (IPTG)	Gibco/BRL
L-glutamine (200 mM)	Gibco/BRL
L-methionine	General Biochemicals
low melting point agarose	Gibco/BRL
ß-mercaptoethanol	BDH, Baker Chemical
2-[N-morpholino]-ethanesulfonic acid (MES)	Sigma

3-[N-morpholino]-propanesulfonic acid (MOPS)	Sigma
non-essential amino acids (100X)	Gibco/BRL
Nonidet P-40	BDH
paraformaldehyde	Sigma
penicillin (5000 U/ml)/ streptomycin (5 mg/ml)	Gibco/BRL
phenol (crystalline)	BDH
polyethylene glycol-8000	Fischer Scientific
PPO (2-5 diphenyloxazole)	Fischer Scientific
protein A-Sepharose	Pharmacia
rubidium chloride	Sigma
salicylic acid	Sigma
sodium citrate (dihydrate)	Caledon Laboratories
sodium dodecyl sulfate (SDS)	BDH, Bio-Rad
sodium deoxycholate	Calbiochem
spermidine	Sigma
sucrose	Gibco/BRL
tetracycline	Sigma
N,N,N',N',-tetramethylethylene-diamine (TEMED)	Gibco/BRL, Bio-Rad
Tris(hydroxymethyl)aminomethane (Tris)	Gibco/BRL
Triton X-100	Sigma, Bio-Rad
vitamin solution (100X)	Gibco/BRL
Xylene cyanole FF	Baker Chemical
urea	Gibco/BRL
yeast extract	Difco

All other common chemicals ($CaCl_2$, NaCl and others) were purchased from BDH.

2.1.2 Radiochemicals

[α-^{35}S]dATP (1000 Ci/mmol)	Du Pont-New England Nuclear
[^{35}S]methionine (1100 Ci/mmol)	Du Pont-New England Nuclear
Na[^{125}I] (carrier-free)	Du Pont-New England Nuclear

2.1.3 Enzymes

Calf intestinal alkaline phosphatase	Gibco/BRL, Boehringer Mannheim
DNA ligase (T4)	Gibco/BRL, Bio-Rad
E. coli DNA polymerase I (Klenow fragment)	Gibco/BRL
DNA polymerase, modified T7 (sequenase)	United States Biochemical
ß-Endoglycosidase H	ICN-Immunologicals
Glucose oxidase	Boehringer Mannheim
Lactoperoxidase	Boehringer Mannheim
Lysozyme chloride	Sigma
Polynucleotide kinase (T4)	Gibco/BRL
Ribonuclease A	Sigma
Restriction endonucleases	Gibco/BRL, Boehringer Mannheim, Pharmacia

2.1.4 Multi-component systems

Gene clean kit	BIO 101
Sequenase sequencing kit	United States Biochemical
Oligonucleotide synthesis kit	Applied Biosystems

2.1.5 Oligodeoxynucleotides

The oligonucleotides used for DNA sequencing and *in vitro* mutagenesis were synthesized on an Applied Biosystems model 381-A DNA synthesizer using ß-cyanoethyl phosphoramidites (Caruthers *et al.*, 1982) according to the directions supplied with the synthesizer by the manufacturer. The basic scheme of the synthesis reaction is as follows. The starting material for the oligonucleotide synthesis is a solid support to which is attached the 3' nucleoside (via its 3'-hydroxyl) of the end product oligonucleotide. Since the growing oligonucleotide is

attached to a solid support column, products in the liquid phase can be removed easily. The 5'-hydroxyl of this nucleoside is protected with a dimethoxytrityl group, and removal of this group with trichloroacetic acid is the first step in the synthesis reaction. The washings from this step can be collected in order to monitor the amounts of dimethoxytrityl removed (by spectrophotometrically monitoring the absorbance of the trityl solution at 498 nm), which is an indication of the efficincy of the reactions. Removal of the protecting group allows access to the 5'-hydroxy of the nucleoside, and the next nucleoside is added to this. Any chains which did not undergo this addition reaction are then capped by acetylation in order to minimize their length, making subsequent removal of these impurities easier. The process is then repeated, again starting with removal of the dimethoxytrityl protecting group, until the oligonucleotide is complete.

Once the synthesis is complete, the oligonucleotide must be cleaved from the solid support column, and this is done using ammonium hydroxide. Next, the protecting groups on the bases must be removed. These groups prevent side reactions with the reactive groups on the bases from ocurring. The final step is purification of the desired oligonucleotide from the shorter impurities that resulted from incomplete reactions. This was done by running the reaction product on

15 to 20 percent polyacrylamide gels (section 2.2.12.3), and the largest band (visualized by UV-shadowing), was isolated and purified (section 2.2.4.1).

Oligonucleotides that were not synthesized (namely, oligonucleotides Δ12 and Δ2, see Table 3.2.1) were purchased from the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University.

2.1.6 Antibodies

The rabbit anti-HSV-1 and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibodies were purchased from the Dako Corporation. The monoclonal antibodies to the gB glycoprotein of HSV-1, 3S (Showalter *et al.*, 1981) and II-105 (Claesson-Welsh and Spear, 1986) were a generous gift of Dr. M. Zweig and Dr. P. Spear respectively. A polyclonal antibody to the gB glycoprotein of HSV-1 was prepared by Mr. R. Gilbert by inoculating rabbits with a recombinant adenovirus containing the gB glycoprotein gene of HSV-1. The rabbit anti-VSV antibody was prepared by Mr M. Butcher (McMaster University) by inoculating rabbits with detergent treated vesicular stomatitis virus particles (VSV) (serotype Indiana).

2.1.7 Growth media and buffers

The composition of frequently used bacteria and phage growth media and buffers is presented in Table 2.1.7.

Table 2.1.7 Frequently used buffers and growth media for bacteria and phage

BUFFER/ MEDIUM	COMPOSITION
5X KGB	1 M K-glutamine, 250 mM Tris-acetate (pH 7.6), 50 mM Mg-acetate, 250 μg/ml bovine serum albumin, 2.5 mM 2-mercaptoethanol
2xYT media	1.6% bactotryptone, 1.0% yeast extract, 86 mM NaCl, pH 7
LB media	1% bactotryptone, 0.5% yeat extract, 86 mM NaCl, pH 7
Minimal media	42.3 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl, 1 mM $MgSO_4$, 0.001% thiamine HCl, 0.2% glucose, pH 7
SOB media	2% bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgS0_4$, pH 7
TAE	40 mM Tris-acetate, 0.11% glacial acetic acid, 2 mM EDTA, pH 8
TBE	89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8.3
TE	10 mM Tris-HCl, 1 mM EDTA, pH 8

2.1.8 Plasmids, cDNAs, bacteriophages and bacteria strains

The eukaryotic expression vectors pXM and p91023(B) (Kaufman, 1985; Yang *et al.*, 1986) were kindly provided by Dr. G. G. Wong (Genetics institute, Cambridge MA) and Dr. R. J. Kaufman (Genetics institute, Cambridge MA) respectively. The vector pXM-G, encoding the VSV G gene, was a generous gift of Mr. Chris Drone (McMaster University). An HSV-1 (KOS) clone containing the glycoprotein gB gene was kindly provided by Dr. J. Smiley (McMaster University). The vector pKBXX (Cai *et al.*, 1987), containing the cDNA for the wild type gB glycoprotein of HSV-1 was a generous gift from Dr. S. Person. Bacteriophage M13mp18 double stranded DNA (replicative form (RF)) was purchased from Gibco/BRL and M13mp18 single stranded DNA was purchased from United States Biochemical.

The following bacterial strains were used: Esherichia coli (*E. coli*) DH5α, purchased from Gibco/BRL; *E. coli* JM109, purchased from Gibco/BRL; *E. coli* BW313 (dut^-,ung^-), was obtained from Dr. N. Skipper, Allelix Laboratories, Mississauga. The bacteria, unless otherwise specified, were grown in liquid LB at 37°C with vigorous agitation in an incubator shaker or on LB agar plates (LB containing 1.5% agar (w/vol)) at 37°C in an incubator. Stocks of bacteria were kept frozen at -70°C in LB containing 15% glycerol.

2.1.9 Molecular weight markers

Two DNA molecular weight (MW) markers were used to determine the size of DNA fragments in agarose gels. The first was *Hind*III digested lambda DNA and the second was the one kb DNA ladder, both purchased from Gibco/BRL. The DNA was visualized by staining the gels with ethidium bromide and using UV light.

Two kinds of protein MW markers were used for SDS-PAGE: a cold marker and a radioactive marker. The cold protein MW marker used was bought from Sigma and was composed of the following proteins: bovine erythrocytes carbonic anhydrase (29 kilodaltons (kDa)), ovalbumin (45 kDa), bovine plasma albumin (66 kDa), rabbit muscle phosphorylase B (97 kDa), *E. coli* ß-galactosidase (116 kDa), rabbit muscle myosin (205 kDa). This marker was visualized by staining with comassie blue. The radioactive protein MW marker consisted of [^{35}S]methionine labelled, detergent treated VSV (serotype Indiana). The following proteins are observed after fluorography of SDS-PAGE of this marker: M (29 kDa), NS (49 kDa), N (50 kDa), G (69 kDa), and L (190 kDa) (Toneguzzo and Ghosh, 1977).

2.1.10 Mammalian cells and viruses

The mutant herpes simplex virus type 1 (HSV-1) K082 and the transformed Vero cell line, designated D6, that expresses HSV-1 glycoprotein gB (Cai *et al.*, 1987; Cai *et al.*, 1988b), constructed in the laboratory of Dr. S. Person, were provided by Drs. M. Levine and J. Glorioso. COS-1 cells, Vero cells and VSV (serotype Indiana) were provided by Mr. M. Butcher (McMaster University).

2.2 Methods

2.2.1 Restriction endonuclease digestion

Restriction endonuclease digestions of DNA were generally carried out at the temperatures and with the buffers recommended by the manufacturers. If multiple enzymes were used, the KGB buffer (Hanish and McClelland, 1988) was generally used due to its ability to support an extremely large range of enzyme requirements.

For partial DNA digestion, either the length of time of digestion was reduced (generally from 1 hour down to 5 or 10 min), or the concentration of restriction endonuclease was greatly reduced, or a combination of both were used. The digestion was stopped by adding an excess of EDTA, by heat inactivation, or by extracting with phenol:chloroform:isoamyl alcohol (50:49:1). The degree of digestion was monitored by agarose gel electrophoresis.

2.2.2 Extraction of DNA

2.2.2.1 Phenol-chloroform extractions

Phenol-chloroform extractions were used to remove contaminating proteins from the nucleic acids. Phenol, phenol:chloroform:isoamyl alcohol (50:49:1) and chloroform:isoamyl alcohol (49:1) were prepared as described in Maniatis *et al.* (1982). To prepare these solutions, crystalline phenol stored at -20°C was melted at 65°C, then 8-

hydroxyquinoline was added to a final concentration of 0.1% (w/vol). Hydroxyquinoline is an antioxidant, a partial inhibitor of RNase, a weak chelator of metal ions, and gives the phenol a light yellow colour, providing a convienent method of identifying the organic phase (Kirby, 1965). The phenol was then extracted with 1 volume of 1 M Tris-HCl (pH 8), followed by several extractions with 1 volume of 0.1 M Tris-HCl (pH 8) containing 0.2% ß-mercaptoethanol (vol/vol). These extractions continued until the pH of the aqueous phase reached 7.4. The equilibrated phenol was kept in the cold room under one volume of 0.1 M Tris-HCl (pH 8) containing 0.2% ß-mercaptoethanol (vol/vol).

Phenol:chloroform:isoamyl alcohol (50:49:1) was prepared by mixing an equal volume of equilibrated phenol with chloroform:isoamyl alcohol (49:1).

To extract DNA, an equal volume of equilibrated phenol was added. After vortexing, the organic and aqueous phases were separated by centrifugation. The aqueous phase was removed to a fresh tube, and an equal volume of phenol:chloroform:isoamyl alcohol (50:49:1) was added, followed by vortexing and centrifugation. Once again, the aqueous phase was transferred to a fresh tube, and an equal volume of chloroform:isoamyl alcohol (49:1) added, followed by vortexing and centrifugation. The final aqueous phase was then transferred to a fresh tube, and the DNA generally

precipitated with ethanol.

2.2.2.2 Ethanol Precipitation

To precipitate DNA, 0.1 volumes of 2.5 M sodium acetate and 2.5 volumes of absolute ethanol were mixed with one volume of DNA solution. After 15 min at -70°C, the DNA was recovered by centrifugation at 10,000 to 16,000xg for 15 min at 4°C. The supernatant was carefully removed, the DNA pellet was resuspended in 1 ml of ice cold 70% ethanol and re-centrifuged at 10,000 to 16,000xg for 5 min. The ethanol was removed, and the DNA pellet was left to dry for 10 min at room temperature, or was dried in a vacuum desiccator.

2.2.3 Analytical agarose gel electrophoresis

Restriction endonuclease digests were analyzed by electrophoresis on 0.6 to 1.5% agarose gels in TBE or TAE buffer containing 0.5 μg/ml ethidium bromide. After the DNA digests were complete, 6X DNA sample dye I (0.25% bromophenol blue (w/vol), 0.25% xylene cyanole (w/vol), 40% sucrose (w/vol)) (Maniatis *et al.*, 1982) was added and 2 to 20 μl of each sample loaded. The gels were run at 100 milliamps until the dye reached about halfway down the gel. The DNA was visualized by exposing the gels to short wave ultraviolet light and photographs were taken using a Polaroid MP-4 Land Camera (Fotodyne).

2.2.4 Preparative agarose gel electrophoresis

In order to subclone fragments of DNA from one vector to another, it was necessary to isolate and purify these specific DNA fragments (inserts and vectors) by preparative agarose gel electrophoresis. In order to do this, digested DNA was run on agarose gels, and the DNA fragment was recovered from the agarose by either of these two methods: (i) using low melting point agarose, or (ii) binding to a silica matrix.

2.2.4.1 Low melting point agarose

Following digestion with restriction endonucleases, the resultant DNA fragments were separated on 1% agarose gels made with low melting point agarose using TBE buffer. These gels were run at 4°C and at a lower voltage than regular agarose gels (typically 50 milliamps). At the end of the electrophoresis, the DNA bands were visualized using long wave ultraviolet light and the desired DNA fragments were excised with a razer blade and transferred into 1.5 ml microcentrifuge tubes. One volume of TE was added, and the agarose was melted at 65°C for 15 min. The agarose was removed by two extractions with phenol, followed by extractions with phenol:chloroform:isoamyl alcohol, and chloroform:isoamyl alcohol. The DNA was then recovered by ethanol precipitation and resuspended in 10 to 20 μl of TE. A small aliquot of this was then run against a known DNA standard on an analytical

agarose gel in order to estimate the concentration of the isolated DNA fragment.

2.2.4.2 Purification of DNA fragments using a silica matrix

The Geneclean Kit (Bio 101 Inc.) contains a specially formulated silica matrix that specifically binds single and double stranded DNA. This kit was used to isolate DNA fragments from agarose gels, and the protocols and reagents needed for this are contained in this kit.

Digested DNA was separated on agarose gels made with TAE buffer as described in section 2.2.3. The advantage of this method is that no special agarose, buffers or gel running conditions are required. Following electrophoresis, the DNA fragments were visualized using long wave ultraviolet light and the desired DNA fragments excised from the gel with a razor blade and transferred into pre-weighed 1.5 ml microcentrifuge tubes. The tubes were then re-weighed to find the weight of the gel slice, and its approximate volume determined from this. Three volumes of 6 M NaI solution (supplied in the kit) were then added to the gel slice, and the tube placed at 45 to 55°C for 5 min to melt the agarose. Once the agarose melted, 5 μl of the silica matrix suspension (GLASSMILK) was added, and the tube placed on ice. After 5 min on ice, the tube was centrifuged in a microcentrifuge for 5 seconds. The supernatant was removed, and the pellet washed

3 times with 0.5 ml of cold NEW WASH (Tris, EDTA, NaCl, ethanol, concentrations unknown since supplied in the kit). After the supernatant from the last wash was removed, the GLASSMILK pellet was resuspended in 10 μl of TE, and placed at 45 to 55°C for 3 min to elute the DNA from the silica matrix. The tube was then spun for 1 min in a microcentrifuge, and the supernatant containing the DNA transferred to a fresh 1.5 ml microcentrifuge tube. A small aliquot of this was then run against a known DNA standard on an analytical agarose gel in order to estimate the concentration of the isolated DNA fragment.

2.2.5 Dephosphorylation of DNA

DNA fragments with similarly digested cohesive ends have a tendency to recircularize during ligations. In order to reduce this, digested DNA fragments were dephosphorylated using calf intestinal alkaline phosphatase (CIP). DNA fragments with protruding 5' termini were dephosphorylated in 5X KGB buffer using 1 unit of CIP, followed by incubation at 37°C for 30 to 60 min. After dephosphorylation, the CIP was heat inactivated by incubating at 68°C in the presence of 0.5% SDS and 1 mM EDTA for 15 min. The CIP was then removed from the DNA fragments by extracting with phenol:chloroform, and the DNA fragments recovered by ethanol precipitation.

2.2.6 Ligation

Once the desired vector and insert fragments were isolated, and their relative concentrations estimated, they could be ligated together to generate the desired construct. To perform these ligations, 4 μl of 5X ligation buffer (125 mM Tris-HCl pH 7.8, 50 mM $MgCl_2$, 2 mM ATP, 10 mM DTT), and 1 μl of T4 DNA ligase was added to a total mass of about 200 to 1000 ng of DNA (insert and vector) at a molar ratio of 2:1 (insert:vector) diluted to 15 μl with H_2O. The reaction mixture was incubated for 18 hours at 15°C and then used to transform competent bacteria.

2.2.7 Preparation of competent cells

Two protocols were used to prepare competent cells. The first method, using magnesium chloride and calcium chloride, was relatively simple and inexpensive, and gave transformation efficiencies in the order of 10^6 transformants per μg of DNA. When high efficiency competent cells were required, a more complex method using rubidium chloride was employed, and this method typically resulted in transformation efficiencies in the order of 10^7 to 10^8 transformants per μg of DNA.

2.2.7.1 Calcium chloride method

This method is based on the procedure of Mandel and Higa (1970) as modified by Maniatis *et al.* (1982). One ml of a saturated overnight culture of DH5α or JM109 cells was used to inoculate 250 ml of LB in a 1 litre flask. This was grown at 37°C with vigourous agitation until mid-log phase growth was reached, as indicated by an OD_{550} of approximately 0.4. When this cell density was achieved, the culture was quickly cooled on ice and subsequently transferred into two pre-chilled 50 ml co-polymer tubes. It is important to carry out all subsequent steps at 4°C to achieve maximum competency. The bacteria was pelleted by centrifugation at 1,000xg for 15 min at 4°C. The cell pellet was resuspended in 50 ml of an ice-cold solution of 0.1 M $MgCl_2$ and incubated on ice. After 15 min, the bacteria was recovered by centrifugation at 1,000xg for 15 min. The cell pellet was resuspended in 100 ml of an ice-cold solution of 0.1 M $CaCl_2$ and incubated on ice. After 30 min on ice, the bacteria was pelleted by centrifugation at 1,000xg for 15 min at 4°C. The cell pellet was resuspended in 12.5 ml of an ice-cold freezing solution (85 mM $CaCl_2$, 15% glycerol) and 200 μl aliquots were transferred into pre-chilled 1.5 ml microcentrifuge tubes and flash frozen in liquid nitrogen or in a dry ice/ethanol bath. The competent cells were stored frozen at -70°C.

2.2.7.2 Rubidium chloride method

Competent cells were prepared using the rubidium chloride procedure as described by Hanahan (1985). Frozen *E. coli* DH5α or JM109 cells were streaked on an SOB plate (SOB media, 1.5% agar) and incubated overnight at 37°C. The next morning, several colonies were picked with a sterile toothpick and dispersed into 1 ml of prewarmed SOB. This was then used to inoculate 100 ml of SOB in a 1 litre flask and the culture was grown at 37°C with vigourous shaking until the OD_{550} of the culture reached 0.4 OD units, indicating that the culture is in mid-log growth phase. The culture was then cooled on ice. At this point it was important to ensure that all the following steps were carried out at 4°C. Once the culture had cooled, it was transferred into pre-chilled 250 ml polypropylene bottles and centrifuged at 1,000xg for 15 min at 4°C to pellet the cells. The cell pellet was resuspended in a total volume of 30 ml of filter sterilized cold RFI solution (100 mM rubidium chloride, 50 mM $MnCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$, 15% glycerol (w/vol), pH adjusted to 5.8 with 0.2 M acetic acid). The cells were incubated for on ice for 15 min and then centrifuged at 1,000xg for 15 min at 4°C. The cell pellet was resuspended in a total volume of 8 ml of filter sterilized cold RFII solution (10 mM MOPS pH 6.8, 10 mM rubidium chloride, 75 mM $CaCl_2$, 15% glycerol (w/vol)). The cell suspension was incubated on ice for 30 min, after which

200 µl aliquots were dispensed into pre-chilled 1.5 ml microcentrifuge tubes and flash frozen in liquid nitrogen or in a dry ice/ethanol bath. The competent cells were stored frozen at -70°C.

2.2.8 Transformation of bacteria

Competent cells were stored frozen at -70°C in 200 µl aliquots in 1.5 ml microcentrifuge tubes. Prior to use, these cells were thawed on ice. Immediately after thawing, the cells were transferred into chilled 15 ml polypropylene tubes, and 5 µl of ligation mixture, or 1 to 10 ng of supercoiled plasmid DNA, was added. The cells were incubated on ice for 30 to 90 min with occasional mixing, then were heat shocked for 90 sec in a 42°C water bath, and returned immediately on ice for 5 min. After the addition of 1 ml of pre-warmed LB, the cells were incubated at 37°C with moderate agitation for 45 min. Following this incubation, the cells were spun down briefly and gently and were resuspended in approximately 200 µl of LB. The bacteria (typically 1 to 100 µl) were then plated on appropriate selection plates, such as ampicillin plates (LB, 1.5% agar (w/vol) and 100 µg/ml of ampicillin) or on tetracycline plates (LB, 1.5% agar (w/vol) and 15 µg/ml of tetracycline), the plates were then inverted and incubated at 37°C overnight.

2.2.9 Small-scale preparation of plasmid DNA

Plasmid DNA was isolated from *E. coli* using one of two methods described by Maniatis *et al.* (1982). The two methods are (i) lysis by boiling and (ii) lysis by alkali. In both instances, cells from a single colony were grown to saturation in LB media that contained ampicillin (100 μg/ml) or tetracycline (15 μg/ml).

2.2.9.1 Lysis by boiling

The boiling method of plasmid DAN isolation described by Maniatis *et al.* (1982) is a modification of the procedure of Holmes and Quigley (1981). Bacteria from 1.5 ml of a saturated liquid culture were pelleted by centrifugation in a microcentrifuge for 2 min. Each cell pellet was resuspended in 300 μl of STET solution (0.1 M NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 5% Triton X-100 (vol/vol)). To this was added 25 μl of a lysozyme solution (10 mg/ml in 10 mM Tris-HCl pH 8) and mixed by vortexing quickly. The tubes were boiled for 90 seconds in a water bath, centrifuged at room temperature for 10 min in a microcentrifuge and the pellets carefully removed with a wooden toothpick. The DNA was precipitated by adding 40 μl of 2.5 M sodium acetate (pH 5.2) and 420 μl of isopropanol, followed incubating for 5 min at room temperature. The DNA was recovered by centrifugation in a microcentrifuge for 5 min at 4°C. The DNA pellet was then

washed with 70% ethanol, re-centrifuged, and dried at room temperature for 5 min. The plasmid DNA was resuspended in 10 to 100 μl of TE containing 20 μg/ml ribonuclease A.

The plasmid DNA was analyzed by digestion with restriction endonucleases as described (section 2.2.1), and, where necessary, further verified by dideoxynucleotide sequencing.

2.2.9.2 Lysis by alkali

This method of plasmid isolation described by Maniatis *et al.* (1982) is a modification of the procedure of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981). It should be noted that this was the method of choice for the isolation of the M13mp18 series of plasmids.

Cells from 1.5 ml of a saturated culture were pelleted by centrifugation in a microcentrifuge for 2 min. The supernatant was carefully removed and the pellet was resuspended in 100 μl of ice-cold solution I (50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA). Cellular DNA was denatured by the addition of 200 μl of freshly prepared solution II at room temperature (0.2 N NaOH, 1% SDS (w/vol)). The tube was then mixed gently by inverting several times and incubated on ice for 5 min. After this incubation, 150 μl of ice-cold Solution III (60 ml 5 M potassium acetate, 11.5 ml acetic acid, 28.5 ml H_2O) was added. The tubes were vortexed for 10

seconds and then incubated on ice for 5 min. The resultant precipitate, consisting of protein, chromosomal DNA, and high molecular weight RNA, was removed by centrifugation for 5 min at 4°C in a microcentrifuge. The supernatant was extracted once with phenol:chloroform:isoamyl alcohol (50:49:1) and once with chloroform:isoamyl alcohol (49:1). The plasmid DNA was recovered by the addition of 2 volumes of ethanol, followed by a 10 min spin in a microcentrifuge at 4°C. Each plasmid DNA pellet was resuspended in 10 to 100 μl of TE buffer containing 20 μg/ml of ribonuclease A.

The plasmid DNA was analyzed by digestion with restriction endonucleases as described (section 2.2.1), and, where necessary, further verified by dideoxynucleotide sequencing.

2.2.10 Large scale preparation of Plasmid DNA

For the preparation plasmid DNA from 100 to 1000 ml of saturated bacterial culture, which would yield one to several mg of plasmid DNA, the procedure of Birnboim and Doly (1979; see section 2.9.2) was used, with all the volumes scaled up and modified accordingly.

A saturated culture was obtained by inoculating 100 to 1000 ml of LB (containing the appropriate selection antibiotic) with 1 ml of saturated culture and growing overnight at 37°C with vigourous agitation. The saturated

cell culture was cooled down on ice for 5 min, transferred into 250 ml polypropylene bottles and centrifuged at 2,500xg in a Sorvall centrifuge using a Sorvall rotor type GSA for 10 min at 4°C.

The supernatant was carefully removed and the cell pellet resuspended in 5 ml of ice-cold solution I (50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA) per 250 ml of cell culture. The cells were resuspended by vortexing, and transferred to 50 ml centrifuge tubes (one tube per 250 ml of cell culture). To this, 10 ml of freshly prepared solution II (0.2 N NaOH, 1% SDS (w/vol)) at room temperature was added, and the tubes mixed by inversion, and placed on ice. After 10 min on ice, 7.5 ml of ice-cold solution III (60 ml of 5 M potassium acetate, 11.5 ml of acetic acid, 28.5 ml of H_2O) was added to each tube, the tubes mixed by inversion and incubated on ice. After 10 min on ice, the tubes were centrifuged at 17,000xg using a Sorvall rotor type SS-34 in a Sorvall centrifuge for 20 min at 4°C.

The supernatant was filtered through four layers of cheesecloth into 50 ml centrifuge tubes and the DNA precipitated by adding 0.6 volumes of isopropanol and incubating at room temperature for 10 min. The DNA was recovered by centrifugation at 17,000xg using a Sorvall rotor type SS-34 in a Sorvall centrifuge for 20 min at room temperature. The DNA pellet was washed with 10 ml of ice cold

70% ethanol, re-centrifuged and dried in a vacuum desiccator, and dissolved in 4 ml of TE.

The DNA plasmids were further purified by CsCl density gradient purification as described by Maniatis *et al.* (1982). To the 4 ml of plasmid DNA solution obtained from the previous steps above, 4.4 g of cesium chloride was added and dissolved. Ethidium bromide (10 mg/ml) was added at the rate of 0.8 ml of ethidium bromide solution for every 10 ml of DNA/CsCl solution. This was transferred into 13 x 51 mm Quick-Seal Beckman centrifuge tubes, and the tubes were balanced and sealed. The tubes were centrifuged in a vertical Beckman rotor type VTi 65 at 55,000 RPM for 18 hours at 25°C.

The closed circular plasmid forms a dark red band about halfway down the tube which, if present in sufficient quantity is readily visible, otherwise can be visualized using long wave ultra violet light. This band was removed using a 5 ml syringe, transferred into 15 ml polypropylene tubes and extracted several times with an equal volume of 1-butanol equilibrated with water to remove the ethidium bromide. The extraction steps were repeated until the organic and aqueous phases became colourless. The DNA was then diluted with five volumes of TE and precipitated by the addition of 2 volumes of ethanol and incubating on ice for 10 min. The DNA was recovered by centrifugation in a Sorvall centrifuge using a Sorvall rotor type SS-34 at 8,000xg, washed with 5 ml of 70%

ice cold ethanol, and re-centrifuged for 10 min. The DNA pellet was dried in a vacuum desiccator and resuspended in 100 to 1000 μl of TE.

The DNA concentration and purity was estimated by measuring the OD_{260} and OD_{280}. For double stranded DNA, an OD_{260} of 1 represents a DNA concentration of 50 μg/ml, and the OD_{260}/OD_{280} ratio should be between 1.8 to 2.0.

2.2.11 Site directed mutagenesis

The deletion mutants used in this study were generated by site directed mutagenesis. These mutants were constructed using a technique developed by Zoller and Smith and modified by Kunkel (Zoller and Smith, 1983; Zoller and Smith, 1984; Kunkel, 1985; Kunkel *et al.*, 1987; Zoller and Smith, 1987).

The basic steps in this procedure involve cloning the desired DNA sequence into the M13mp18 vector; growing this DNA in *E. coli* BW313 (dut^- ung^-) cells to generate single stranded DNA containing uracil; isolation of this DNA; annealing the primer to the DNA, followed by extending the primer and ligating this second strand; transformation of competent *E. coli* JM109 cells with the reaction mixture; and finally, screening of the resultant phage to identify desired mutations.

The *Sal*I-*Eco*RI fragment of the HSV-1 gB DNA, which encompasses the carboxy terminal hydrophobic region of

interest, was previously cloned into M13mp18 to generate a plasmid designated M13mp18-gB (Raviprakash *et al.*, 1990). An M13 phage stock previously prepared from this was the starting point for the construction of the deletion mutants.

2.2.11.1 Growth of uracil-containing phage

A frozen stock of *E. coli* BW313 cells was streaked on minimal agar plates (minimal media, 15% agar), and grown at 37°C until distinct colonies appeared. A saturated culture of *E. coli* BW313 cells was prepared by inoculating 5 ml of 2xYT media with one of these colonies and grown at 37°C with shaking overnight. The next morning, 10 ml of 2xYT media was inoculated with 100 µl of the overnight BW313 culture, and this was incubated for 90 min at 37°C with vigourous shaking. The bacteria was then sedimented by centrifugation at 5,000xg for 5 min in a Sorvall rotor type SS-34. The cells were resuspended in 10 ml 2xYT containing 0.5 µg/ml uridine, and this was infected with 10 µl of the recombinant M13mp18-gB phage stock described in section 2.2.11. At the same time, another 10 ml of 2xYT media was inoculated with 100 µl of the overnight BW313 culture. Both of these cultures were grown for 3 hours at 37°C with vigourous shaking.

After 3 hours of growth, 1 ml from the infected culture was mixed with the 10 ml BW313 culture, and this was placed into 90 ml of fresh 2xYT media. This culture was then grown

at 37°C with vigourous shaking for 6 hours.

At the end of 6 hours of growth, the culture was cooled down on ice, transferred into a 250 ml polypropylene bottle, and centrifuged in a Sorvall rotor type GSA at 5,000xg for 5 min at 4°C. The cell pellet was used for the isolation of double stranded phage DNA (referred to as RF, replicative form, DNA), using the alkaline lysis procedure described in section 2.2.9.2. The supernatant, containing the recombinant phage, was transferred into a fresh 250 ml bottle and the centrifugation step was repeated to ensure complete removal of the bacterial cells.

At this point, the phage stock was titered before proceeding with the isolation of single stranded phage DNA. Since the object of this procedure is to have uracil containing DNA, titering the phage stock on BW313 (deficit in uracil-N-glycosidase and dUTPase activity), and on JM109 cells, will indicate if uracil has been incorporated into the phage DNA. Ideally, a good preparation of uracil-containing phage will have a titer of at least 10^4 orders higher on BW313 cells.

To titer the phage stock, overnight cultures of both BW313 and JM109 cells are needed. Serial dilutions (from 10^4 to 10^{10}) of the phage stock were prepared in 2xYT media. In a 15 ml polypropylene tube, 0.1 ml of the diluted phage, 0.3 ml of BW313 or JM109 overnight culture, and 2.5 ml of top agar

(2xYT, 0.75% agar) at 45°C was combined and immediately poured onto pre-warmed (37°C) 2xYT plates (2xYT, 1.5% agar). The top agar was allowed to solidify, then the plates inverted and placed at 37°C overnight. The plaques were then counted, and the phage titer on BW313 and JM109 cells determined. If the titers were sufficiently different, indicating uracil incorporation, single stranded DNA was isolated from the phage.

2.2.11.2 Isolation of single stranded uracil-containing DNA

To isolate single stranded DNA from the phage stock, the phage was precipitated by the addition of 19.3 ml of 2.5 M NaCl containing 20% (w/vol) of polyethylene glycol 8000 to the solution of uracil-containing phage (about 90 ml in a 250 ml polypropylene bottle). This was incubated for 30 to 45 min at room temperature. After the 30 to 45 min incubation, the phage was pelleted by centrifugation in a Sorvall rotor type GSA at 10,000xg for 15 min. The supernatant was carefully removed, and the bottle re-centrifuged in order to remove any residual polyethylene glycol on the side of the bottle. It is very important to ensure complete removal of the polyethylene glycol. The small white phage pellet was resuspended in 500 μl of TE and transferred to a 1.5 ml microcentrifuge tube. After a 10 min incubation on ice, the tube was centrifuged for 2 min in a microcentrifuge, and the supernatant was

transferred into a fresh 1.5 ml microcentrifuge tube and stored at 4°C.

The 500 µl phage solution was extracted twice with phenol, once with phenol:chloroform:isoamyl alcohol, and once with chloroform:isoamyl alcohol (section 2.2.2.1). The single stranded DNA was precipitated by the addition of 0.1 volumes of 3 M sodium acetate and 2.5 volumes of ethanol, and incubated for 30 min at -70°C. The DNA was recovered by centrifugation for 15 min at 4°C in a microcentrifuge. The DNA pellet was washed with ice-cold 80% ethanol, re-centrifuged for 10 min, dried in a vacuum desiccator and resuspended in 50 µl of TE. In order to estimate the approximate DNA concentration, small (1 to 5 µl) aliquots were run on a 1% agarose gel along with a known amount of single stranded DNA as a standard.

2.2.11.3 Primer phosphorylation

Once a good stock of uracil-containing ssDNA was obtained, the oligonucleotides used as primers for the mutagenesis reaction were phosphorylated using T4 polynucleotide kinase. The mutagenic primers were diluted in TE to give a final concentration of 10 pmol/µl. To 16 µl of primer (between 100 to 200 pmol) in a 1.5 ml tube, was added 2 µl of 10x kinase solution (1 M Tris-HCl pH 8.3, 100 mM $MgCl_2$, 100 mM DTT), 1 µl of 10 mM ATP and 1 µl T4

polynucleotide kinase (5 units/μl). This was incubated at 37°C for 1 hour, then at 65°C for 10 min to stop the reaction. The phosphorylated primer is stable for at least one week at -20°C.

2.2.11.4 Primer annealing

For optimum results, a molar ratio of primer to single stranded DNA template of 20:1 was used. In a 1.5 ml microcentrifuge tube, 0.5 to 2 μg of uracil-containing single stranded DNA template, phosphorylated primer, 1 μl 10x annealing buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 500 mM NaCl, 10mM DTT), and water to a final volume of 10 μl was combined. This was heated for 5 min to 90 to 95°C in a water bath and then slowly cooled down to room temperature. The solution was then centrifuged for 30 seconds in a microcentrifuge to collect the contents to the bottom of the tube.

2.2.11.5 Primer extension

To the annealed primer-template solution, 1 μl of 10x extension buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 100 mM DTT), 4 μl of a 2.5 mM dNTP mix, 1 μl of 10 mM ATP, 6 units of T4 DNA ligase, 2 units of *E. coli* DNA polymerase I Klenow fragment, and water was added to give a total volume of 20 μl. This was incubated at 12 to 15°C overnight.

2.2.11.6 Screening for mutants

The next day, 2 to 15 μl of the extension reaction was used to transform high efficiency competent JM109 cells (section 2.2.7.2). These transformed cells were mixed with 200 μl of saturated JM109 culture and added to 2.5 ml top agar (2xYT, 0.75% agar) at 50°C and plated on 2xYT plates (2xYT, 1.5% agar). Well isolated plaques obtained from this transformation were picked using a sterile pasteur pipette and resuspended in 50 μl of 2xYT, and 10 μl of this was used to inoculate 5 ml of 2xYT containing 200 μl of saturated JM109 culture. These cultures were grown for 4 to 6 hours at 37°C with vigourous shaking.

After the 4 to 6 hour growth, 1.5 ml of each culture was spun in a microcentrifuge for 2 min. The supernatant was kept at 4°C, and double stranded RF DNA was isolated from the infected cells using the alkaline lysis method of plasmid preparation (section 2.2.4.2). Typically 24 plaques were initially screened for each mutation. The RF DNA was the digested with *SalI* and *Eco*RI, which cuts out the gB gene insert, and the digests were run on a 1% agarose gel. Since the mutants being constructed were deletion mutants, plasmids harbouring potential mutants should have a smaller *SalI*- *Eco*RI gB fragment than the wild type plasmids.

Once several clones containing the required insert had been identified, single stranded DNA was isolated from the

corresponding phage supernatants that had been kept at 4°C. The single stranded DNA was isolated by centrifuging the phage supernatant in a microcentrifuge for 2 min. To 1.2 ml of the supernatant was added 0.3 ml of 2.5 M sodium acetate containing 20% polyethylene glycol 8000 (w/vol), and was incubated on ice for 15 min. After 15 min, the phage were pelleted by centrifugation for 10 min in a microcentrifuge. The supernatant was removed, and the tubes spun again briefly to remove any liquid on the side of the tubes. The phage pellet was resuspended in 200 μl of TE, and extracted twice with phenol:chloroform:isoamyl alcohol, and once with chloroform:isoamyl alcohol. The DNA was recovered by the addition of 0.1 volumes of 3 M sodium acetate and 2.5 volumes of ethanol, incubating at -70°C for 30 min, and centrifuging for 15 min in a microcentrifuge. The single stranded DNA was then sequenced to identify the mutants. Once the desired mutants were identified, they were subcloned into the appropriate vector.

2.2.12 DNA Sequencing

The nucleotide sequence of the mutant constructs was determined using the dideoxy chain termination method (Sanger *et al.*, 1977). Both single stranded and double stranded DNA was sequenced. In both instances, modified T7 DNA polymerase (Sequenase™) (Tabor and Richardson, 1987) was used.

2.2.12.1. Double stranded DNA preparation

Purified plasmid DNA was denatured prior to sequencing (Zhang et al., 1988) as follows: 3 μg of purified plasmid was placed in a 1.5 ml microcentrifuge tube in a volume of 18 μl and 1 μl of 4 N NaOH and 1 μl of 4 mM EDTA were added and the tube incubated for 5 min at room temperature. After 5 min, the solution was neutralized by the addition of 2 μl of 2 M ammonium acetate pH 4.6 and the tube was placed on ice. The DNA was precipitated with 2.5 volumes of ethanol, and recovered by a 5 min centrifugation in a microcentrifuge. The dried DNA pellet was dissolved in TE.

2.2.12.2 Sequencing reaction

In a 1.5 ml microcentrifuge tube, 1 μg of single stranded DNA (prepared as described in section 2.2.11.6) or 3 μg of denatured double stranded DNA (see section 2.2.12.1) were mixed with 0.5 to 1 pmol of primer, 2 μl of annealing buffer (200 mM Tris-HCl pH 7.7, 100 mM $MgCl_2$, 250 mM NaCl), and the volume adjusted to 10 μl with water. The tube was then heated for 2 min at 65°C, and slowly cooled down to room temperature over a period of about 1 hour.

To the annealed template-primer was added 1 μl of 0.1 M DTT, 2 μl of diluted labelling mix (dGTP, dCTP and dTTP, 1.5 μM of each), 0.5 μl of [α-^{35}S]dATP and 2 μl of modified T7 DNA polymerase (Sequenase™, 1.6 units/μl). This was incubated for

5 min at room temperature. Immediately after this incubation, 3.5 µl were transferred into each of 4 tubes containing 2.5 µl of termination mix (50 mM NaCl, 80 µM each of dATP, dCTP, dGTP and dTTP, and 8 µM ddATP for the A termination mix, 8 µM ddTTP for the T terminaton mix, 8 µM ddGTP for the G termination mix, 8 µM ddCTP for the C termination mix). The four tubes were incubated for 5 min at 37°C and the reaction was stopped by the addition of 4 µl of stop solution (95% formamide (vol/vol), 20 mM EDTA pH 8, 0.05% Bromophenol Blue (w/vol), 0.05% Xylene cyanole FF (w/vol)).

2.2.12.3 Electrophoresis of sequencing products

The sequencing samples were denatured immediately prior to electrophoresis by heating for 2 min at 80°C, and then quickly placing on ice. For each reaction, 1 to 2 µl were loaded and the fragments separated by electrophoresis on 6% polyacrylamide gels (40:1, acrylamide:bisacrylamide, 7 M urea in TBE (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8.3)) run at 60 watts for 2 hours, or until the first dye reaches the bottom of the gel. After electrophoresis, the gels were fixed for 15 min in a solution containing 10% acetic acid (vol/vol) and 12% methanol (vol/vol), transferred onto filter paper (Watman no. 1), and dried at 80°C for 30 to 45 min on a gel dryer. The gels were then exposed to Kodak XAR-5 film at room temperature.

2.2.13 Mammalian cells and culture conditions

COS-1 cells (Gluzman, 1981), were grown in high glucose Dulbecco's modified Eagle's Medium (DME) (Gibco/BRL) supplemented with 10% calf serum (vol/vol). Vero cells (African green monkey kidney cells) were grown in DME supplemented with 7% fetal bovine serum (vol/vol). The D6 cell line used was derived from Vero cells, and as such, was treated as Vero cells. The DME growth medium was also supplemented with 4 mM glutamine, 45 mM $NaHCO_3$, 10 mM HEPES, 1X MEM non-essential amino acids (Gibco/BRL), 1X MEM vitamins (Gibco/BRL), 100 units/ml penicillin (Gibco/BRL), and 100 μg/ml penicillin/streptomycin (Gibco/BRL).

Monolayer cultures of COS-1, Vero or D6 cells were routinely grown in plastic culture dishes at 37°C under an atmosphere of 5% CO_2, and were usually passaged every three days (at a ratio ranging from 1:4 up to 1:20) using trypsin-EDTA (Gibco/BRL).

For long term storage, frozen cell stocks were stored in liquid nitrogen in DME medium supplemented with 10% calf serum or 7% fetal bovine serum and 10% dimethyl sulfoxyde (vol/vol). Alternatively, these cell stocks could be stored at -80°C for up to 3 months.

2.2.14 Transfection of COS-1 and Vero cells

For transient expression of the wild type and mutant proteins, subconfluent monolayers of COS-1 and Vero cells were transfected using one of two methods: (i) the calcium phosphate method (Graham and Van Der Eb, 1973) or (ii) the DEAE-dextran method (Sompayrac and Danna, 1981). A description of each method follows.

For the calcium phosphate transfections, COS-1 cells at about 60% confluency in 60 mm culture dishes were used for transfection. For each 60 mm dish to be transfected, 10 μg of plasmid DNA was mixed with 250 μl of 0.25 M $CaCl_2$ in a 1.5 ml microcentrifuge tube. This DNA solution was added with slow agitation to 250 μl of HeBS buffer (1% HEPES (w/vol), 1.6% NaCl (w/vol), 0.075% KCl (w/vol), 0.025% Na_2HPO_4 (w/vol), 0.2% dextrose (w/vol), pH adjusted to 7.1) in a 15 ml clear polystyrene tube to form a fine precipitate. The DNA solution was incubated at room temperature for 30 to 45 min, during which time the DME growth media on the COS-1 cells was removed and replaced with fresh DME growth medium containing 10% calf serum. After the incubation at room temperature, the DNA precipitate was added to the COS-1 cells. The cells were then incubated at 37°C for about 4 hours. Following this, the media was removed, and the cells were glycerol shocked by adding 0.5 ml of serum free DME medium containing 15% glycerol (vol/vol). After 2 min, 10 ml of serum free DME medium was

added to dilute the glycerol. The medium was then removed, and the cells washed twice with 10 ml of serum free DME medium, and incubated with DME medium containing 10% calf serum at 37°C for 40 to 48 hours.

Subconfluent monolayers of Vero cells at about 60% confluency were transfected using the DEAE-dextran protocol (Sompayrac and Danna, 1981). In this procedure, for each 60 mm plate to be transfected, 0.5 to 3 μg of plasmid DNA was added to 1 ml of serum free DME medium containing 100 μg/ml DEAE-dextran. This was incubated at room temperature for 30 min, during which time the Vero cells in 60 mm plates were washed twice with serum free DME medium. The medium containing the DNA and DEAE-dextran was added to the cells, and incubated at 37°C for 2 to 4 hours, after which the medium was removed, the cells washed twice with serum free DME medium, and finally 5 ml DME medium containing 7% fetal bovine serum added and cells incubated at 37°C.

2.2.15 Metabolic labelling of transfected cells

For metabolic labelling of the expressed proteins, the transfected COS-1 cells were incubated at 37°C for 40 to 48 hours after transfection. After this incubation, the media was removed from the plates, the cells were washed with phosphate-buffered saline (PBS: 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH adjusted to 7.4) and starved for

1 hour by incubating with 0.5 ml DME medium without methionine at 37°C. The proteins were labelled by the addition of 0.5 ml of DME medium containing 50 to 100 μCi of [^{35}S]methionine (specific activity 1100 Ci/mmol) and the cells were left for the specified time at 37°C. For pulse-chase experiments, the cells were labelled for the required amount of time, and were then chased by adding 0.5 ml of DME medium containing 2.5 mM cold methionine, and the cells were then harvested at the desired time point.

2.2.16 Immunoprecipitation

At the end of the labelling or chase period, the 1 ml of labelling or chase medium was removed from the cells and was either discarded, or if needed, was placed in a 1.5 ml microcentrifuge tube and an equal volume of cold 2X RIPA lysis buffer added (2% Nonidet P-40 (vol/vol), 0.8% sodium deoxycholate (w/vol), 132 mM EDTA, 20 mM Tris-HCl (pH 7.4), 2 mM phenylmethylsulfonyl fluoride (PMSF) and 200 units/ml aprotinin). The cells were washed with cold PBS, and scraped into 0.5 ml cold 1X RIPA lysis buffer (1% Nonidet P-40 (vol/vol), 0.4% sodium deoxycholate (w/vol), 66 mM EDTA, 10 mM Tris-HCl (pH 7.4), 1 mM PMSF and 100 units/ml aprotinin). The cell lysate was then transferred into 1.5 ml microcentrifuge tubes, and particulate matter removed from both the media and cell lysates by centrifugation for 2 min in a microcentrifuge,

and the supernatants were then transferred into fresh 1.5 ml microcentrifuge tubes.

To this lysate was added 1 to 5 μl of the appropriate antibody (anti-HSV-1, monoclonal anti-gB, or anti-VSV), and the tubes were rotated at 4°C for 1 to 4 hours, after which 50 to 100 μl of a 10% protein A-Sepharose (w/vol) suspension in 1X RIPA lysis buffer was added and the tubes rotated at 4°C for another 1 to 4 hours. The protein A-Sepharose beads were then pelleted by centrifugation for 2 min at 4°C in a microcentrifuge, and the supernatant was discarded. The pellet was washed 4 times with 1 ml of cold 1X RIPA lysis buffer containing 0.3% SDS (w/vol). After the final wash, the beads were resuspended in 10 to 100 μl of 1X RIPA lysis buffer containing 0.3% SDS (w/vol), and an equal volume of 2X sample loading buffer (0.125 M Tris-HCl (pH 6.8), 4% SDS (w/vol), 20% glycerol (vol/vol), 0.05% bromophenol blue (w/vol) and 10% ß-mercaptoethanol (vol/vol)) was added. To elute the antibody/protein complex from the protein-A sepharose beads, the samples were placed in a boiling water bath for 3 min or, if heat denaturation of the protein was not desired, the samples were incubated at 37°C for 15 min with occasional vortexing, and the samples subsequently analyzed by SDS-PAGE (see section 2.2.17).

2.2.17 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated by electrophoresis using the discontinuous system of Laemmli (1970). The resolving gels contained 0.375 M Tris-HCl (pH 8.8), 0.1% SDS (w/vol), 0.1% ammonium persulfate (w/vol), 0.07% TEMED (vol/vol), and 7 to 10% acrylamide (acrylamide:bisacrylamide, 40:1). For larger proteins, the lower acrylamide concentrations were used, and vice versa for smaller proteins. The stacking gel contained 0.125 M Tris-HCl (pH 6.8), 0.1% SDS (w/vol), 0.05% ammonium persulfate (w/vol), 0.05% TEMED (vol/vol), 3.89% acrylamide (w/vol), and 0.11% bisacrylamide (w/vol). The gels were electrophoresed using gel running buffer (0.02 M Tris, 0.192 M glycine, 0.1% SDS (w/vol), pH 6.8). An equal volume of 2X sample buffer (0.125 M Tris-HCl (pH 6.8), 4% SDS (w/vol), 20% glycerol (vol/vol), 0.05% bromophenol blue (w/vol) and 10% ß-mercaptoethanol (vol/vol)) was generally added to samples, and the samples heated in a boiling water bath prior to electrophoresis to denature the proteins. The dyes present in the sample buffer allow the electrophoresis to be monitored, and the gels were generally run until the bromophenol blue dye reached the bottom of the gel.

After electrophoresis, the gels were stained and fixed by incubating at room temperature with gentle shaking in staining solution (0.125% Comassie Blue (w/vol), 50% methanol (vol/vol), 10% acetic acid (vol/vol)) for 10 to 30 min. The

gels were then destained in destaining solution (50% methanol (vol/vol), 10% acetic acid (vol/vol)). Following this, the gels were processed for fluorography.

2.2.18 Fluorography of polyacrylamide gels

Two methods of fluorography, impregnation with diphenyl oxazole (Bonner and Laskey, 1974) or impregnation with sodium salicylate (Chamberlain 1979), were used to enhance detection of radioactivity in the polyacrylamide gels.

2.2.18.1 Impregnation with diphenyl oxazole (PPO)

The gels were stained and fixed as described, then placed under running water for 30 min. The gels were then placed in 200 ml of dimethyl sulfoxide (DMSO) for 30 min, after which the DMSO was replaced with fresh DMSO and the gel left for a further 30 min. The gels were then placed in 200 ml of 20% PPO (w/vol) in DMSO, for 1.5 to 3 hours. The PPO/DMSO was then removed, and the gels washed under running water for 1 hour. The gels were then placed onto filter papers (Watman no.1) and dried at 60°C on a gel dryer for about 2 hours, or until the gel was dry. The gels were then exposed to Kodak XAR-5 film at -70°C.

2.2.18.2 Impregnation with sodium salicylate

After staining and fixing, the gels were placed under running water for 30 min, and then placed in a solution of 1 M salicylic acid, 1 M NaOH (pH adjusted to 5-7 with NaOH) for 30 min. The gels were then placed onto filter papers, dried and exposed as described in section 2.18.1.

2.2.19 Tunicamycin treatment of transfected cells

Duplicate sets of COS-1 cells grown in 60 mm plates were transfected with pXM or p91023 expression plasmids containing the deletion mutants or the wild type gB gene as described in section 2.2.14. The cells were then incubated at 37°C for 40 to 45 hours. Following this incubation, one plate of each set was starved for 1 hour using 0.5 ml of DME medium lacking methionine, and the duplicate plate was similarly starved using 0.5 ml of DME medium lacking methionine and supplemented with 2 μg/ml of tunicamycin. The cells were then labelled by adding 50 μCi of [^{35}S]methionine to each plate and incubating for 2 to 4 hours at 37°C. Both the media and cell lysates were then harvested (section 2.2.16) and immunoprecipitated (section 2.2.16) with anti-HSV-1 antiserum (Dako Corp.), and subsequently analyzed by SDS-PAGE (section 2.2.17).

2.2.20 Indirect immunofluorescence

COS-1 cells grown on coverslips in 60 mm plates were transfected as described in section 2.2.14. At 40 to 48 hours post-transfection, the cells were washed twice with cold PBS and treated for either cell surface or internal immunofluorescence.

For surface immunofluorescence, the washed cells were fixed in a fresh solution of 2% paraformaldehyde (w/vol) in PBS. The cells were fixed for 5 to 10 min at 4°C, then washed twice with PBS, and the coverslips were carefully transferred into porcelain vertical racks and immersed in 100 ml of PBS in a 500 ml beaker.

For internal immunofluorescence, the washed coverslips were carefully transferred into porcelain vertical racks, and immersed in acetone at -20°C, and placed in a -20°C freezer for 15 to 20 min. The coverslips were then immersed in 100 ml of PBS in a 500 ml beaker.

From this point on, both sets of coverslips (internal and surface) were treated the same. The coverslips in the porcelain vertical racks were transferred from the 100 ml PBS to a 500 ml beaker containing 100 ml of PBS with 0.5% BSA (w/vol). After 10 to 30 min, the coverslips were carefully laid out cell side up on moist paper towels placed in a 100 mm diameter petri dishes. Then 30 μl of anti-HSV-1 antiserum (Dako Corp.), which had been pre-absorbed with COS-1 cells and

diluted 1:30 in PBS, was added onto each coverslip, the petri dish then covered and placed at 37°C for 45 min.

The coverslips were then washed in 100 ml of PBS, then incubated in 100 ml of PBS containing 0.5% BSA for 10 to 30 min, and then again laid out cell side up on moist paper towels, and 30 μl of fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (1:20 dilution in PBS) was added to the cells, the petri dish was covered and incubated at 37°C for 45 min. The coverslips were then washed with 100 ml of PBS containing 0.5% BSA, and then with a further 100 ml of PBS. The coverslips were then placed cell side up on dry paper towels and allowed to air dry, then were mounted on microscope slides using 50% glycerol (vol/vol) in PBS. The cells were examined under a Zeiss fluorescence microscope equipped with phase contrast optics, and photographs of both fluorescence and phase contrast were taken using Kodak black and white T-max 400 film.

2.2.21 Endoglycosidase H digestion

Plates of COS-1 cells were transfected with pXM or p91023 expression plasmids containing the deletion mutants or the wild type gB gene as described in section 2.2.14. At 40 to 45 hours post-transfection, the medium was removed from the plates, the cells were washed with PBS and were starved by adding 0.5 ml of DME without methionine. The cells were

incubated at 37°C for 1 hour, after which 75 to 100 μCi of [^{35}S]methionine was added, and the cells were pulse-labelled by incubating at 37°C for 15 min. Following the 15 min pulse label, the medium was removed, the cells were washed with PBS and chased with DME supplemented with 2.5 mM cold methionine. The cells were then incubated for 0, 30, 60, 120, or 180 min at 37°C. At the end of the chase period, the cells were lysed in 1X RIPA lysis buffer (1% Nonidet P-40 (vol/vol), 0.4% sodium deoxycholate (w/vol), 66 mM EDTA, 10 mM Tris-HCl (pH 7.4), 1 mM PMSF and 100 units/ml aprotinin) and immunoprecipitated as described in section 2.2.16. The immunoprecipitate was resuspended in 20 μl of 50 mM Tris-HCl (pH 6.8) containing 1% SDS (w/vol) and heated for 2 min in a boiling water bath. The supernatants were divided into 2 aliquots of 10 μl each. To one aliquot was added 1 μl of endoglycosidase H (endo H) (30 μg/ml, 45 units/mg) (ICN Immunobiologicals) and both aliquots were incubated at 37°C for 16 hours. The samples were then mixed with 10 μl of 2X sample loading buffer (0.125 M Tris-HCl pH 6.8, 4% SDS (w/vol), 20% glycerol (vol/vol), 0.05% bromophenol blue (w/vol) and 10% ß-mercaptoethanol (vol/vol)) and analyzed by SDS-PAGE (section 2.2.17). The bands on the resultant autoradiograms were quantified by scanning with a densitometer in the linear exposure range of the films, and the results used to determine the rate of acquisition of endo H resistance

by determining the percentage of endo H resistant species at each time point.

2.2.22 Lactoperoxidase-catalyzed cell-surface iodination

Lactoperoxidase-catalyzed cell surface iodination was performed essentially as described by Guan *et al.* (1984). COS-1 cells grown in 100 mm dishes were transfected with the appropriate DNA as described in section 2.2.14. At 40 to 45 hours post-transfection, the cells were washed twice with phosphate-buffered NaCl (PBCl: 10 mM Na_2HPO_4, 150 mM NaCl, pH adjusted to 7.4), and 3 ml of PBCl added to each plate. To each plate was then added 135 μl of 2% glucose in PBCl, 60 μg lactoperoxidase (Boehringer Mannheim), and 250 μCi of $Na^{125}I$ (carrier-free, Du Pont-New England Nuclear). The reaction was initiated by adding 0.5 units of glucose-oxidase (Boehringer Mannheim). The cells were incubated at room temperature for 40 min with occasional rocking. The cells were then washed twice with phosphate-buffered NaI (PBI: 10 mM Na_2HPO_4, 150 mM NaI, pH adjusted to 7.4), followed by 5 washings with PBCl. Following the washings, the cells were lysed in 1X RIPA (section 2.2.16), immunoprecipitated (section 2.2.16), and analyzed by SDS-PAGE (section 2.2.17).

2.2.23 Subcellular fractionation of transfected COS-1 cells

Fractionation of cells into nuclear and post-nuclear fractions were carried out according to the procedure of Bos *et al.* (1989). Plates of COS-1 cells (100 mm diameter) were transfected as described in section 2.2.14. At 40 to 45 hours post-transfection, the medium was removed from the plates, the cells were washed with PBS and 1 ml of DME lacking methionine was added. The cells were incubated at 37°C for 1 hour, and then 250 μCi of [^{35}S]methionine was added to each plate, and the cells incubated for 30 min at 37°C. Following this labelling, the cells were chased by washing with PBS and adding 5 ml of DME supplemented with 2.5 mM cold methionine, followed by incubation at 37°C for 1.5 hours.

At the end of the chase, the cells were scraped into 2 ml of cold PBS, transferred into 15 ml polystyrene tubes, and centrifuged at 1,000xg for 5 min at 4°C. The cells were then washed with 5 ml of cold hypotonic buffer (25 mM Tris-HCl (pH 7.8), 1 mM $MgCl_2$, 5 mM KCl), resuspended in 1 ml of the same hypotonic buffer, and incubated on ice for 5 min. An equal volume of hypotonic buffer containing 1% Nonidet P-40 (vol/vol) was then added, and after an additional 5 min on ice, the nuclei were recovered by centrifugation at 1,000xg for 5 min. The pelleted nuclei were washed with the hypotonic buffer containing 0.5% Nonidet P-40 (vol/vol), and the washings were combined with the supernatant and was used as

the post-nuclear fraction. The nuclear pellet was lysed by resuspending in nuclear lysis buffer (25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Nonidet P-40 (vol/vol), 0.5% sodium deoxycholate (w/vol), 0.5% SDS (w/vol)), and the lysed nuclei passed through a 25-gauge needle to shear the DNA. The post-nuclear fraction was adjusted to the same salt and detergent concentration as the nuclear fraction. Both fractions were centrifuged at 12,000xg for 5 min to pellet cell debris, and the supernatants were then immunoprecipitated (section 2.2.16) and analyzed by SDS-PAGE (section 2.2.17).

The bands on the resultant autoradiograms were quantified by scanning with a densitometer in the linear exposure range of the films, and the results used to determine the percentage of protein found in each fraction.

2.2.24 Oligomerization assay

The oligomeric state of the gB-1 glycoprotein was determined by a method based on the observation that the HSV-1 gB glycoprotein forms heat-dissociable but detergent-stable dimers (Claesson-Welsh and Spear, 1986; Sarmiento and Spear, 1979).

COS-1 cells grown in 100 mm plates were transfected with the appropriate DNA as described in section 2.2.14, and 40 to 45 hours post-transfection the cells were starved for 1 hour in 1 ml of DME medium lacking methionine. The cells were then

labelled with the addition of 100 μCi of [^{35}S]methionine per 100 mm plate, and the cells incubated at 37°C for 2 hours. The cells were lysed with 1 ml of a modified RIPA lysis buffer (10 mM Tris-HCl (pH 7.4), 0.5% Nonidet P-40 (vol/vol), 0.5% sodium deoxycholate (w/vol), 150 mM NaCl, 1 mM PMSF, 100 units/ml aprotinin). The cell lysates were then immunoprecipitated (section 2.2.16) with the following modifications: (i) the cleared lysate supernatants were immunoprecipitated with monoclonal anti-gB 3S (Showalter *et al.*, 1981) or II-105 (Claesson-Welsh and Spear, 1986) antibodies and (ii) the immunoprecipitates were extracted with sample buffer containing 50mM Tris-HCl (pH 6.8), 0.3% SDS (w/vol), 0.1% ß-mercaptoethanol (vol/vol) without heating by incubating at 37°C for 15 min with vigorous vortexing after every 2 min. The immunoprecipitates were analyzed by SDS-PAGE on a 7.5% polyacrylamide gel (section 2.2.17).

2.2.25 Complementation assay

Subconfluent monolayers of Vero cells in 60 mm plates were transfected using the DEAE-dextran protocol (section 2.2.14). Seventeen hours post-transfection, each plate was infected with 10^6 pfu of K082 virus (Cai *et al.*, 1987; Cai *et al.*, 1988b) in 0.2 ml serum free DME medium, and incubated at 37°C for 2 hours. Unadsorbed virions were removed by treating with a glycine buffer (8 g NaCl, 0.38 g KCl, 0.1 g $MgCl_2 \cdot 6H_2O$,

0.1 g $CaCl_2 \cdot 2H_2O$, 7.5 g glycine per litre, pH adjusted to 3.0) followed by 2 washings with PBS. The cultures were incubated at 37°C for 24 hours, then harvested and viral stocks prepared and titered on both D6 (Cai *et al.*, 1987; Cai *et al.*, 1988b) and Vero cells.

To prepare the viral stocks, the infected cells in 60 mm plates were scraped off the plates into the media using a rubber policeman. This was transferred into 15 ml clear polystyrene tubes, and the cells pelleted by centrifugation at 1,000xg for 5 min at 4°C. The cells were then resuspended in 0.5 ml of cold PBS per 60 mm plate, and frozen at -70°C. The cells were then thawed quickly in a 37°C water bath, and then again frozen at -70°C. After 2 more of these freeze-thaw cycles, the cells were thawed and sonicated for 15 seconds, placed on ice to cool, then sonicated once more for 15 seconds. The cell debris was pelleted by centrifugation at 1,000xg for 5 min at 4°C, and the supernatant, containing the virus stock, was aliquoted and stored at -70°C.

To titre the virus stocks, duplicate 6 well culture plates were set up with $5x10^5$ Vero or D6 cells per well. Serial dilutions (from 10^{-2} to 10^{-8}) of the prepared virus stocks were made in serum free DME media, and 0.5 ml of each dilution was added to each of 2 duplicate wells. This was left at 37°C for 1.5 hours with periodic rocking of the plates. After the incubation, 2 ml of DME medium containing

1% FBS and 0.05% human gamma globulin was added to each well, and the plates left at 37°C with minimal disturbance for 2 to 4 days until plaques were clearly visible. Once the plaques were formed, the medium was removed, and 1 ml of crystal violet (0.1% crystal violet (w/vol) in 25 % ethanol) was added and left on the cells for 1 hour. The crystal violet was then removed, and the plaques counted.

3.0 RESULTS

3.1 Description of HSV-1 Glycoprotein gB

The HSV-1 (KOS) glycoprotein gB has been cloned and sequenced, with the nucleotide and predicted amino acid sequence shown in Figure 3.1.1 (Bzik *et al.*, 1984; Bzik *et al.*, 1986). The glycoprotein is 904 amino acids in length, with 6 potential N-linked glycosylation sites (Asn-X-Thr/Ser) and has a molecular weight of approximately 94 kDa when non-glycosylated, whereas the molecular weight of the glycosylated form is around 108 kDa (Claesson-Welsh and Spear, 1987). As shown in Figure 1.4.3.2 (section 1.4.3), the protein can be divided into 3 distinct regions: an extracytoplasmic region of 726 amino acids; a transmembrane domain of 69 amino acids which is predicted to span the lipid bilayer three times (Bzik *et al.*, 1984; Pellet *et al.*, 1985b); and a long cytoplasmic domain of 109 amino acids. As well, HSV-1 gB has a cleaved signal sequence of 30 amino acids which directs the protein to the rough endoplasmic reticulum. The protein is localized in both the inner nuclear membrane (from where the virus buds) and the plasma membrane (Compton and Courtney, 1984; Koga *et al.*, 1986; Ali *et al.*, 1987; Pachl *et al.*, 1987; Cai *et al.*, 1988; Torrisi *et al.*, 1992; Gilbert and Ghosh, 1993), and has been shown to exist as a homodimer (Sarmiento and Spear, 1979;

Figure 3.1.1 The nucleotide and predicted amino acid sequence of HSV-1 glycoprotein gB.

The DNA sequence of HSV-1 gB (Bzik *et al.*, 1984; Bzik *et al.*, 1986) is shown, along with the corresponding predicted amino acid sequence. The nucleotide and amino acid residue numbers are shown to the left of the sequence, with the amino acid number placed in parenthesis. The carboxy terminal hydrophobic domain, consisting of the 69 amino acids from residue 727 to 795 inclusive, is underlined.

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1       GTCAACGGGCCCCTCTTTGATCACTCCACCCACAGCTTCGCCCAGCCCCCCAACACCGCGCTGTATTACAGCGTCGAGAACGTGGGGCTCCTGCCGCACCTGAAGGAGG
110     AGCTCGCCCGGTTCATCATGGGGGCGGGGGGCTCGGGTGCTCATTGGGCCGTCAGCGAATTTCAGAGGTTTTACTGTTTTGACGGCATTTCCGGAATAACGCCCACTCA
219     GCGCGCCGCCTGGCGATATATTCGCGAGCTGATTATCGCCACCACACTCTTTGCCTCGGTCTACCGGTGCGGGGAGCTCGAGTTGCGCCGCCCGGACTGCAGCCGCCCG
328     ACCTCCGAAGGTCGTTACCGTTACCGCCCGGCGTATATCTCACGTACGACTCCGACTGTCCGCTGGTGGCCATCGTCGAGAGCGCCCCCGACGGCTGTATCGGCCCCC
437     GGTCGGTCGTGGTCTACGACGCCGACGTTTTCTCGATCCTCTACTCGGTCCTCCAGCACCTCGCCCCCAGGCTACCTGACGGGGGGCACGACGGGCCCCCGTAGTCCCGCC

548     ATG CAC CAG GGC GCC CCC TCG TGG GGG CGC CGG TGG TTC GTC GTA TGG GCG CTC TTG GGG TTG ACG CTG GGG GTC CTG GTG GCG
(1)     Met His Gln Gly Ala Pro Ser Trp Gly Arg Arg Trp Phe Val Val Trp Ala Leu Leu Gly Leu Thr Leu Gly Val Leu Val Ala

632     TCG GCG GCT CCG AGT TCC CCC GGC ACG CCT GGG GTC GCG GCC GCG ACC CAG GCG GCG AAC GGG GGC CCT GCC ACT CCG GCG CCG
(29)    Ser Ala Ala Pro Ser Ser Pro Gly Thr Pro Gly Val Ala Ala Ala Thr Gln Ala Ala Asn Gly Gly Pro Ala Thr Pro Ala Pro

716     CCG CCC CTT GGC GCC GCC CCA ACG GGG GAC CCG AAA CCG AAG AAG AAC AAA AAA CCG AAA AAC CCA ACG CCA CCA CGC CCC GCC
(57)    Pro Pro Leu Gly Ala Ala Pro Thr Gly Asp Pro Lys Pro Lys Lys Asn Lys Lys Pro Lys Asn Pro Thr Pro Pro Arg Pro Ala

800     GGC GAC AAC GCG ACC GTC GCC GCG GGC CAC GCC ACC CTG CGC GAG CAC CTG CGG GAC ATC AAG GCG GAG AAC ACC GAT GCA AAC
(85)    Gly Asp Asn Ala Thr Val Ala Ala Gly His Ala Thr Leu Arg Glu His Leu Arg Asp Ile Lys Ala Glu Asn Thr Asp Ala Asn

884     TTT TAC GTG TGC CCA CCC CCC ACG GGC GCC ACG GTG GTG CAG TTC GAG CAG CCG CGC CGC TGC CCG ACC CGG CCC GAG GGT CAG
(113)   Phe Tyr Val Cys Pro Pro Pro Thr Gly Ala Thr Val Val Gln Phe Glu Gln Pro Arg Arg Cys Pro Thr Arg Pro Glu Gly Gln

968     AAC TAC ACG GAG GGC ATC GCG GTG GTC TTC AAG GAG AAC ATC GCC CCG TAC AAG TTC AAG GCC ACC ATG TAC TAC AAA GAC GTC
(141)   Asn Tyr Thr Glu Gly Ile Ala Val Val Phe Lys Glu Asn Ile Ala Pro Tyr Lys Phe Lys Ala Thr Met Tyr Tyr Lys Asp Val

1052    ACC GTT TCG CAG GTG TGG TTC GGC CAC CGC TAC TCC CAG TTT ATG GGG ATC TTT GAG GAC CGC GCC CCC GTC CCC TTC GAG GAG
(169)   Thr Val Ser Gln Val Trp Phe Gly His Arg Tyr Ser Gln Phe Met Gly Ile Phe Glu Asp Arg Ala Pro Val Pro Phe Glu Glu

1136    GTG ATC GAC AAG ATC AAC GCC AAG GGG GTC TGT CGG TCC ACG GCC AAG TAC GTG CGC AAC AAC CTG GAG ACC ACC GCG TTT CAC
(197)   Val Ile Asp Lys Ile Asn Ala Lys Gly Val Cys Arg Ser Thr Ala Lys Tyr Val Arg Asn Asn Leu Glu Thr Thr Ala Phe His

1220    CGG GAC GAC CAC GAG ACC GAC ATG GAG CTG AAA CCG GCC AAC GCC GCG ACC CGC ACG AGC CGG GGC TGG CAC ACC ACC GAC CTC
(225)   Arg Asp Asp His Glu Thr Asp Met Glu Leu Lys Pro Ala Asn Ala Ala Thr Arg Thr Ser Arg Gly Trp His Thr Thr Asp Leu

1304    AAG TAC AAC CCC TCG CGG GTG GAG GCG TTC CAC CGG TAC GGG ACG ACG GTA AAC TGC ATC GTC GAG GAG GTG GAC GCG CGC TCG
(253)   Lys Tyr Asn Pro Ser Arg Val Glu Ala Phe His Arg Tyr Gly Thr Thr Val Asn Cys Ile Val Glu Glu Val Asp Ala Arg Ser

1388    GTG TAC CCG TAC GAC GAG TTT GTG CTG GCG ACT GGC GAC TTT GTG TAC ATG TCC CCG TTT TAC GGC TAC CGG GAG GGG TCG CAC
(281)   Val Tyr Pro Tyr Asp Glu Phe Val Leu Ala Thr Gly Asp Phe Val Tyr Met Ser Pro Phe Tyr Gly Tyr Arg Glu Gly Ser His

1472    ACC GAA CAC ACC ACG TAC GCC GCC GAC CGC TTC AAG CAG GTC GAC GGC TTC TAC GCG CGC GAC CTC ACC ACC AAG GCC CGG GCC
(309)   Thr Glu His Thr Thr Tyr Ala Ala Asp Arg Phe Lys Gln Val Asp Gly Phe Tyr Ala Arg Asp Leu Thr Thr Lys Ala Arg Ala

1556    ACG GCG CCG ACC ACC CGG AAC CTG CTC ACG ACC CCC AAG TTC ACC GTG GCC TGG GAC TGG GTG CCA AAG CGC CCG TCG GTC TGC
(337)   Thr Ala Pro Thr Thr Arg Asn Leu Leu Thr Thr Pro Lys Phe Thr Val Ala Trp Asp Trp Val Pro Lys Arg Pro Ser Val Cys

1640    ACC ATG ACC AAG TGG CAG GAA GTG GAC GAG ATG CTG CGC TCC GAG TAC GGC GGC TCC TTC CGA TTC TCC TCC GAC GCC ATA TCC
(365)   Thr Met Thr Lys Trp Gln Glu Val Asp Glu Met Leu Arg Ser Glu Tyr Gly Gly Ser Phe Arg Phe Ser Ser Asp Ala Ile Ser

1724    ACC ACC TTC ACC ACC AAC CTG ACC GAG TAC CCG CTC TCG CGC GTG GAC CTG GGG GAC TGC ATC GGC AAG GAC GCC CGC GAC GCC
(393)   Thr Thr Phe Thr Thr Asn Leu Thr Glu Tyr Pro Leu Ser Arg Val Asp Leu Gly Asp Cys Ile Gly Lys Asp Ala Arg Asp Ala

1808    ATG GAC CGC ATC TTC GCC CGC AGG TAC AAC GCG ACG CAC ATC AAG GTG GGC CAG CCG CAG TAC TAC CTG GCC AAT GGG GGC TTT
(421)   Met Asp Arg Ile Phe Ala Arg Arg Tyr Asn Ala Thr His Ile Lys Val Gly Gln Pro Gln Tyr Tyr Leu Ala Asn Gly Gly Phe

1892    CTG ATC GCG TAC CAG CCC CTT CTC AGC AAC ACG CTC GCG GAG CTG TAC GTG CGG GAA CAC CTC CGA GAG CAG AGC CGC AAG CCC
(449)   Leu Ile Ala Tyr Gln Pro Leu Leu Ser Asn Thr Leu Ala Glu Leu Tyr Val Arg Glu His Leu Arg Glu Gln Ser Arg Lys Pro

1976    CCA AAC CCC ACG CCC CCG CCG CCC GGG GCC AGC GCC AAC GCG TCC GTG GAG CGC ATC AAG ACC ACC TCC TCC ATC GAG TTC GCC
(477)   Pro Asn Pro Thr Pro Pro Pro Pro Gly Ala Ser Ala Asn Ala Ser Val Glu Arg Ile Lys Thr Thr Ser Ser Ile Glu Phe Ala

2060    CGG CTG CAG TTT ACG TAC AAC CAC ATA CAG CGC CAT GTC AAC GAT ATG TTG GGC CGC GTT GCC ATC GCG TGG TGC GAG CTA CAG
(505)   Arg Leu Gln Phe Thr Tyr Asn His Ile Gln Arg His Val Asn Asp Met Leu Gly Arg Val Ala Ile Ala Trp Cys Glu Leu Gln

2144    AAT CAC GAG CTG ACC CTG TGG AAC GAG GCC CGC AAG CTG AAC CCC AAC GCC ATC GCC TCG GTC ACC GTG GGC CGG CGG GTG AGC
(533)   Asn His Glu Leu Thr Leu Trp Asn Glu Ala Arg Lys Leu Asn Pro Asn Ala Ile Ala Ser Val Thr Val Gly Arg Arg Val Ser

2228    GCG CGG ATG CTC GGC GAC GTG ATG GCC GTC TCC ACG TGC GTG CCG GTG GCC GCG GAC AAC GTG ATC GTC CAA AAC TCG ATG CGC
(561)   Ala Arg Met Leu Gly Asp Val Met Ala Val Ser Thr Cys Val Pro Val Ala Ala Asp Asn Val Ile Val Gln Asn Ser Met Arg

2312    ATC AGC TCG CGG CCC GGG GCC TGC TAC AGC CGC CCC CTG GTC AGC TTT CGG TAC GAA GAC CAG GGC CCG TTG GTC GAG GGG CAG
(589)   Ile Ser Ser Arg Pro Gly Ala Cys Tyr Ser Arg Pro Leu Val Ser Phe Arg Tyr Glu Asp Gln Gly Pro Leu Val Glu Gly Gln

2396    CTG GGG GAG AAC AAC GAG CTG CGG CTG ACG CGC GAT GCG ATC GAG CCG TGC ACC GTG GGA CAC CGG CGC TAC TTC ACC TTC GGT
(617)   Leu Gly Glu Asn Asn Glu Leu Arg Leu Thr Arg Asp Ala Ile Glu Pro Cys Thr Val Gly His Arg Arg Tyr Phe Thr Phe Gly

2480    GGG GGC TAC GTG TAC TTC GAG GAG TAC GCG TAC TCC CAC CAG CTG AGC CGC GCC GAC ATC ACC ACC GTC AGC ACC TTC ATC GAC
(645)   Gly Gly Tyr Val Tyr Phe Glu Glu Tyr Ala Tyr Ser His Gln Leu Ser Arg Ala Asp Ile Thr Thr Val Ser Thr Phe Ile Asp

2564    CTC AAC ATC ACC ATG CTG GAG GAT CAC GAG TTT GTC CCC CTG GAG GTG TAC ACC CGC CAC GAG ATC AAG GAC AGC GGC CTG CTG
(673)   Leu Asn Ile Thr Met Leu Glu Asp His Glu Phe Val Pro Leu Glu Val Tyr Thr Arg His Glu Ile Lys Asp Ser Gly Leu Leu

2648    GAC TAC ACG GAG GTC CAG CGC CGC AAC CAG CTG CAC GAC CTG CGC TTC GCC GAC ATC GAC ACG GTC ATC CAC GCC GAC GCC AAC
(701)   Asp Tyr Thr Glu Val Gln Arg Arg Asn Gln Leu His Asp Leu Arg Phe Ala Asp Ile Asp Thr Val Ile His Ala Asp Ala Asn

2732    GCC GCC ATG TTC GCG GGC CTG GGC GCG TTC TTC GAG GGG ATG GGC GAC CTG GGG CGC GCG GTC GGC AAG GTG GTG ATG GGA CTC
(729)   Ala Ala Met Phe Ala Gly Leu Gly Ala Phe Phe Glu Gly Met Gly Asp Leu Gly Arg Ala Val Gly Lys Val Val Met Gly Leu

2816    GTG GGC GGC GTG GTA TCG GCC GTG TCG GGC GTG TCC TCC TTC ATG TCC AAC CCC TTT GGG GCG CTG GCC GTG GGT CTG TTG GTC
(757)   Val Gly Gly Val Val Ser Ala Val Ser Gly Val Ser Ser Phe Met Ser Asn Pro Phe Gly Ala Leu Ala Val Gly Leu Leu Val

2900    CTG GCC GGC CTG GCG GCG GCC TTC TTC GCC TTT CGT TAC GTC ATG CGG CTG CAG AGC AAC CCC ATG AAG GCC CTG TAC CCT CTA
(785)   Leu Ala Gly Leu Ala Ala Ala Phe Phe Ala Phe Arg Tyr Val Met Arg Leu Gln Ser Asn Pro Met Lys Ala Leu Tyr Pro Leu

2984    ACC ACC AAG GAG CTC AAG AAC CCC ACC AAC CCG GAC GCG TCC GGG GAG GGC GAG GAG GGC GGC GAC TTT GAC GAG GCC AAG CTA
(813)   Thr Thr Lys Glu Leu Lys Asn Pro Thr Asn Pro Asp Ala Ser Gly Glu Gly Glu Gly Gly Asp Phe Asp Glu Ala Lys Leu

3068    GCC GAG GCC AGG GAG ATG ATA CGG TAC ATG GCC CTG GTG TCG GCC ATG GAG CGC ACG GAA CAC AAG GCC AAG AAG AAG GGC ACG
(841)   Ala Glu Ala Arg Glu Met Ile Arg Tyr Met Ala Leu Val Ser Ala Met Glu Arg Thr Glu His Lys Ala Lys Lys Lys Gly Thr

3152    AGC CGG CTG CTC AGC GCC AAG GTC ACC GAC ATG GTC ATG CGC AAG CGC CGC AAC ACC AAC TAC ACC CAA GTT CCC AAC AAA GAC
(869)   Ser Arg Leu Leu Ser Ala Lys Val Thr Asp Met Val Met Arg Lys Arg Arg Asn Thr Asn Tyr Thr Gln Val Pro Asn Lys Asp

3236    GGT GAC GCC GAC GAG GAC GAC CTG TGA CGGGGGGTTTGTTGTAAATAAAAACCACGGGTGTTAAACCGCATGCGCATCTTTTGGTTTTTTTGTTTGGTCAGC
(897)   Gly Asp Ala Asp Glu Asp Asp Leu End

3320    CTTTTGTGTGTGTGGGAAGAAAGAAAAAGGAACACATAAACTCCCCCGGGTGTCCGCGGCCTGTTTCCTCTTTCCTTTCCCGTGACAAAACGGACCCCCTTGGTCAGTG
3431    CCGATTTCCTCCCCCCCACGCCTTCCTCCACGTCAAAGGCTTTTGCATTGTAAAGCTACCCGCCTACCCGCCCCTCCCAATAAAAAAAAAAAGAACATACACCAATGGGTCT
3542    TATTTGGTATTACCTGGTTTATTTAAAAAGATATACAGTAAGACATCCCATGGTACCAAAGACCGGGGCGAATCAGCGGGCCCCCATCATCTGAGAGACGAACAAATCGGC
3653    GGCGCGGGCCGTGTCAACGTCCACGTGTGCTGCGCTGCTGGCGTTGACAAGGCCCCGGCCTCCGCGTTGGATGCTCCGGTTGGGATCC
```

Claesson-Welsh and Spear, 1986; Zhu and Courtney, 1988; Ali, 1990; Highlander, *et al.*, 1991).

The HSV-1 glycoprotein gB has been expressed transiently in COS-1 and Vero cells using cloned DNA in eucaryotic expression vectors (Ali *et al.*, 1987; Cai *et al.*, 1987; Pachl *et al.*, 1987). This transiently expressed protein is fully glycosylated, is transported to both the plasma membrane and nuclear envelope and is biologically active (it can induce cell fusion in the absence of other viral glycoproteins) (Ali *et al.*, 1987). Since this transient expression closely resembles the wild type expression of HSV-1 glycoprotein gB, it can be used as a model system to study structure and function relationships in the HSV-1 glycoprotein gB.

The majority of viral spike glycoproteins, which are integral membrane proteins, span the lipid bilayer only once (Stephens and Compans, 1988). Studies performed on the hydrophobic transmembrane sequences of these membrane glycoproteins implicate these hydrophobic domains in membrane anchoring and also in intracellular transport (Guan and Rose, 1984; Adams and Rose, 1985; Perez *et al.*, 1987; Chan and Ester, 1988; Doms *et al.*, 1988; Lazarovitz *et al.*, 1990). In the case of the coronavirus E1 glycoprotein, a specific transmembrane domain is required for retention of the glycoprotein in the Golgi (Machamer and Rose, 1987). Based on these results, which indicate that the transmembrane domain

may do more than just anchor the protein in the membrane, and the somewhat unique nature of the carboxy terminal transmembrane hydrophobic domain of HSV-1 glycoprotein gB, site directed mutagenesis was performed on the gB hydrophobic domain in order to determine its role in membrane anchoring and intracellular transport.

3.2 Construction of the mutant HSV-1 gB glycoproteins

The hydrophobic region of HSV-1 gB stretches from residue 727 to 795 (Bzik *et al.*, 1984; Bzik *et al.*, 1986). This large hydrophobic region can be further subdivided into 3 smaller hydrophobic segments each consisting of 20 to 22 residues. This is shown in Figure 3.1.1. These 3 segments are designated segment 1, 2 and 3, with segment 1 comprised of residues 727 to 746, segment 2 comprised of residues 752 to 772 and segment 3 comprised of residues 775 to 795. It is convenient to remember that segment 3 is closest to the carboxy terminus and segment 1 is closest to the amino terminus of the glycoprotein.

Deletions in the hydrophobic domain of HSV-1 gB were generated by site directed mutagenesis. In order to construct the mutants, the gB gene had to be subcloned into the M13mp18 plasmid using *Sal*I and *Eco*RI. Once the mutants were obtained, however, they had to be subcloned back into the pXM or p91023(B) vectors (for transient expression in mammalian

cells, see section 3.3) using the same restriction sites. A general scheme for this is depicted in Figure 3.2.1. Table 3.2.1 lists the oligonucleotides used for mutagenesis, as well as the protein sequences deleted along with the hydrophobicities of the remaining segments. A line diagram, showing the amino acids deleted as well as the residues remainig in the mutant proteins is shown in Figure 3.2.2.

As indicated, all of the oligonucleotides used for mutagnesis (except for Δ12 and Δ2) were synthesized on an Applied Biosystems model 381-A DNA synthesizer. Since only a small amount of the oligonucleotide was needed, small scale synthesis were performed. Typical yields obtained from the synthesis ranged from 60 to 180 μg of purified oligonucleotide. Monitoring the amount of trityl cation released from each addition cycle allows the determination of the coupling efficiency of each step, and from this the overall stepwise yield can be calculated. Typical overall stepwise yields obtained ranged from 75 to 91 percent.

Site directed mutagenesis was performed using the Kunkel method for site directed mutagenesis (Zoller and Smith, 1983; Zoller and Smith, 1984; Kunkel, 1985; Kunkel *et al.*, 1987; Zoller and Smith, 1987). Screening was performed by simply picking 6 to 12 phage plaques for each reaction, and growing 5 ml cultures of each plaque. Replicative form (double stranded) DNA was isolated from the infected bacteria, and

Figure 3.2.1 Construction of mutant HSV-1 gB genes.

A schematic representation of oligonucleotide-directed mutagenesis and cloning of mutant gB genes in the expression vectors pXM and p91023(B). p91023-gB (p91023(B) containing the full length HSV-1 gB gene) was digested with *Eco*RI and *Sal*I and the 2.1 kb fragment was cloned into *Eco*RI and *Sal*I digested M13mp18. The M13mp18-gB single stranded phage DNA was used as template for site-directed mutagenesis. The replicative form (RF) DNA from the mutant phage was digested with *Eco*RI and *Sal*I and the 2.1 kb fragment was ligated back into the pXM-gB or p91203-gB digested with *Eco*RI and *Sal*I. The plasmids containing the mutant gB-1 genes are designated as Δ1, Δ12, Δ13, Δ2, Δ23, Δ3 and Δ123 (indicating which segment(s) are deleted). The gap shows the amino acids deleted and the numbers indicate the first and the last residue number of the peptide that has been deleted in the mutant.

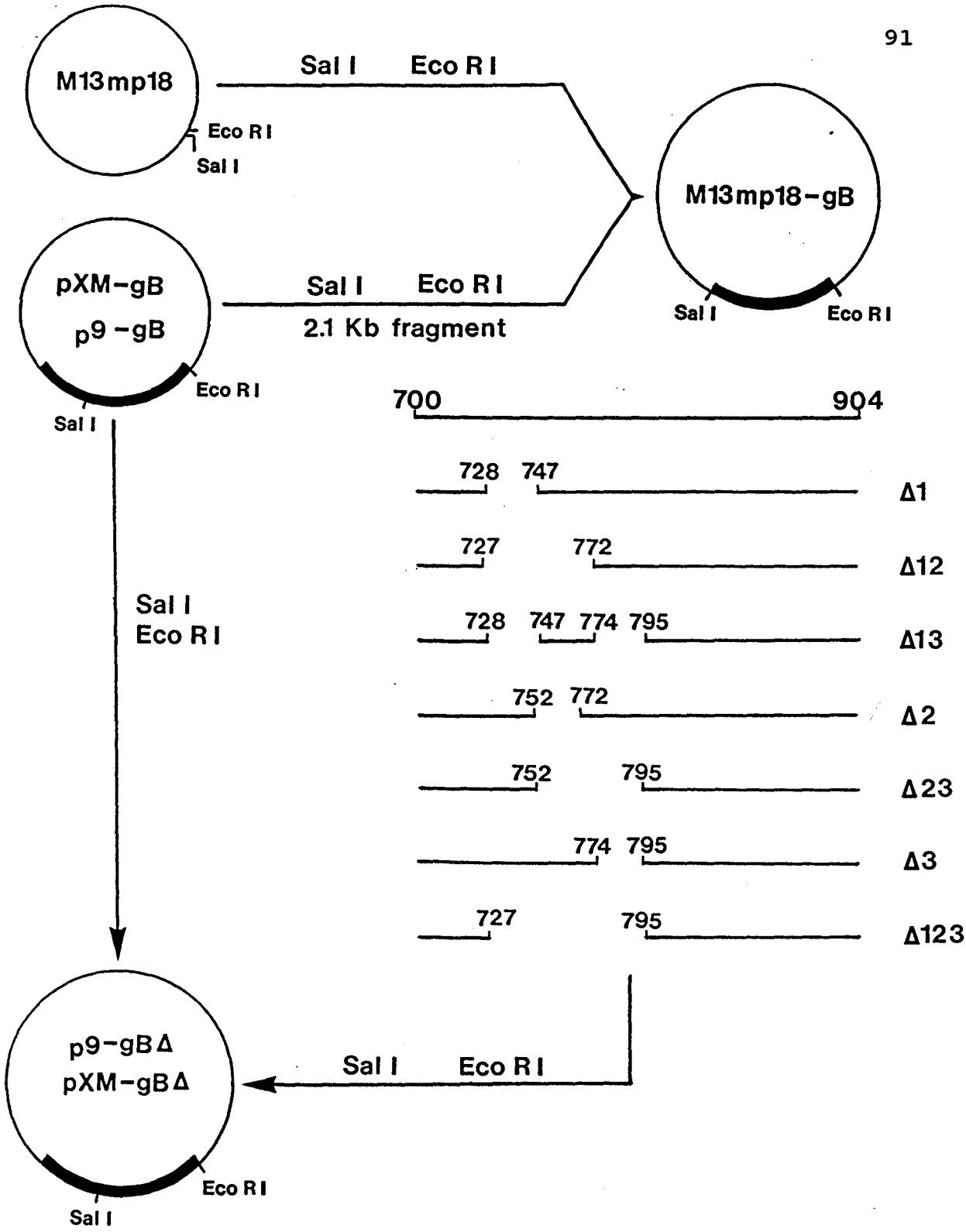

M13mp18
Eco R I
Sal I
Sal I
Eco R I
M13mp18-gB
Sal I
Eco R I
pXM-gB
p9 -gB
Sal I
Eco R I
Sal I
Eco R I
2.1 Kb fragment
700
904
728
747
Δ1
727
772
Δ12
728
747
774
795
Δ13
752
772
Δ2
752
795
Δ23
774
795
Δ3
727
795
Δ123
Sal I
Eco R I
p9-gBΔ
pXM-gBΔ
Sal I
Eco R I

Figure 3.2.2 Linear representation of the HSV-1 gB glycoprotein and the mutants constructed in this work.

The box at the top of the figure is a linear representaion of the HSV-1 glycoprotein gB molecule. The large open area represents the extracytoplasmic domain (E), the dark area is the 69 residue hydrophobic domain (H), and the shaded area is the cytoplasmic domain (C). The amino and carboxy terminals are also indicated.

Directly below is shown the wild type (WT) amino acid sequence of the carboxy terminal hydrophobic domain. The numbers shown correspond to the amino acid residue numbers as present in the HSV-1 gB glycoprotein sequence deduced from nucleotide sequence data (Bzik *et al.*, 1984; Bzik *et al.*, 1986). The three proposed hydrophobic segments are underlined, and the number of each segment (1, 2, or 3) is indicated below the sequence. The amino acid sequence of the deletion mutants is shown below that of the wild type, with the mutant designation indicated to the right of each sequence. The amino acid residues deleted in each mutant are replaced in the figure with a dot (.), with the remaining residues shown in the single letter code.

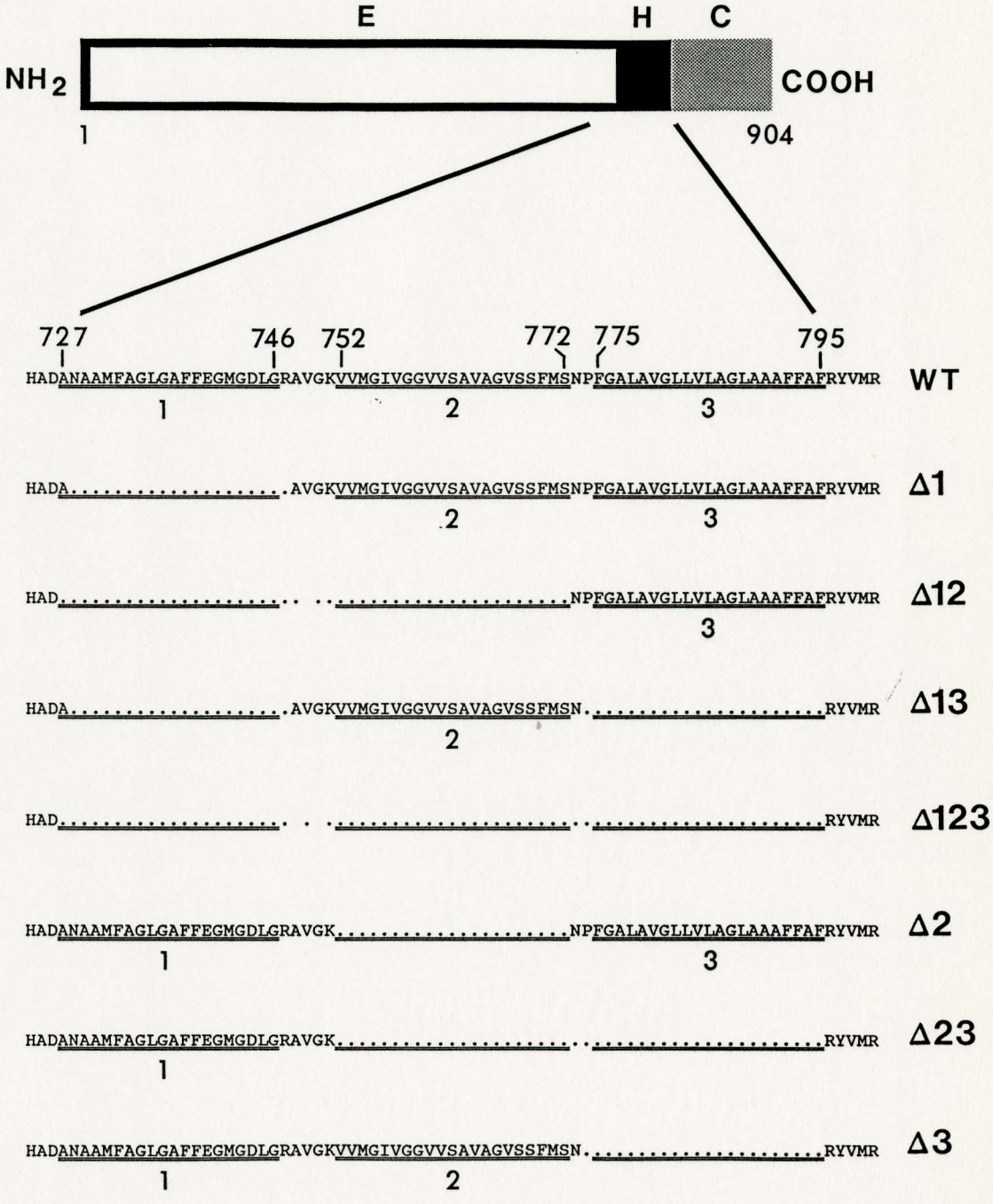

E
H
C
NH2
COOH
1
904
727
746
752
772
775
795
HADANAAMFAGLGAFFEGMGDLGRAVGKVVMGIVGGVVSAVAGVSSFMSNPFGALAVGLLVLAGLAAAFFAFRYVMR
WT
1
2
3
HADA.....AVGKVVMGIVGGVVSAVAGVSSFMSNPFGALAVGLLVLAGLAAAFFAFRYVMR
Δ1
2
3
HAD.....NPFGALAVGLLVLAGLAAAFFAFRYVMR
Δ12
3
HADA.....AVGKVVMGIVGGVVSAVAGVSSFMSN.....RYVMR
Δ13
2
HAD.....RYVMR
Δ123
HADANAAMFAGLGAFFEGMGDLGRAVGK.....NPFGALAVGLLVLAGLAAAFFAFRYVMR
Δ2
1
3
HADANAAMFAGLGAFFEGMGDLGRAVGK.....RYVMR
Δ23
1
HADANAAMFAGLGAFFEGMGDLGRAVGKVVMGIVGGVVSAVAGVSSFMSN.....RYVMR
Δ3
1
2

Table 3.2.1 Oligonucleotides used to construct the site directed deletion mutations in the HSV-1 glycoprotein gB.

Mutant	Mutagenic oligonucleotide sequence (5' to 3')	Nucleotide residues deleted	Amino Acid residues deleted	Hydrobicity of remaining segment
Δ1	CACGCCGACGCCGCGGTCGGCAA	2729-2788	728-747	2.1
Δ1,2	GTCATCCACGCCGACAACCCCTTTGGGGCGCT	2726-2863	727-772	2.4
Δ1,3	oligo Δ1 and oligo Δ3 used	2729-2788 2867-2932	728-747 774-795	1.7
Δ2	CGCGCGGTCGGCAAGAACCCCTTTGGGGCGCT	2801-2863	752-772	1.5
Δ2,3	CGCGCGGTCGGCAAGCGTTACGTCATGCGG	2801-2932	752-795	0.8
Δ3	TCCTTCATGTCCAACCGTTACGTCATGCGG	2867-2932	774-795	1.2
Δ1,2,3	GTCATCCACGCCGACCGTTACGTCATGCGG	2726-2932	727-795	-

The oligonucleotides used to construct the deletion mutants as well as the nucleotides and amino acids deleted for each mutant are shown. The nucleotide and amino acid numbers shown were derived from the published sequence of HSV-1 gB gene (Bzik *et al.*, 1984; Bzik *et al.*, 1986). The hydrophobicity of the segments were calculated according to Kyte and Doolittle (1982).

digested with *Sal*I and *Eco*RI and run on agarose gels. Since the mutations are deletions, the restiction digest fragments would be slightly smaller than the wild type and this could be visualized on the agarose gels. The single stranded DNA was then isolated from the corresponding M13 phage, and the region around the mutation site sequenced, and the results for the confirmed mutants are shown in Figures 3.2.3 and 3.2.4. The confirmed mutants were then subcloned back into pXM-gB or p91023-gB to generate plasmids harboring the desired mutation in HSV-1 gB as outlined in Figure 3.2.1. These deletion mutants were then used to study the role of the hydrophobic segments in transmembrane anchoring, intracellular transport and subcellular localization.

3.3 Expression, glycosylation and secretion of mutant HSV-1 gB glycoproteins

Transient expression of the wild type and mutant gB glycoproteins in mammalian cells was performed using the shuttle vectors pXM (Wong *et al.*, 1985; Kaufman *et al.*, 1989) and p91023(B) (Kaufman, 1985; Yang *et al.*, 1986). The first mutants obtained (Δ123, Δ23 and Δ3) were cloned into p91023(B), as was the wild type gB gene. The pXM vector was derived from the vector P91023(B), and was used for all the later mutants for a simple reason: p91023(B) contains a *Sal*I site within the tetracycline resistance gene, and this meant

Figure 3.2.3 Dideoxynucleotide sequence analysis of the wild type and the constructed mutants of HSV-1 glycoprotein gB.

The sequence of the region directly about the mutations engineered in the HSV-1 glycoprotein gB is shown. The mutants Δ2 and Δ23 are shown along with the entire sequence of the wild type region (WT). The lanes were loaded in the order shown above each sequence. The nucleotide residue number for the start and end of each sequence is shown to the right of each sequence. For the wild type, therefore, the sequence from nucleotide residue 2717 to 2967 is shown. The arrow at the right of the deletion mutant sequences depicts the junction point of the mutation, which is the point where two distal squences in the WT were by removal of the intervening sequences. Thus, for example, the arrow to the right of Δ2 is where segment 1 directly joins segment 3, thereby deleting segment 2. The sequence of the remaining mutants is shown in Figure 3.2.4.

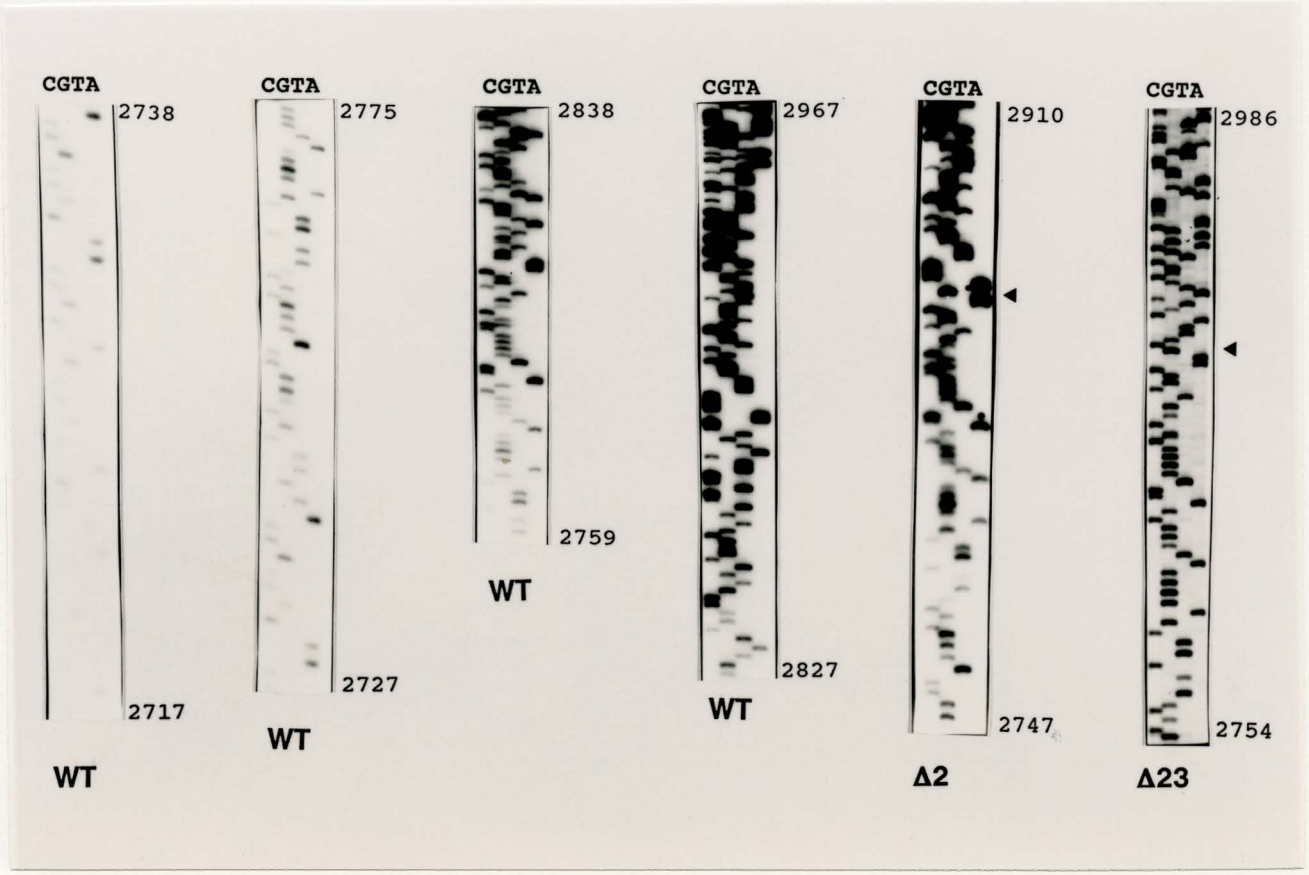
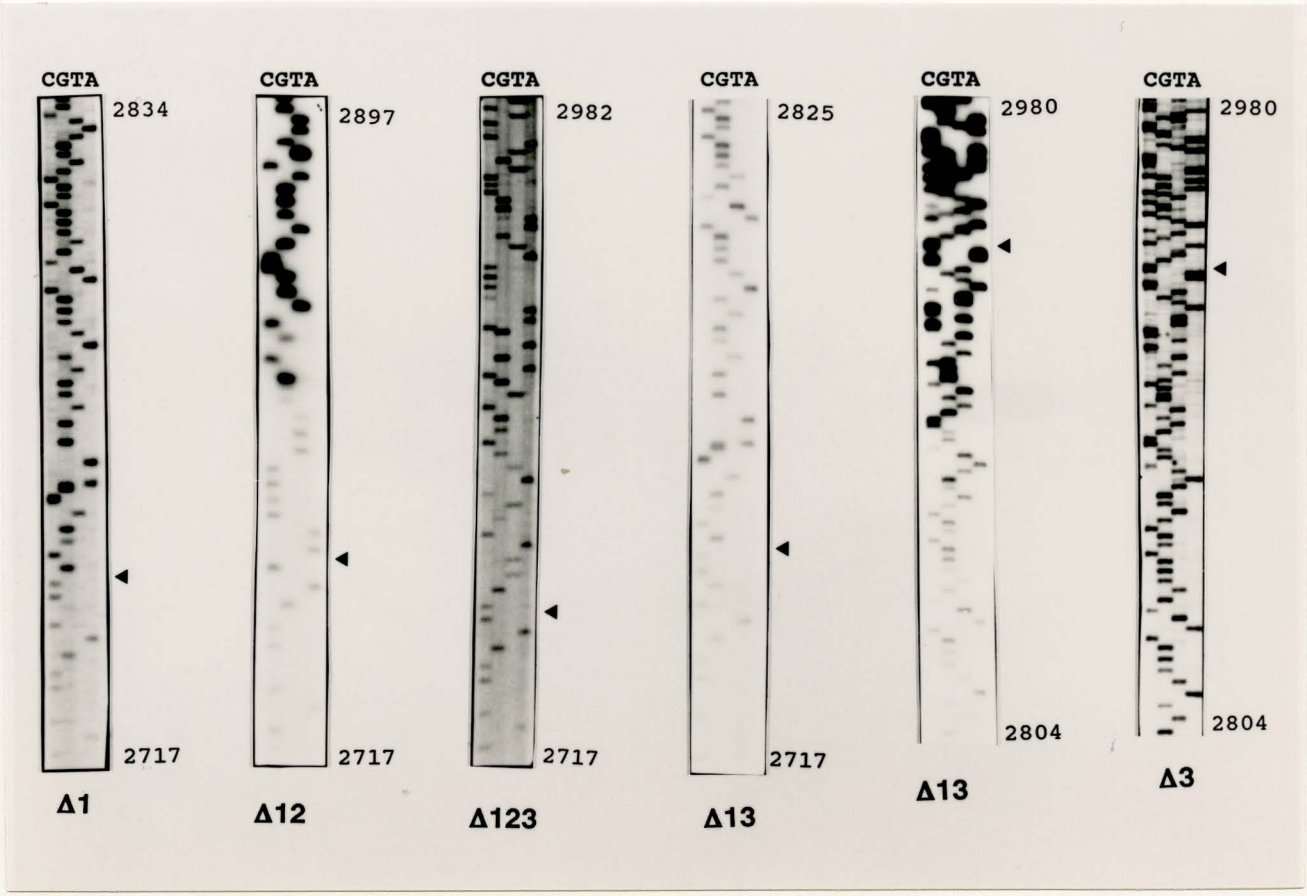

CGTA
2738
2717
WT
CGTA
2775
2727
WT
CGTA
2838
2759
WT
CGTA
2967
2827
WT
CGTA
2910
2747
Δ2
CGTA
2986
2754
Δ23

Figure 3.2.4 Dideoxynucleotide sequence analysis of the constructed mutants of HSV-1 glycoprotein gB.

The sequence of the region directly about the mutations engineered in the HSV-1 glycoprotein gB is shown. The mutants Δ1, Δ12, Δ123, Δ13 and Δ3 are shown. The lanes were loaded in the order shown above each sequence. The nucleotide residue number of the start and end of each sequence is shown to the right of each sequence. For Δ13, two sequences are shown: the first corresponds to the removal of segment 1, and the second corresponds to the removal of segment 3 (the sequences were too far apart to read using only 1 sqeuncing run). The arrow at the right of the mutant sequences depicts the junction point of the mutation (see Figure 3.2.3).


CGTA
2834
2717
Δ1
CGTA
2897
2717
Δ12
CGTA
2982
2717
Δ123
CGTA
2825
2717
Δ13
CGTA
2980
2804
Δ13
CGTA
2980
2804
Δ3

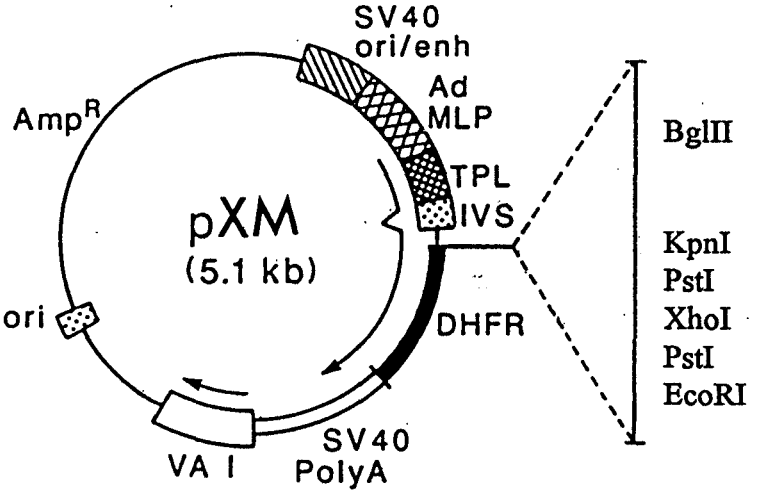
that a partial *SalI* digest was required in order to subclone the fragments from M13mp18-gB back into p91023-gB. The pXM vector, however, lacks this site since it contains the ampicillin resistance gene. Transient expression levels were similiar regardless of the vector used.

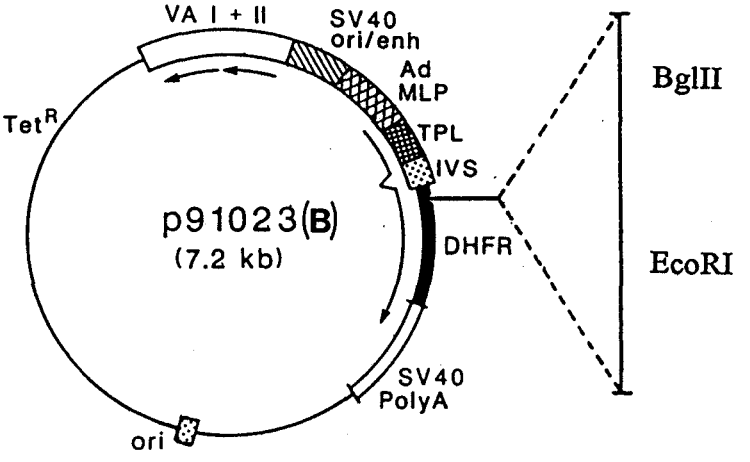
The pXM and p91023(B) plasmid maps are shown in Figure 3.3.1. These vectors contain the pBR origin of replication (Bolivar *et al.*, 1977) as well as the SV40 origin of replication and enhancer sequences. Replication from the SV40 origin is dependent on the SV40 large T-antigen. COS-1 cells are a simian cell line which constituently expresses the large T-antigen. The expressed T-antigen acts in trans to drive the replication of pXM from the SV40 origin of replication. The vectors also have a multiple cloning site downstream of an adenovirus type 2 major late promotor (AdMLP). This places the transcription (and hence expression) of the cloned cDNA under the control of a constitutive eucaryotic promoter in COS-1 cells. Other sequences present in the vectors include: SV40 polyadenylation sequences downstream of the multiple cloninig site; two copies of the 72 base pair enhancer sequence from SV40; adenovirus VA genes and tripartite leader sequences which are required for the efficient translation of mRNA made from the AdMLP (Kaufman, 1985; Schneider *et al.*, 1985; Kaufman *et al.*, 1989) and ampicillin or tetracycline resistance genes. All of these sequences result in a shuttle

Figure 3.3.1 The eucaryotic expression plasmids pXM and p91023.

The eucaryotic expression vectors pXM and p91023(B) used for transient expression of the wild type and mutant HSV-1 gB glycoproteins in COS-1 cells are shown.

Symbols: SV40 ori/enh: SV40 origin of replication and enhancer; Ad MLP: adenovirus major late promoter; TPL: tripartite leader sequence; IVS: intervening sequence; DHFR: dihydrofolate reductase coding sequence; SV40 poly A: SV40 early polyadenylation signal; ori: pBR322 origin of replication; VA I + II: adenovirus VA I and VA II genes; Amp^R: ampicillin resistance gene; Tet^R: tetracycline resistance gene. Restriction enzyme sites in the cloning site are as indicated. Adapted from Kaufman *et al.* (1989).


SV40
ori/enh
Ad
MLP
TPL
IVS
Amp^R
pXM
(5.1 kb)
ori
DHFR
SV40
PolyA
VA I
BglII
KpnI
PstI
XhoI
PstI
EcoRI


VA I + II
SV40
ori/enh
Ad
MLP
TPL
IVS
Tet^R
p91023(B)
(7.2 kb)
DHFR
SV40
PolyA
ori
BglII
EcoRI

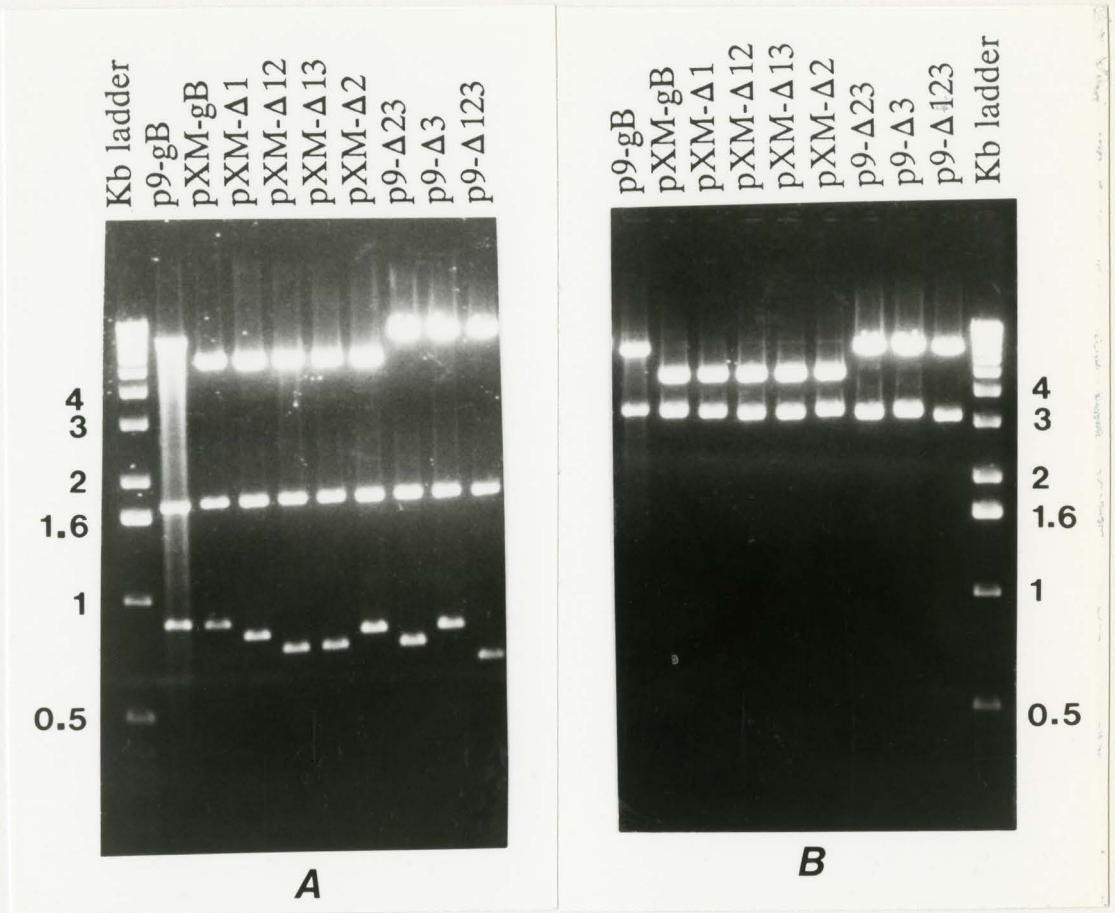
vector that yields high expression of cloned genes in COS-1 cells.

The wild type gB gene was cloned into the *Bgl*II/*Eco*RI sites of p91023(B) to generate p9-gB (Ali *et al.*, 1987). This *Bgl*II/ *Eco*RI fragment was then taken from p9-gB and cloned into the *Bgl*II/ *Eco*RI of pXM to generate pXM-gB. *Pst*I and *Bgl*II/*Eco*RI digestions of the wild type and mutant constructs are shown in Figure 3.3.2. For the *Pst*I digests, fragments of about 0.8 and 1.7 and 5.9 Kb (for pXM) or 0.8, 1.7 and 8.0 Kb (for p91023) are expected. The 0.8 Kb fragment encompasses the deletion sites, and a size shift reflecting the deletions in the mutants is seen. For the *Bgl*II/*Eco*RI digests, fragments of 3.4 and 5.0 Kb (for pXM) and 3.4 and 7.1 Kb (for p91023) are expected. The 3.4 Kb fragment is the entire gB gene, and slight size differences are seen in this fragment due to the deletion of sequences in the mutants.

To determine if the mutant glycoproteins are synthesized and glycosylated, subconfluent monolayers of COS-1 cells were transfected using the calcium phosphate method (Graham and Van Der Eb, 1973; Raviprakash *et al.*, 1990) with the mutant and wild type HSV-1 gB constructs in the pXM or p91023(B) expression vectors. The transfected cells were incubated at 37°C for 40 to 45 hours and then starved in methionine deficient media for 1 hour. A parallel set of transfections were performed with the addition of tunicamycin (2 μg/ml) in

Figure 3.3.2 Restriction enzyme analysis of the wild type and mutant HSV-1 gB genes constructed in this work and cloned into the pXM and p91023(B) plasmids.

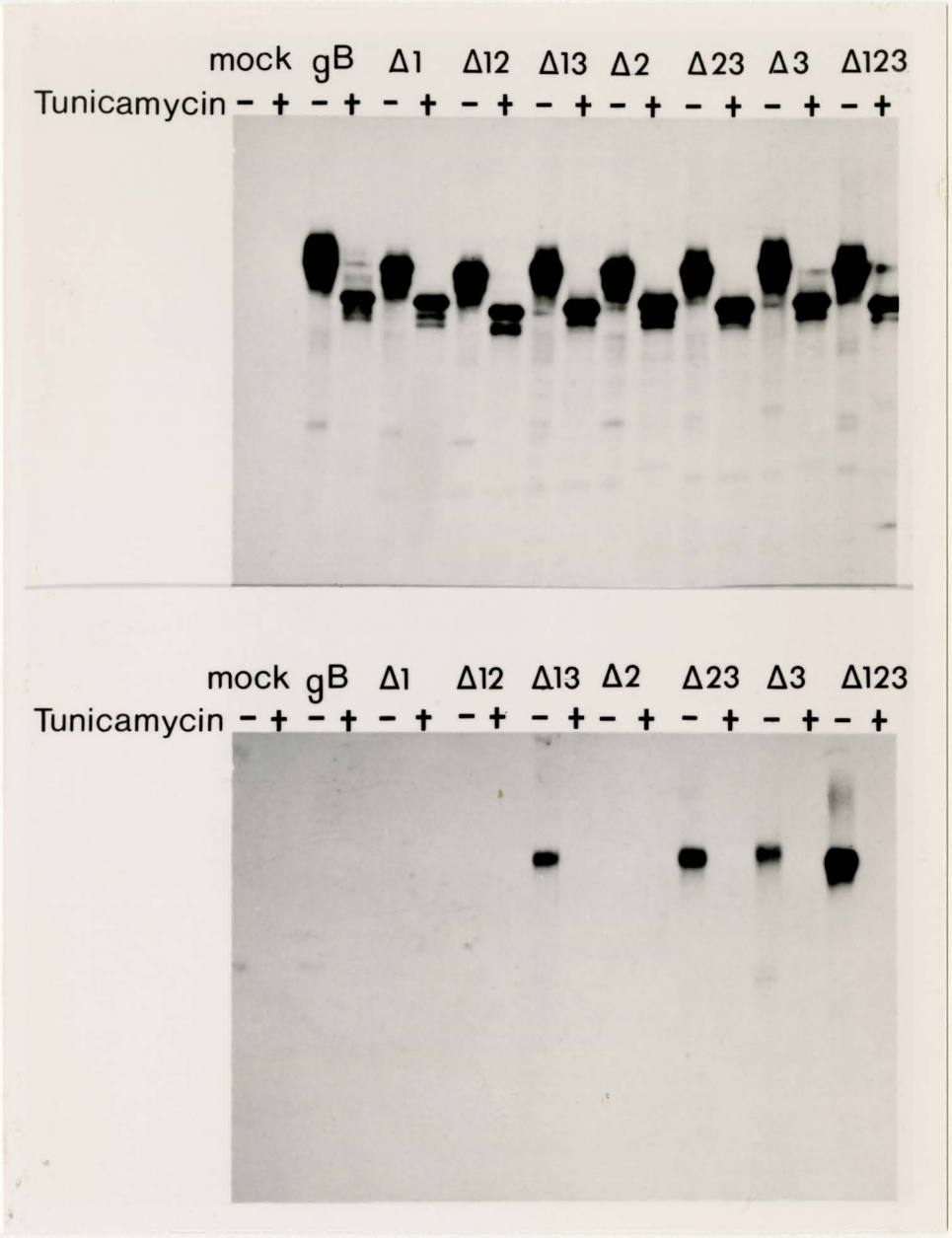
A *Pst*I (panel A) and *Bgl*II/*Eco*RI (panel B) restriction digests are shown. The kb ladder lane is indicated, and the size of the marker fragments are inicated up to 4 kb, with each band above this indicating an increase in size of 1 kb. See text for more details.


Kb ladder
p9-gB
pXM-gB
pXM-Δ1
pXM-Δ12
pXM-Δ13
pXM-Δ2
p9-Δ23
p9-Δ3
p9-Δ123
4
3
2
1.6
1
0.5
A
p9-gB
pXM-gB
pXM-Δ1
pXM-Δ12
pXM-Δ13
pXM-Δ2
p9-Δ23
p9-Δ3
p9-Δ123
Kb ladder
4
3
2
1.6
1
0.5
B

the starving media. Tunicamycin inhibits N-linked glycosylation by blocking the first step in the lipid-linked pathway of glycosylation (Struck and Lennarz, 1980). Thus, proteins expressed in the presence of tunicamycin will not be glycosylated and this will be reflected on SDS-polyacrylamide gels by their decreased molecular weight as compared to proteins expressed in the absence of tunicamycin and hence glycosylated. After starving, both sets of transfections were metabolically labelled for 3 hours with [^{35}S]methionine and proteins from the cell lysates and media immunoprecipitated with anti-HSV-1 antiserum. These immunoprecipitates were then analyzed on SDS-polyacrylamide gels (see Figure 3.3.3). It is clearly seen from Figure 3.3.3 that all the mutant constructs express proteins which are recognized specifically by anti-HSV-1 antiserum, and as expected, these constructs exhibit a molecular size similar to or slightly less than wild type HSV-1 gB. The mutant glycoproteins are also immunoprecipitated by a gB specific monoclonal antibody (Rasile *et al.*, 1993; also see section 3.9 and Figure 3.9.1) as well as with an antibody that recognizes the cytoplasmic tail of gB glycoprotein (D. Snoddy, personal communication). Furthermore, all the mutant constructs are glycosylated as indicated by a decrease in molecular weight in the presence of tunicamycin. The multiple bands observed for both the wild type and mutant gB glycoproteins represent glycoproteins with various degrees of

Figure 3.3.3 Expression, glycosylation and secretion of mutant HSV-1 gB glycoproteins.

Subconfluent monolayers of COS-1 cells were transfected with the mutant gB constructs in the pXM expression vector. The cells were then starved in methionine deficient media for 1 hour in the absence (- lanes) or in the presence (+ lanes) of tunicamycin (2 ug/ml) and labelled with [^{35}S]methionine for 3 hours. Cell lysates (top gel) and media (bottom gel) were immunoprecipitated with anti-HSV-1 antiserum and the immunoprecipitates subjected to electrophoresis on a 10% polyacrylamide SDS gel. The gels were fluorgraphed, dried and exposed at -70°C. For each construct, protein expressed in the absence of tunicamycin is run beside protein expressed in the presence of tunicamycin. The mock lanes represent COS-1 cells transfected with non-recombinant pXM vector.


mock gB Δ1 Δ12 Δ13 Δ2 Δ23 Δ3 Δ123
Tunicamycin - + - + - + - + - + - + - + - + - +
mock gB Δ1 Δ12 Δ13 Δ2 Δ23 Δ3 Δ123
Tunicamycin - + - + - + - + - + - + - + - + - +

glycosylation and processing.

One of the major roles of a transmembrane region generally is membrane anchoring. In the case of wild type gB glycoprotein, the protein is stably anchored and is not secreted from the virion or virus infected cells. The topographical model of HSV-1 gB suggests that there are 3 membrane spanning regions in the carboxy terminal hydrophobic domain of HSV-1 gB. However, the exact role of each of these spanning domains is not known. It has not been determined which segment or segments are required for membrane anchoring. Examination of the immunoprecipitates of the cell media (Figure 3.3.3 panel B) shows that the mutants Δ13, Δ23, Δ3, and Δ123 are secreted into the media. Secretion is dependent upon glycosylation, since secretion is abolished in the prescence of tunicamycin. It thus appears that removal of segment 3, which is the most hydrophobic of the three segments, affects the stable membrane association of the resultant glycoprotein, and that segments 1 and 2 are not required for the stable membrane anchoring of the glycoprotein, as seen by the lack of secretion of the mutant glycoproteins Δ1, Δ2, and Δ12.

3.4 Analysis of secretion of the mutant HSV-1 gB glycoproteins

Although Figure 3.3.3 establishes that the mutants Δ13, Δ23, Δ3 and Δ123 are secreted, it gives no information as to the relative rates and amounts secreted. A time course analysis experiment was performed to obtain this information. COS-1 cells were transfected with the mutant constructs Δ13, Δ23, Δ3 and Δ123. The cells were subsequently metabolically labelled with [^{35}S]methionine for 30 min and chased with methionine-rich media for 0, 30, 90, 180, or 300 min. Cell lysates and media were immunoprecipitated with anti-HSV-1 antiserum and analyzed on SDS-polyacrylamide gels. Figure 3.4.1 shows the results of these gels, and it is clearly seen that the proportion of protein secreted into the media increases as the chase time increases. Densitometric analyses of the gels over the linear exposure range of the film shows that after 300 minutes of chase, the proportion of the protein secreted was 29% for Δ3, 53% for Δ13, 56% for Δ23, and 73% for Δ123. The amount of glycoprotein secreted was dependent on the hydrophobicity of the segments remaining in each mutant: the proteins with the least hydrophobic regions are secreted the most, and vice versa. Table 3.4.1 lists the results of the densitometric scans. A plot of the percentage of total protein secreted at different time intervals (Figure 3.4.2) shows that the glycoproteins containing the least hydrophobic

Figure 3.4.1 Kinetics of secretion of mutant HSV-1 gB glycoproteins.

Subconfluent monolayers of COS-1 cells were transfected with the secreted HSV-1 gB mutant constructs Δ13, Δ23, Δ3 and Δ123. Forty-eight hours post transfection the cells were starved for 1 hour in medium lacking methionione, pulse-labelled with [^{35}S]methionine for 15 min, then chased with methionine rich medium for 0, 30, 90, 180 or 300 min. Extracts from the both the cell lysate (intracellular) and medium (secreted) were immunoprecipitated with anti-HSV-1 antiserum and run on SDS polyacrylamide gels. For densitometric quantitation, exposures in the linear range of the film were used, and these results are summarized in Table 3.4.1.

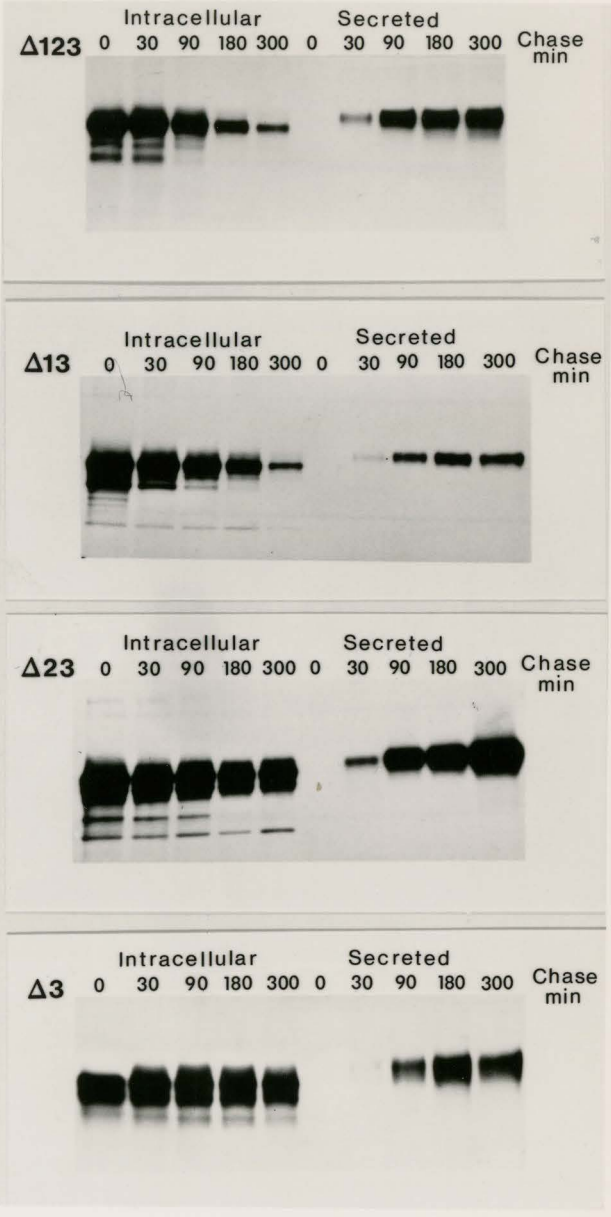

Δ123
Intracellular
Secreted
0 30 90 180 300 0 30 90 180 300 Chase min
Δ13
Intracellular
Secreted
0 30 90 180 300 0 30 90 180 300 Chase min
Δ23
Intracellular
Secreted
0 30 90 180 300 0 30 90 180 300 Chase min
Δ3
Intracellular
Secreted
0 30 90 180 300 0 30 90 180 300 Chase min

Table 3.4.1 Percentage of expressed HSV-1 glycoprotein gB secreted from cells.

	Percent of Protein Secreted at Chase Time (min)			
Mutant	30	90	180	300
Δ13	4 ± 0.7	19 ± 1.9	35 ± 1.7	53 ± 2.8
Δ23	8 ± 0.9	35 ± 2.2	45 ± 1.6	58 ± 2.9
Δ3	5 ± 0.6	16 ± 1.1	26 ± 1.3	31 ± 1.6
Δ123	7 ± 0.9	34 ± 1.3	57 ± 1.2	74 ± 1.9

The error is expressed as the standard deviation of the average results from two independent experiments. The distribution of the protein was determined by scanning the autoradiograms in Figure 3.4.1 with a densitometer in the linear exposure range of the films.

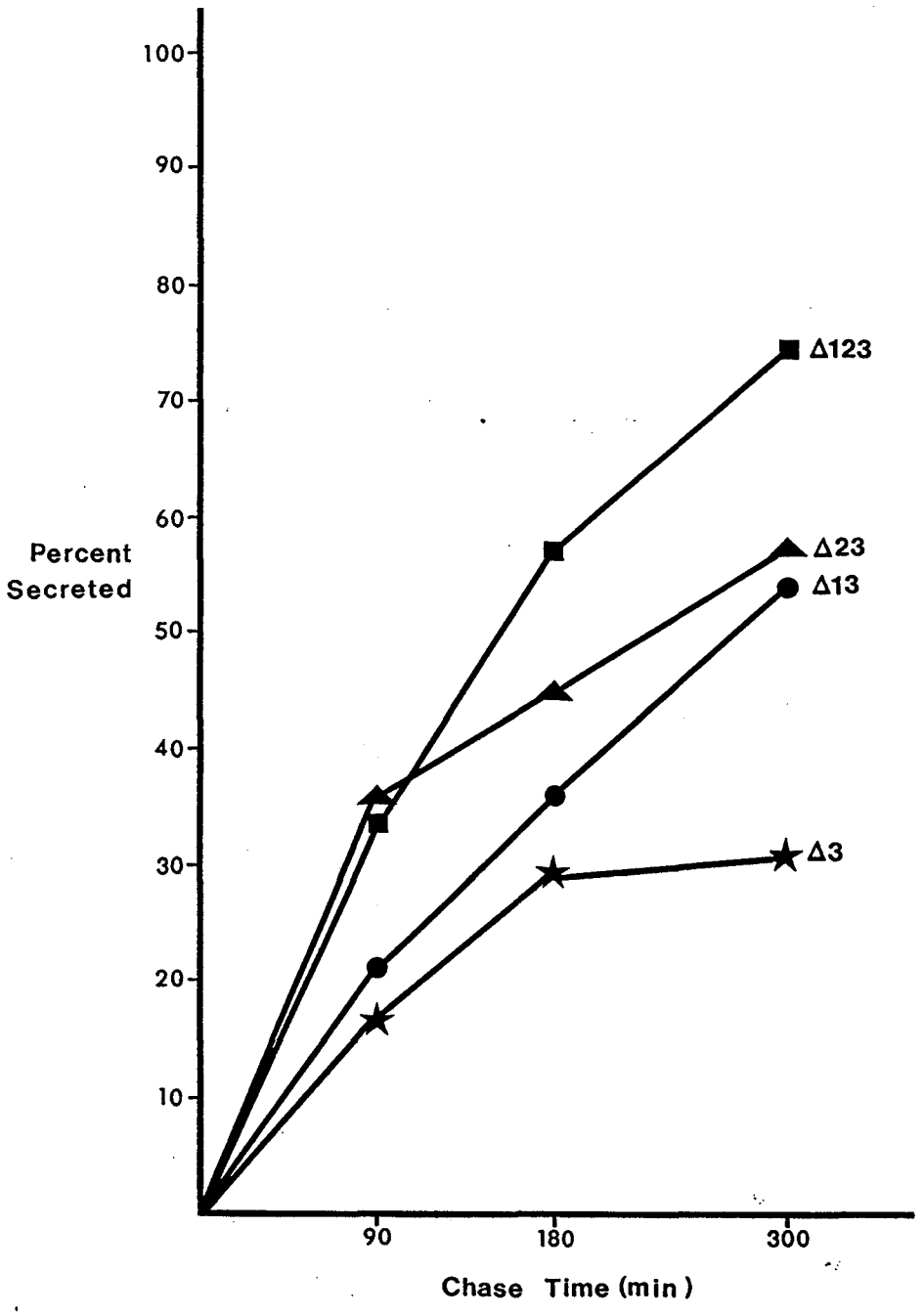


Figure 3.4.2 A plot of percentage of protein secreted over time. The data is from Table 3.4.1, and the mutants are indicated.

segments (mutants Δ123 and Δ23) are secreted to a greater extent than the glycoproteins containing the more hydrophobic segments (mutants Δ3 and Δ13) (see Table 3.2.1 for the hydrophobicities of the mutant glycoproteins).

3.5 Intracellular transport of mutant HSV-1 gB glycoproteins from the endoplasmic reticulum to the Golgi complex

HSV-1 glycoprotein gB contains 6 potential N-linked glycosylation sites (Asn-X-Thr/Ser) (Bzik *et al.*, 1984; Bzik *et al.*, 1986). Some of these sites are glycosylated cotranslationally in the endoplasmic reticulum (ER) by the addition of high mannose containing carbohydrates. These carbohydrate moeities are sensitive to digestion with endoglycosidase H (endo H). Once the protein moves from the ER to the Golgi, these carbohydrate moieties are further processed from their simple high mannose form to their complex form. This processing abolishes the sensitivity of the carbohydrates to digestion by endo H (Tarentino and Maley, 1974; Kornfeld and Kornfeld, 1985). Hence, the acquisition of resistance to digestion by endo H can be used to measure the transport of glycoproteins from the ER to the Golgi complex. To examine the rate of transport of the mutant and wild type HSV-1 glycoproteins from the ER to the Golgi complex, a series of experiments were performed. COS-1 cells were transfected with plasmids containing the genes for the mutant and wild

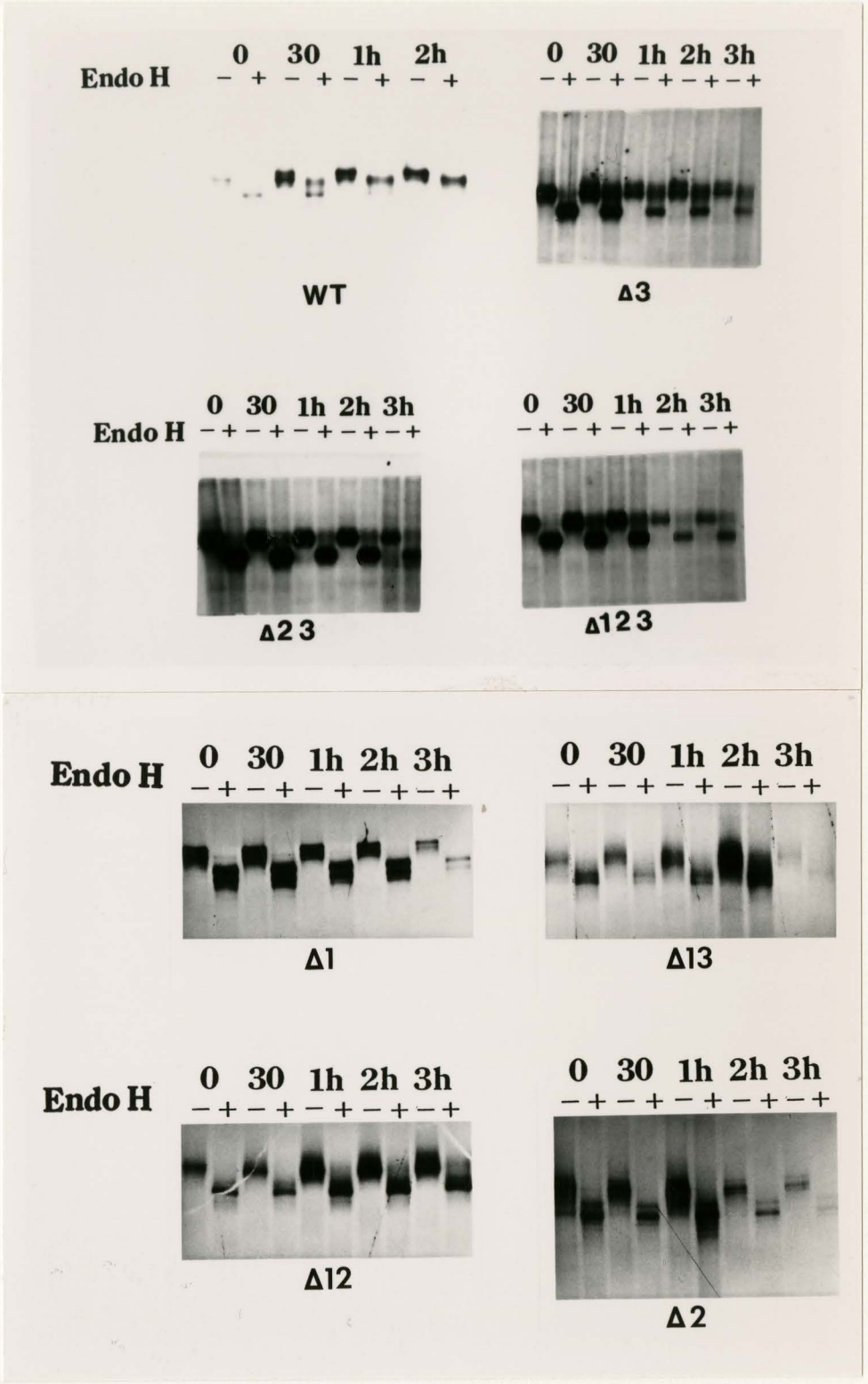
type glycoproteins, and the proteins were subsequently metabolically pulse-labelled with [^{35}S]methionine for 15 min, and then chased with methionine-rich media for 0, 30 min, 1, 2 or 3 hours. The cell lysates were immunoprecipitated with anti-HSV-1 antiserum and divided into 2 aliquots, only one of which was digested with endo H. Both samples (digested and undigested) were run on an SDS-polyacrylamide gel, and the results are shown in Figure 3.5.1. It is clearly seen that Δ1, Δ12, Δ13, Δ2, Δ23, and Δ123 do not exhibit any noticeable resistance to endo H digestion even after a 3 hour chase. Only Δ3 and the wild type HSV-1 gB glycoproteins exhibit significant maturation of the oligosaccharides. The rate of aquisition of resistence to endo-H digestion for these two proteins was determined by densitometric scanning of the gels in the linear exposure range of the film, and the $t_{\frac{1}{2}}$ calculated for wild type glycoprotein gB was 27 minutes while the $t_{\frac{1}{2}}$ calculated for the mutant Δ3 was 135 minutes.

3.6 Intracellular localization of mutant HSV-1 gB glycoproteins by indirect immunofluorescence

The wild type HSV-1 gB glycoprotein has been shown to be localized in the ER, Golgi complex, cell surface and nuclear envelope using immunofluoresecence, immunoelectronmicroscopy and cellular fractionation (Compton and Courtney, 1984; Koga *et al.*, 1986; Ali *et al.*, 1987; Pachl *et al.*, 1987; Cai *et*

Figure 3.5.1 Intracellular transport of wild type and mutant HSV-1 gB glycoproteins as determined by endo H digestion.

Subconfluent monolayers of COS-1 cells transfected with the wild type and mutant HSV-1 gB contructs were starved for methionine then pulse-labelled with [^{35}S]methionine for 15 min, then chased with methionine-rich media for 0, 30 min, 1, 2 and 3 hours. Cell lysates were immunoprecipitated with anti-HSV-1 antiserum, resuspended in sodium phosphate buffer and an aliquot subjected to digestion with endo H as described. For each chase time indicated on the figure, an undigested sample (- lanes) was run alongside an endo H digested aliquot (+ lanes) on an SDS-PAGE in order to visualize any size difference between the two samples. The mutant constructs are indicated below each gel, with WT being wild-type HSV-1 gB. The quantitaion of the bands by densitometry was done using exposures in the linear range of the films.


Endo H
0 30 1h 2h
− + − + − + − +
WT
0 30 1h 2h 3h
− + − + − + − + − +
Δ3
Endo H
0 30 1h 2h 3h
− + − + − + − + − +
Δ2 3
0 30 1h 2h 3h
− + − + − + − + − +
Δ1 2 3
Endo H
0 30 1h 2h 3h
− + − + − + − + − +
Δ1
0 30 1h 2h 3h
− + − + − + − + − +
Δ13
Endo H
0 30 1h 2h 3h
− + − + − + − + − +
Δ12
0 30 1h 2h 3h
− + − + − + − + − +
Δ2

al., 1988; Torrisi *et al.*, 1992; Gilbert and Ghosh, 1993). In order to obtain qualitative data regarding the intracellular localization of mutant and wild type HSV-1 gB glycoproteins, indirect immunofluorescence was performed. This procedure uses antibodies labelled with a fluorescent dye to localize the desired protein in the cell. Due to the nature of this experiment, only qualitative data is generated, meaning that one can establish the presence of the particular protein but cannot determine the amounts of the protein present.

COS-1 cells grown on coverslips were transfected with the wild type and mutant HSV-1 gB constructs. The transfected cells were fixed with cold acetone (to examine internal localization) or with paraformaldehyde (to examine surface localization). The cells were then reacted sequentially with anti-HSV-1 rabbit IgG and FITC-labelled goat anti-rabbit IgG and then examined under a fluorescence microscope (Quinlan *et al.*, 1984; Raviprakash *et al.*, 1990). The resulting micrographs are shown in Figure 3.6.1 (surface localization) and Figures 3.6.2 and 3.6.3 (internal localization). Examination of Figure 3.6.1 reveals that only wild type HSV-1 gB and the mutant Δ3 exhibit noticeable cell surface fluorescence, with all the other mutants (Δ1, Δ12, Δ13, Δ2, Δ23 and Δ123) exhibiting surface fluorescence similar to that shown for Δ1. With respect to internal fluorescence, most of the mutant constructs show peri-nuclear fluorescence similar

Figure 3.6.1 Surface localization of wild type and mutant HSV-1 gB glycoproteins by indirect immunofluorescent staining.

Subconfluent monolayers of COS-1 cells were transfected with the wild type and mutant HSV-1 gB glycoprotein gene constructs and were fixed in paraformaldehyde. The cells were then reacted sequentially with anti-HSV-1 rabbit IgG and FITC-labeled goat anti-rabbit IgG. Fluorescence was examined using a Zeiss fluorescence microscope equipped with phase contrast optics. Right panels are phase contrast. WT represents wild-type HSV-1 gB, mock represents cells transfected with non-recombinant pXM vector. The other mutant constructs not shown do not exhibit any surface flourescence (they are identical to Δ1).

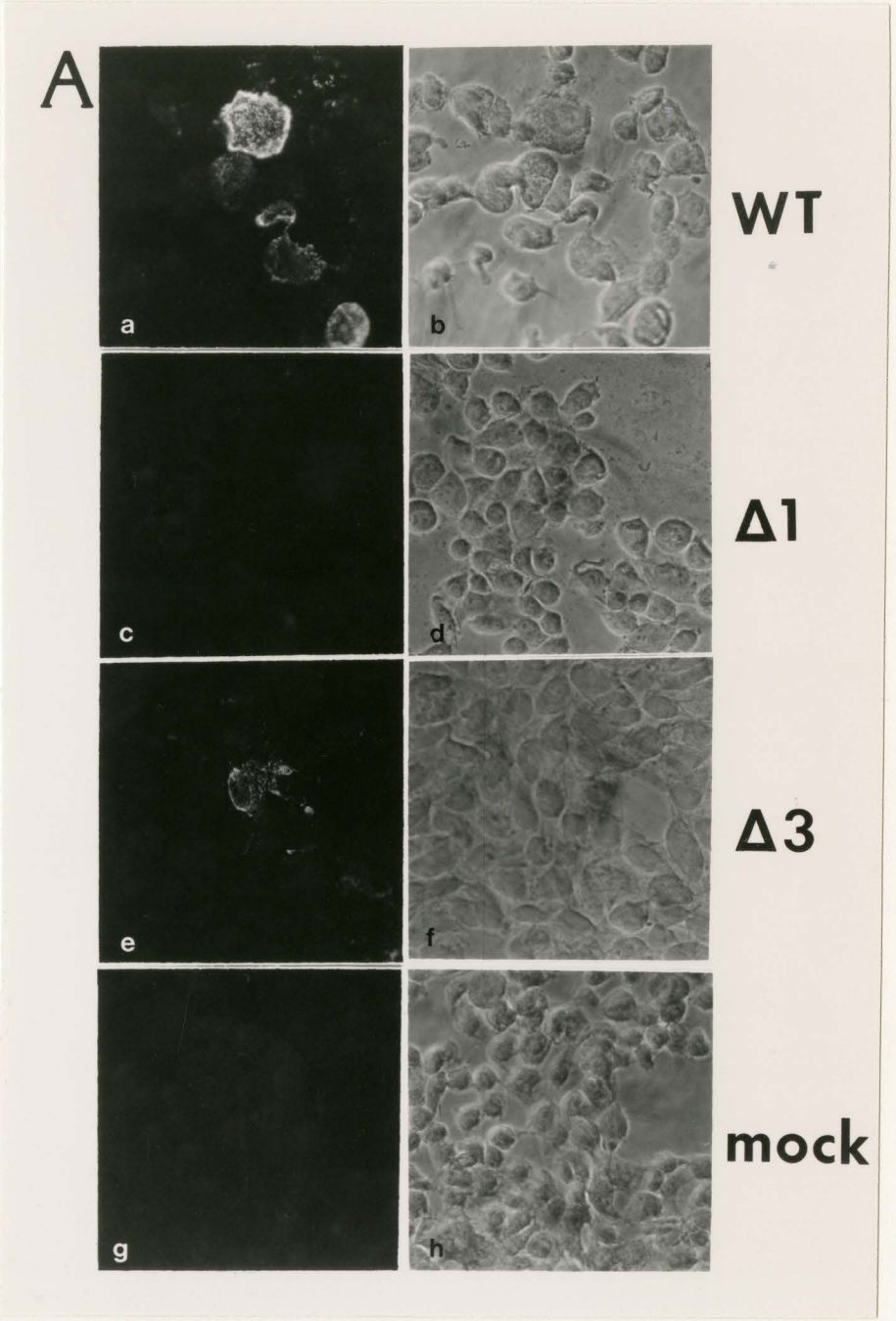

A
a
b
WT
c
d
Δ1
e
f
Δ3
g
h
mock

Figure 3.6.2 Intracellular localization of wild type and mutant HSV-1 gB glycoproteins by indirect immunofluorescent staining.

Subconfluent monolayers of COS-1 cells were transfected with the wild type and mutant HSV-1 gB glycoprotein gene constructs and were fixed in acetone at -20°C. The cells were then reacted sequentially with anti-HSV-1 rabbit IgG and FITC-labeled goat anti-rabbit IgG. Fluorescence was examined using a Zeiss fluorescence microscope equipped with phase contrast optics. Right panels are phase contrast. WT represents wild-type HSV-1 gB. The remaining mutants are shown in Figure 3.6.3.

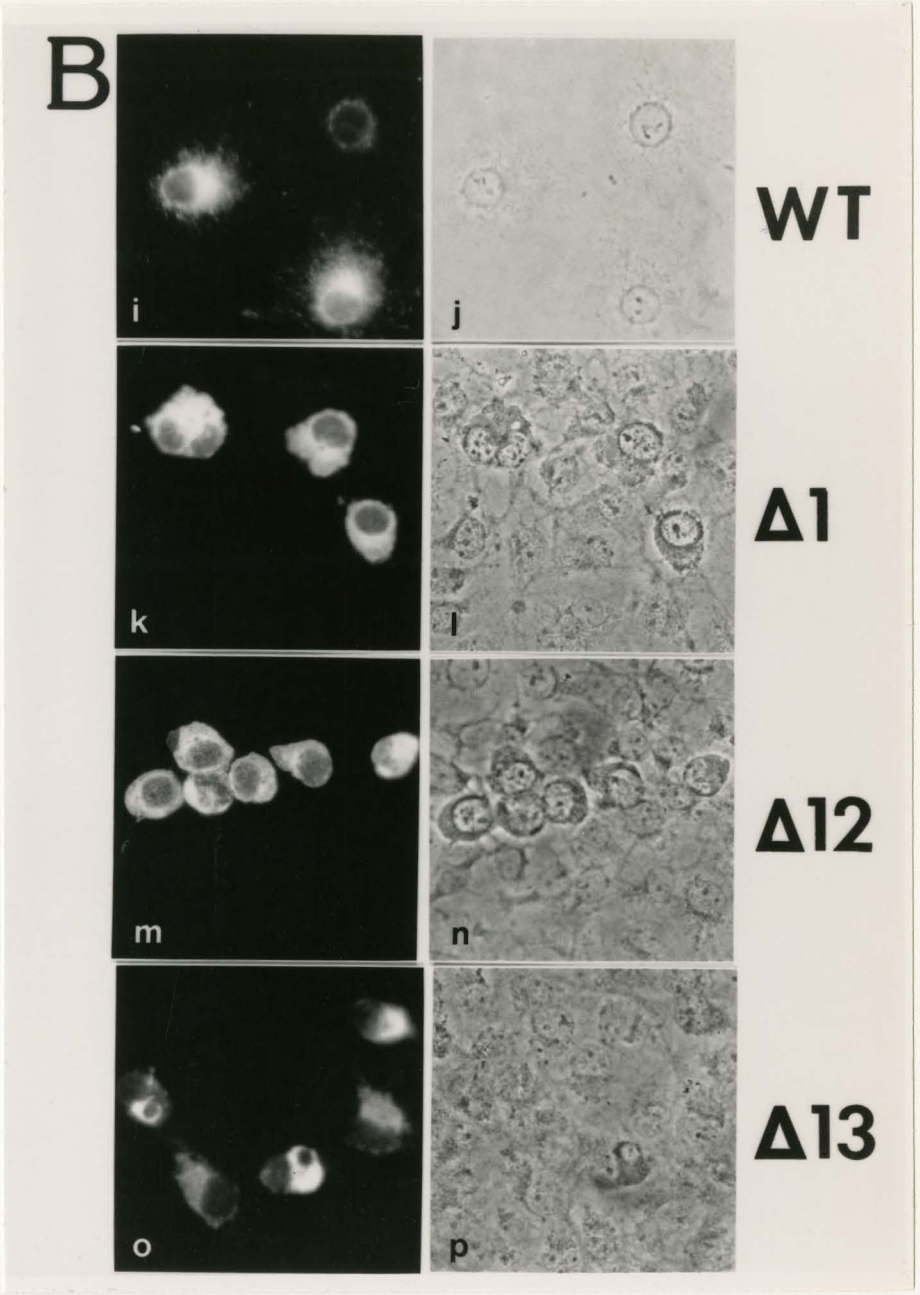
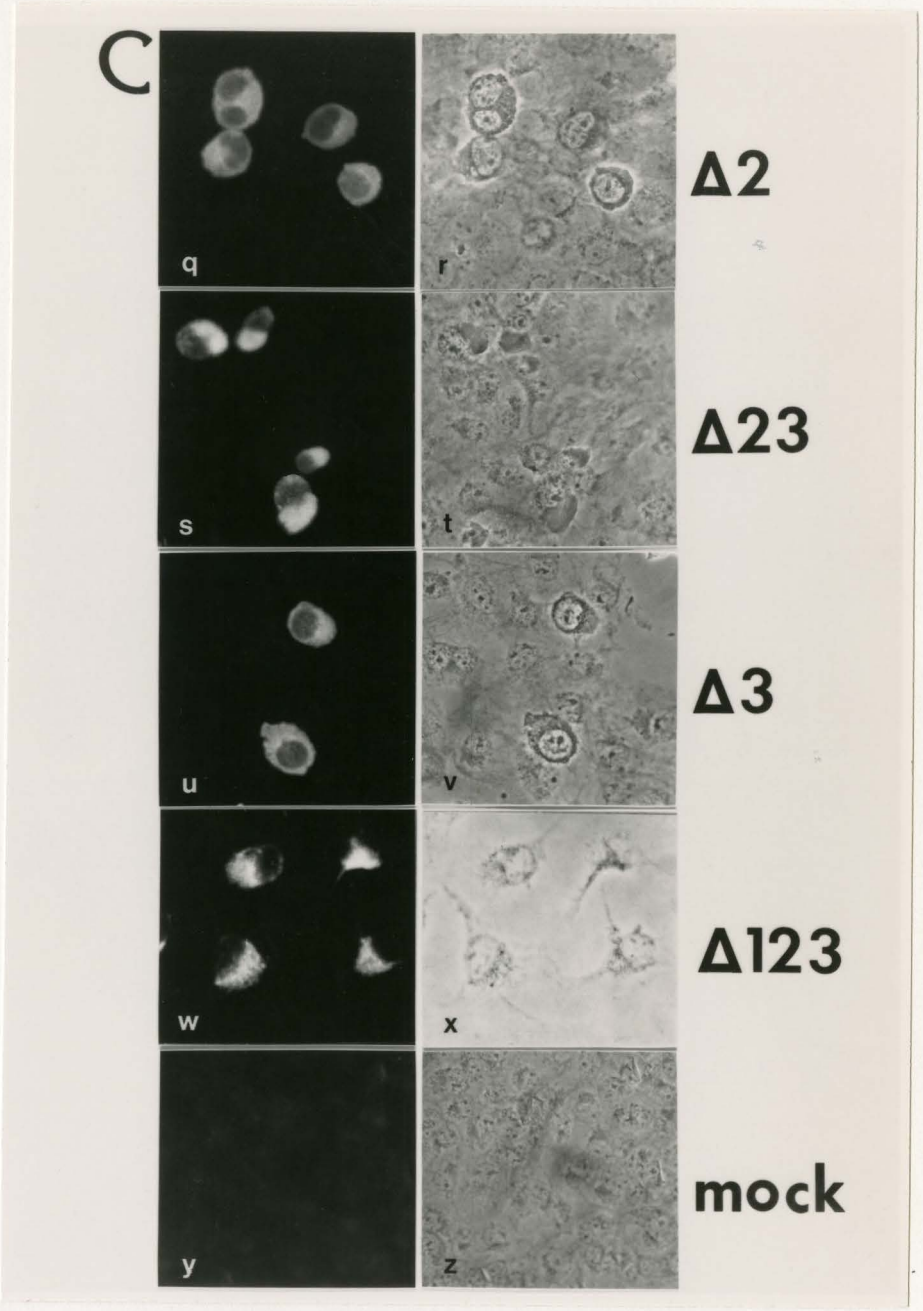

B
i
j
WT
k
l
Δ1
m
n
Δ12
o
p
Δ13

Figure 3.6.3 Intracellular localization of mutant HSV-1 gB glycoproteins by indirect immunofluorescent staining.

Subconfluent monolayers of COS-1 cells were transfected with the mutant HSV-1 gB glycoprotein gene constructs and were fixed in acetone at -20°C. The cells were then reacted sequentially with anti-HSV-1 rabbit IgG and FITC-labeled goat anti-rabbit IgG. Fluorescence was examined using a Zeiss fluorescence microscope equipped with phase contrast optics. Right panels are phase contrast. Mock represents cells transfected with non-recombinant pXM vector.


C
q
r
Δ2
s
t
Δ23
u
v
Δ3
w
x
Δ123
y
z
mock

to that of wild type HSV-1 gB (Figures 3.6.2 and 3.6.3). However, the internal staining patterns for the mutants Δ13 (Figure 3.6.2, panel o), Δ23 (Figure 3.6.3, panel s) and Δ123 (Figure 3.6.3, panel w) were different than that of the wild type gB glycoprotein. Further experiments (namely cell-surface iodination and cellular fractionation) were performed to obtain more quantitative data with respect to the subcellular localization of HSV-1 glycoprotein gB.

3.7 Cell surface localization of mutant HSV-1 gB glycoproteins by cell surface iodination

To further determine cell-surface localization of the mutant HSV-1 gB glycoproteins, cell-surface iodination was performed. Proteins exposed at the cell surface can be labeled with ^{125}I as follows: lactoperoxidase, in the presence of hydrogen peroxide, oxidizes the iodide to a reactive, enzyme bound form, and this activated species can iodinate tyrosine and histidine residues on exposed proteins. Proteins within the cell are not labelled because the activated form of iodine is bound to the lactoperoxidase enzyme, which cannot penetrate the cell membrane (Alberts *et al.*, 1983).

COS-1 cells were transfected with the mutant and wild type HSV-1 gB constructs in pXM or p91023, and subsequently radioiodinated with ^{125}I in a lactoperoxidase catalyzed reaction (Guan and Rose, 1984). Cell lysates were then

immunoprecipitated and run on an SDS-polyacrylamide gel, and this is shown in Figure 3.7.1. From Figure 3.7.1 it is clearly evident that only the wild type and the mutant Δ3 gB glycoprotein exhibit cell-surface labelling. This result supports the immunofluorescence data, where only wild type and Δ3 gB glycoprotein show surface fluorescence.

3.8 Intracellular localization of mutant HSV-1 gB glycoproteins by cellular fractionation

Biochemical cellular fractionation was done to provide a more quantitative determination of the nuclear localization of the wild type and mutant HSV-1 gB glycoproteins. Biochemical cellular fractionation allows one to metabolically label the proteins, fractionate the cells into nuclear and post-nuclear fractions, and then quantitate the amount of protein in each fraction. This allows the determination of the proportion of the expressed protein in the nuclear membranes. This is important because HSV-1 buds from the nuclear membrane, and as such this is the critical targeting organelle.

COS-1 cells were transfected with the mutant and wild type gB constructs, and metabolically labelled with [^{35}S]methionine for 30 min followed by a 1.5 hour chase with methionine-rich medium. The cell lysates were then fractionated into nuclear and post-nuclear fractions as described (Bos *et al.*, 1988; Raviprakash *et al.*, 1990), and

Figure 3.7.1 Surface localization of wild type and mutant HSV-1 gB glycoproteins by cell surface iodination.

Subconfluent monolayers of COS-1 cells were transfected with the wild type and mutant HSV-1 gB glycoprotein constructs and radioiodinated with ^{125}I in a lactoperoxidase catalyzed reaction as described. Cell lysates were immuniprecipitated and analyzed by SDS-PAGE. Mock represents cells transfected with non-recombinant pXM vector, and gB represents wild-type HSV-1 gB.

each fraction then immunoprecipitated with anti-HSV-1 antiserum. The immunoprecipitates were then run on an SDS-polyacrylamide gel, and the resultant autoradiogram is shown in Figure 3.8.1. Densitometric scanning of the gels exposed in the linear range of the film was performed and the results summarized in Table 3.8.1. From Table 3.8.1 it is evident that the wild type gB glycoprotein and all the mutants in which segment 3 is still present (mutants Δ1, Δ12, Δ2), or segment 3 alone is deleted (mutant Δ3), have from 30 to 35% of the glycoprotein associated with the nuclear fraction. However, when segment 3 is deleted in combination with another segment, the amount of protein associated with the nuclear fraction greatly decreases, with the mutants Δ13, Δ23, and Δ123 having 18%, 17%, and 14% of the expressed glycoprotein associated with the nuclear fraction respectively. As a control, vesicular stomatitis virus glycoprotein G (VSV G) was used. Previous studies showed that less than 10% of this predominantly surface protein is associated with the nuclear fraction (Garoff, 1985; Stephens and Compans, 1988). When COS-1 cells were transfected with pXM encoding the VSV G glycoprotein, only 7% of the total VSV G protein expressed was found associated with the nuclear fraction (Figure 3.8.1 and Table 3.8.1).

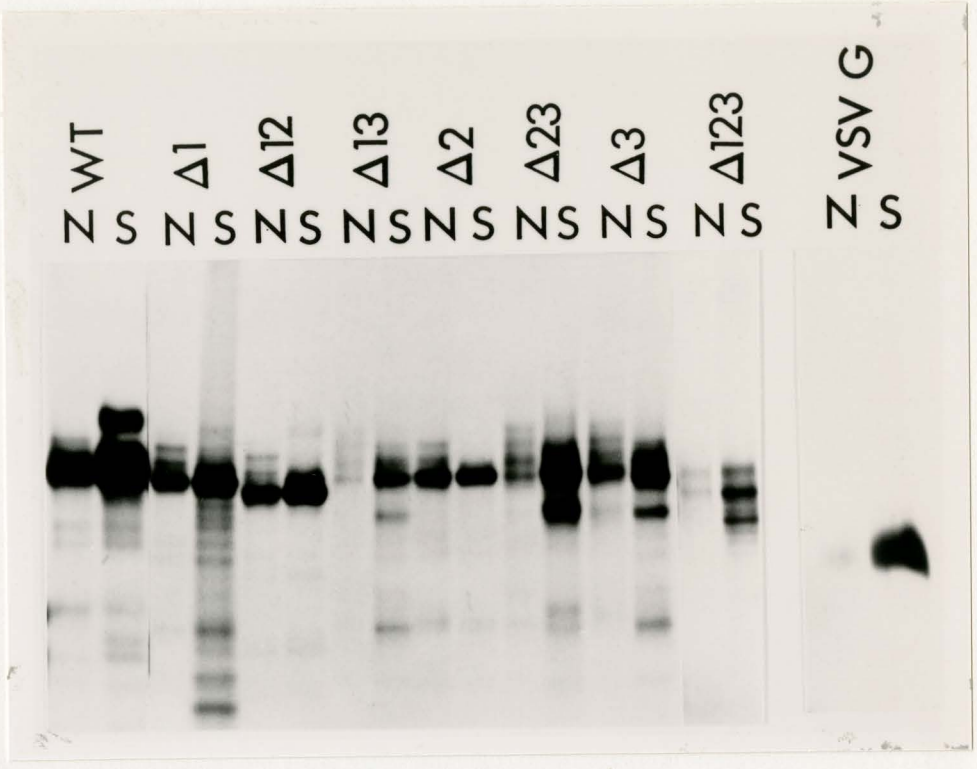


Figure 3.8.1 Intracellular localization of wild type and mutant HSV-1 gB glycoproteins by cellular fractionation.

Subconfluent monolayers of COS-1 cells were transfected with the wild type and mutant HSV-1 gB glycoprotein constructs, starved, and pulse-labelled with [^{35}S]methionine for 30 min and then chased with methionine-rich medium for 90 min. The cell lysates were fractionated into the muclear fraction (N lanes) and post-nuclear fraction (S lanes) as described. Each fraction was subjected to immunoprecipitation with anti-HSV-1 antiserum and analyzed by SDS-PAGE. For each construct, the nuclear and post-nuclear fractions were run alongside each other on the gel. The control, VSV G glycoprotein, is also shown. The bands were quantitated by densitometry using the linear exposure range of the film, and the results are summarized in Table 3.8.1.

Table 3.8.1 Distribution of the constructed mutant HSV-1 gB glycoproteins in the subcellular fractions[a]

Mutant	Nuclear Fraction (%)	Post-nuclear Fraction (%)
WT-gB*	31 ± 4.2	69 ± 4.2
Δ1	30 ± 3.8	70 ± 3.8
Δ1,2	35 ± 2.7	65 ± 2.7
Δ1,3	18 ± 2.9	82 ± 2.9
Δ2*	30 ± 0.4	70 ± 0.4
Δ2,3	17 ± 1.3	83 ± 1.3
Δ3	32 ± 4.1	68 ± 4.1
Δ1,2,3	14 ± 2.0	86 ± 2.0
VSV G*	7 ± 1.9	93 ± 1.9

[a] The distribution of the mutant glycoproteins in the nuclear and post-nuclear fractions were determined by scanning the autoradiograms of the fractionation gels (Figure 3.8.1) with a Hoeffer densitometer. For densitometry, the linear exposure range of the film was used. The experiment was repeated 2 times for each mutant and the values shown represent the averaged results from four independent scans of each lane with the error expressed as the standard deviation of the averaged values.

* for wild-type gB and VSV G, 4 independent fractionations were performed, while for Δ2 the values were obtained from only 1 fractionation. The VSV glycoprotein G was expressed using the expression plasmid pSVGL (Guan and Rose, 1984).

3.9 Oligomerization of mutant HSV-1 gB glycoproteins

Aquisition of the correct tertiary and quaternary structure is a requirement for most proteins to exit the ER and be transported to the cell surface. It has been shown that mutants that do not fold or oligomerize properly can form aggregates and are subsequently retained in the ER (Hurtley and Helenius, 1989; Kreis and Lodish, 1986; Pelham, 1989; Rose and Doms, 1988). Since HSV-1 gB glycoprotein forms dimers soon after synthesis in the ER, and is subsequently transported out of the ER to the nuclear envelope and plasma membrane, it is improtant to determine the oligomeric state of the mutant constructs.

In order to determine the oligomeric nature of the gB deletion mutants, COS-1 cells were transfected with the mutant constructs and subsequently metabolically pulse-labelled with [^{35}S]methionine for 15 min followed by a 2 hour chase with methionine-rich medium. The cell lysates were then immunoprecipitated with monoclonal 3S anti-gB antibody (Showalter *et al.*, 1981) and divided into 2 aliquots. Since the gB dimers are sensitive to dissociation with heat (Claesson-Welsh and Spear, 1986), one aliquot was boiled for two minutes to give only the monomer upon SDS-PAGE. The second aliquot was not heated prior to electrophoresis, and would thus contain gB dimers if present. The resulting autoradiogram (Figure 3.9.1) shows that all the mutants except

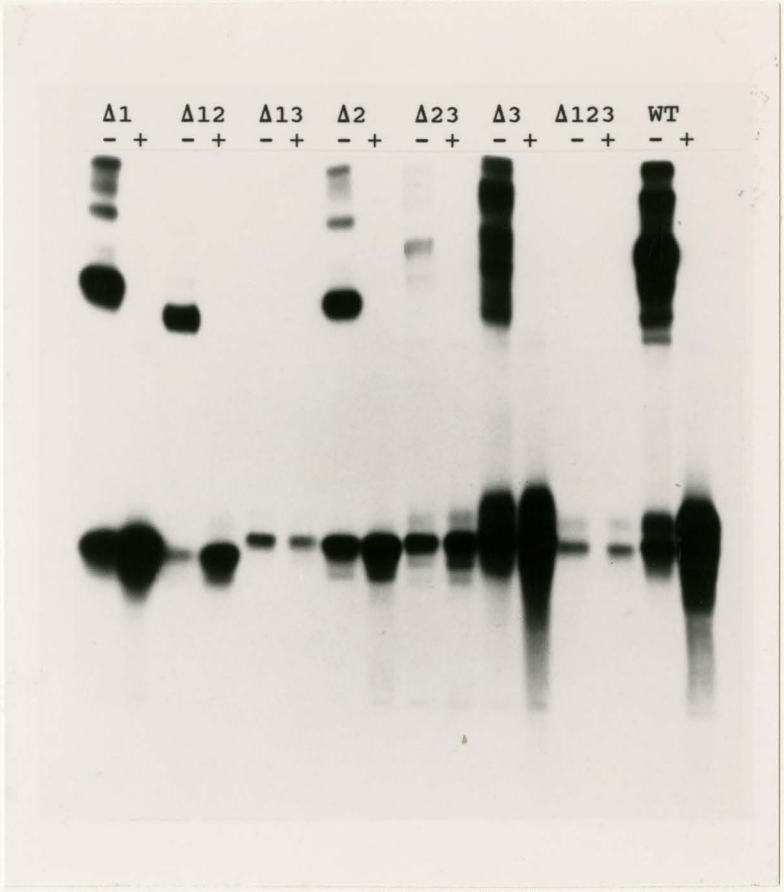


Figure 3.9.1 Analysis of oligomer formation of wild type and mutant HSV-1 gB glycoproteins by SDS-polyacrylamide gel electrophoresis.

COS-1 cells transfected with wild type and mutant gB constructs were labelled with [^{35}S]methionine at 40 hours post transfection and cell extracts were obtained under nondenaturing conditions. The samples were divided into two aliquots, and only one of these was heated. The unheated (- lanes) and heated (+ lanes) samples were analyzed on a polyacrylamide gel containing 0.3% SDS.

for Δ13, Δ23 and Δ123 form dimers which were dissociated upon heating. Higher molecular weight multimers of the wild type gB glycoprotein, and some of the deletion mutants are also seen in Figure 3.9.1. Similiar oligomeric species of the gB glycoproetin have been reported in HSV-1 virions and HSV-1 infected cells (Claesson-Welsh and Spear, 1986). These dimerization results for the gB glycoprotein mutants have been confirmed using sucrose gradient sedimentation analysis of labelled cell extracts transfected with the mutant gB constructs (Rasile *et al.*, 1993).

3.10 Complementation of a gB-null virus by the mutant HSV-1 gB glycoproteins

To determine the ability of the mutant gB glycoproteins to complement the gB defective virus K082 (Cai *et al.*, 1987; Cai *et al.*, 1988), a complementation assay using the wild type and mutant constructs was performed. In order to perform this experiment the plasmid pKBXX was used (Cai *et al.*, 1987; Cai *et al.*, 1988). This plasmid contains a 4 kb HSV-1 fragment cloned into the pUC9 vector (Viera and Messing, 1982). This 4 kb HSV-1 fragment contains the 2.7 kb gB coding region as well as 790 bp of 5' and 498 bp of 3'-flanking sequences. This puts the control of gB expression under its own (HSV-1) promoter.

In order to test the mutant constructs, they first had to

be subcloned from pXM or p91023(B) into pKBXX, and this was done using *Sna*BI and *Kpn*I. This generated pKBXX plasmids harbouring the desired deletion mutant. A *Pst*I digest of the resultant plasmids is shown in Figure 3.10.1. The expected fragment sizes from this digest are 3.5, 1.7, 0.8 and 0.6 Kb. The 0.8 Kb fragment encompasses the deletion sites, and a size shift reflecting the size of the deletions in the mutants is clearly evident.

Vero cells were transfected with pKBXX plasmids encoding the wild type and mutant constructs, and these transfected cells were subsequently infected with K082 virus, which contains a nonsense mutation at residue 43 of the gB glycoprotein. This virus can only grow on gB producing cells, such as D6 cells (Cai *et al.*, 1987; Cai *et al.*, 1988). Virus stocks obtained from transfected Vero cells after infection with K082 virus were titered on both Vero and D6 cells. If the mutant gB construct has the ability to complement the gB defect in K082, then titers on the D6 cells will be much greater (at least 100 fold) than titers on Vero cells (Cai *et al.*, 1988b). It was found that none of the mutant constructs had the ability to complement the K082 virus (Table 3.10.1). However, when the complementation assay was performed using pKBXX plasmid containing the wild type gB glycoprotein, the virus titer on D6 cells was about 150-fold higher than the titer on Vero cells. This is due to complementation of the gB

Figure 3.10.1 Restriction enzyme analysis of the wild type and mutant HSV-1 gB genes constructed in this work and cloned into the pKBXX plasmid.

A *Pst*I restriction digests is shown. The kb ladder lane is indicated, and the size of the marker fragments are inicated up to 4 kb, with each band above this indicating an increase in size of 1 kb. See text for more details.

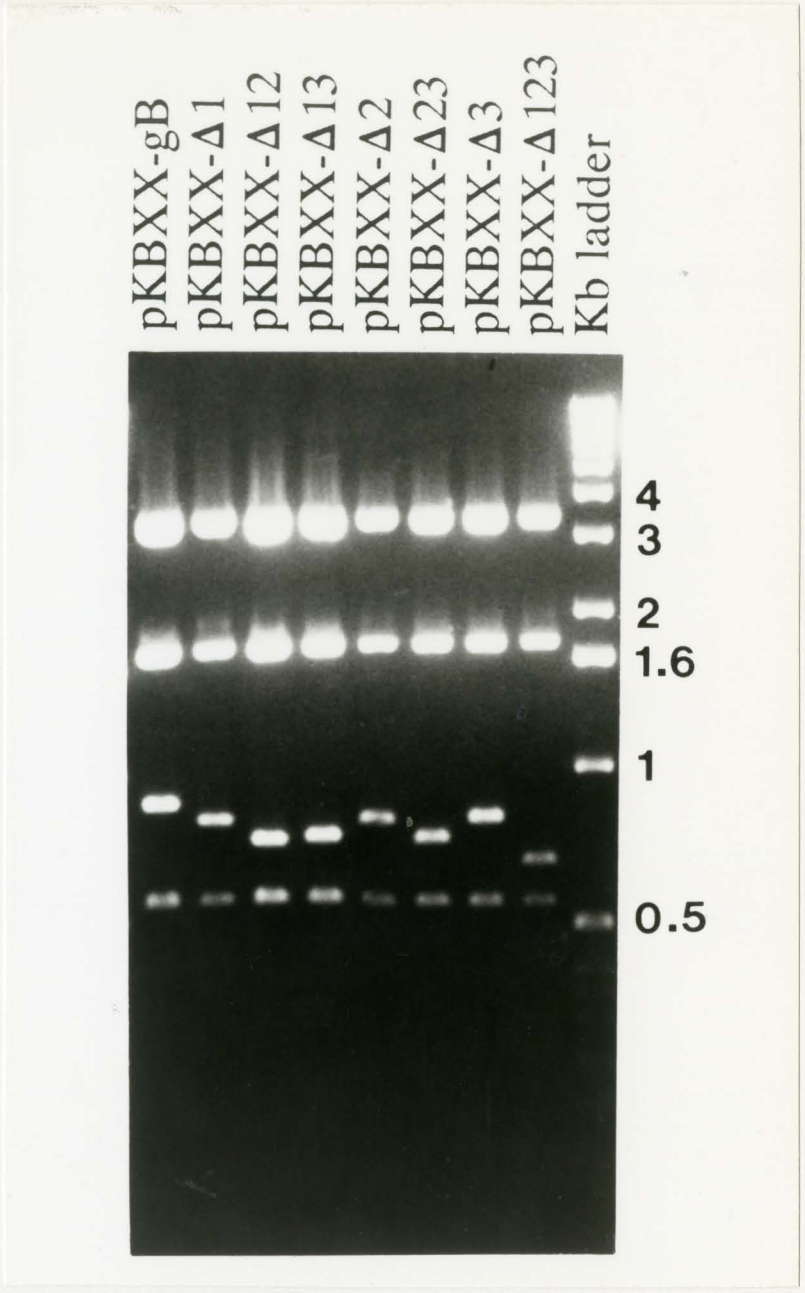

pKBXX-gB
pKBXX-Δ1
pKBXX-Δ12
pKBXX-Δ13
pKBXX-Δ2
pKBXX-Δ23
pKBXX-Δ3
pKBXX-Δ123
Kb ladder
4
3
2
1.6
1
0.5

Table 3.10.1 Complementation of K082 virus by transfection with plasmids containing the constructed mutants in HSV-1 gB glycoprotein.

Transfecting plasmids	Virus titer on cell lines pfu/ml[a] Vero	D6	Plaquing efficiency in Vero cells[b]	Complementation efficiency %[c]
gB wild type	$2.3 \times 10^4 \pm 3 \times 10^3$[d]	$3.5 \times 10^6 \pm 6 \times 10^5$	150	100
Δ1	$2.4 \times 10^4 \pm 2 \times 10^3$	$2.8 \times 10^4 \pm 2 \times 10^3$	1.20	0.80
Δ12	$2.8 \times 10^4 \pm 4 \times 10^3$	$2.4 \times 10^4 \pm 4 \times 10^3$	0.86	0.57
Δ13	$2.6 \times 10^4 \pm 4 \times 10^3$	$2.2 \times 10^4 \pm 4 \times 10^3$	0.85	0.57
Δ2	$2.7 \times 10^4 \pm 3 \times 10^3$	$3.2 \times 10^4 \pm 3 \times 10^3$	1.20	0.80
Δ23	$2.8 \times 10^4 \pm 5 \times 10^3$	$2.7 \times 10^4 \pm 5 \times 10^3$	0.96	0.64
Δ3	$2.5 \times 10^4 \pm 2 \times 10^3$	$2.9 \times 10^4 \pm 2 \times 10^3$	1.20	0.80
Δ123	$1.5 \times 10^4 \pm 2 \times 10^3$	$1.4 \times 10^4 \pm 2 \times 10^3$	0.93	0.62
None	$2.2 \times 10^4 \pm 1 \times 10^3$	$2.7 \times 10^4 \pm 1 \times 10^3$	1.21	0.81

Vero cells were transfected with pKBXX plasmids containing the wild type gB or mutant gB constructs. Transfected cells were infected with the K082 virus at a m.o.i. of at 37°C for 24 h. Virus stock was isolated 24 h later and titered on both vero and D6 cells.

a Titers are average of at least two sets of separate complementation experiments.

b Plaquing efficiency of complemented virus is expressed as the ratio of titre on vero cells with the D6 cells.

c Complementation efficiency is expressed as percentage of plaquing efficiency of virus produced by transfection with wild type gB plasmid.

d Standard error of the mean

defect in K082 by the wild type gB glycoprotein.

4.0 DISCUSSION

The work presented in this thesis deals with the structure and function relationships in the HSV-1 glycoprotein gB. Specifically, the 69 amino acid long carboxy hydrophobic membrane anchoring domain was examined, with the goal being the delineation of the role of this domain in the membrane anchoring, transport, processing, subcellular targeting, oligomerization and viral infectivity. In order to achieve this, site-directed mutagenesis was employed to generate specific deletion mutants within this hydrophobic domain. These mutants were then expressed using transient expression systems, and assays designed to evaluate the various properties of these mutant HSV-1 gB glycoproteins were performed.

4.1 Construction of the deletion mutants

The HSV-1 glycoprotein gB is a transmembrane glycoprotein which can be subdivided into 3 distinct domains: a membrane spanning domain of 69 amino acids separating a carboxyl terminus cytoplasmic domain of 109 amino acids from a large amino terminus extracytoplasmic domain, or ectodomain of 726 amino acids. It has been predicted that the hydrophobic membrane spanning domain could traverse the lipid bilayer

three times. Several deletion mutations encompassing segments of this hydrophobic region were made in order to evaluate the functions of these specific segments of the hydrophobic domain. The 69 residue hydrophobic domain can be further subdivided into 3 distinct potential membrane spanning segments of about 20 residues each. In order to study the function of this domain, mutants were constructed in which one, two or all three of these potential membrane spanning segments were deleted in every combination possible.

The construction of the deletion mutants, described in section 3.2, posed some problems, due mainly to the fact that the HSV-1 gB gene is very G + C rich (Bzik *et al.*, 1984). The first problem posed by this was the difficulty in designing the primers to be used for mutagenesis and for sequencing. The primers used for mutagenesis have to bind uniquely to the desired regions, and the base composition of the gene made this difficult. This is because the primers, being of necessity G + C rich, shared significant homology with several other G + C rich regions. Since G and C nucleotides have a higher melting temperature than A or T nucleotides (Gilliam *et al.*, 1975), this resulted in the primers binding fairly stably to different regions of the gB gene. To combat this, three things were done. Firstly, only a fragment of the gB gene was cloned into M13mp18, thereby minimizing the amount of G + C rich DNA in the vector being used for mutagenesis. Secondly,

every attempt was made to design the mutagenesis primers with 3' terminal A or T nucleotides. The reason for this was to minimize the stability of 3' terminal hybridizations. If the 3' end of the primer is not hybridized, then the primer, even if stably hybridized at the 5' end, cannot prime DNA synthesis. Thirdly, every primer was carefully selected to minimize the amount of homology to regions outside the desired binding sites. This was done by varying the overall size of the primer, along with varying the exact internal sequence of the primer by shifting the binding points up or down one or two codon triplets. This is why not all the same break points are used for the deletions, which is why, for example, Δ1 has residues 728 to 747 deleted, but Δ12 has residues 727 to 772 instead of residues 728 to 772 deleted.

4.2 Delineation of the membrane anchoring domain of the HSV-1 gB glycoprotein

The deletion mutants constructed were tested for transient expression in mammalian cells, and all the mutants were expressed and glycosylated in a manner similiar to the wild type gB glycoprotein. Further, the mutants Δ13, Δ23, Δ3 and Δ123 were found to be secreted from the cells, and this secretion is dependent upon glycosylation of the protein. All these secreted mutants have one thing in common: they each lack segment 3 of the 69 residue hydrophobic domain. This

segment encompasses residues 774 to 795 of the gB glycoprotein, and is the segment closest the carboxy terminus of the protein. All the mutants that contained segment 3, including the mutant Δ12, which contains only segment 3, are not secreted from the cells. This strongly suggests that segment 3 alone is sufficient for membrane anchoring, and may in fact be the membrane anchoring domain.

Secretion kinetic experiments, in which the amount of protein secreted over time is determined, indicates that not all four of the secreted mutant glycoproteins exhibit the same secretion profile. In fact, the mutant Δ123 exhibits the most and fastest secretion rate, followed by the mutants Δ23, Δ13 and Δ3, in that order. The hydrophobicity value of segment 1 is 0.8, of segment 2 is 1.7 and of segment 3 is 2.4, and the amounts of mutant glycoprotein secreted is dependent on the hydrophobicity value of the hydrophobic segments remaining in each mutant: the proteins containing the least hydrophobic regions are secreted the most, and vice versa. The secretion of mutants Δ3 and Δ13 was less than the secretion observed for the mutant Δ23, suggesting that perhaps segments 1 and 2 together, or segment 2 alone, may posses some membrane anchoring ability, but not nearly as much as segment 3. In fact, both segments 2 and 3 meet the minimum criteria for a membrane spanning domain: a contiguous sequence of at least 19 residues with a hydrophobicity value of 1.65 or more (Kyte and

Doolittle, 1982). Segment 1 on its own appears to have no membrane anchoring activity since the mutant Δ23 behaves similar to the mutant Δ123.

These results have been confirmed by two other methods: alkaline extraction of membranes containing the expressed mutant constructs; and by a protease protection assay of microsomes (Rasile *et al.*, 1993). In these studies it was found that mutants lacking only segment 3, which are secreted from cells, are also resistant to protease digestion, suggesting translocation through the membranes and into the microsomes. Again, the mutants Δ3 and Δ13 also had protease sensitive species present, indicating partial membrane anchoring activity present in these mutants. A similar result was obtained with alkaline extraction of membranes: the mutants lacking segment 3 were sensitive to alkaline extraction, however, the mutants Δ3 and Δ13 were both sensitive and resistant to alkaline extraction. As in the case of protease sensitivity and secretion, the mutant Δ3 exhibited stronger membrane anchoring activity than the mutant Δ13.

These results are in line with those presented by other researchers. A number of truncation mutants have been created to date, and the longest secreted mutant has a chain termination codon inserted at residue 737 of the gB molecule. This is within segment 1 (residues 727 to 747) of the

hydrophobic domain. Other chain termination mutants upstream of this are also secreted, while chain termination mutants at residue 810 or downstream are not secreted (Ali *et al.*, 1987; Cai *et al.*, 1987; Cai *et al.*, 1988b; Ali, 1990; Raviprakash *et al.*, 1990; Qadri *et al.*, 1991; Navarro *et al.*, 1993). These are all downstream of the 69 residue hydrophobic domain (residues 727 to 795). Like the deletion mutants presented here, secretion of these other mutants is also dependant upon glycosylation of the protein.

The results presented here create the need to modify the topological model of the gB glycoprotein as presented by Pellet *et al.*, (1985b). In their model, they predicted that the 69 residue hydrophobic domain spanned the lipid bilayer three times (see section 1.4.3). This would indicate that any one of the 3 segments in this domain could serve to anchor the protein, which is clearly not the case. In fact, the results presented here would suggest that the gB glycoprotein, like most other viral glycoproteins (Schlessinger and Schlessinger, 1987), is anchored to the membrane by a single span of hydrophobic sequences, which would include residues 775 to 795 (segment 3). The function of the other two segments is not entirely clear. They could be interacting with the lipid bilayer, but not in a transmembrane orientation. This could explain the behaviour of the mutants Δ3 and Δ13: in the absence of segment 3, segment 2 could then become a

transmembrane domain. Similar results have been observed with the rotavirus nonstructural protein NS28. This protein contains 3 putative hydrophobic membrane anchoring domains, but it has been shown that only one of these spans the lipid bilayer, while the other two regions are peripherally associated with the membrane (Chan *et al.*, 1988; Bergmann *et al.*, 1989).

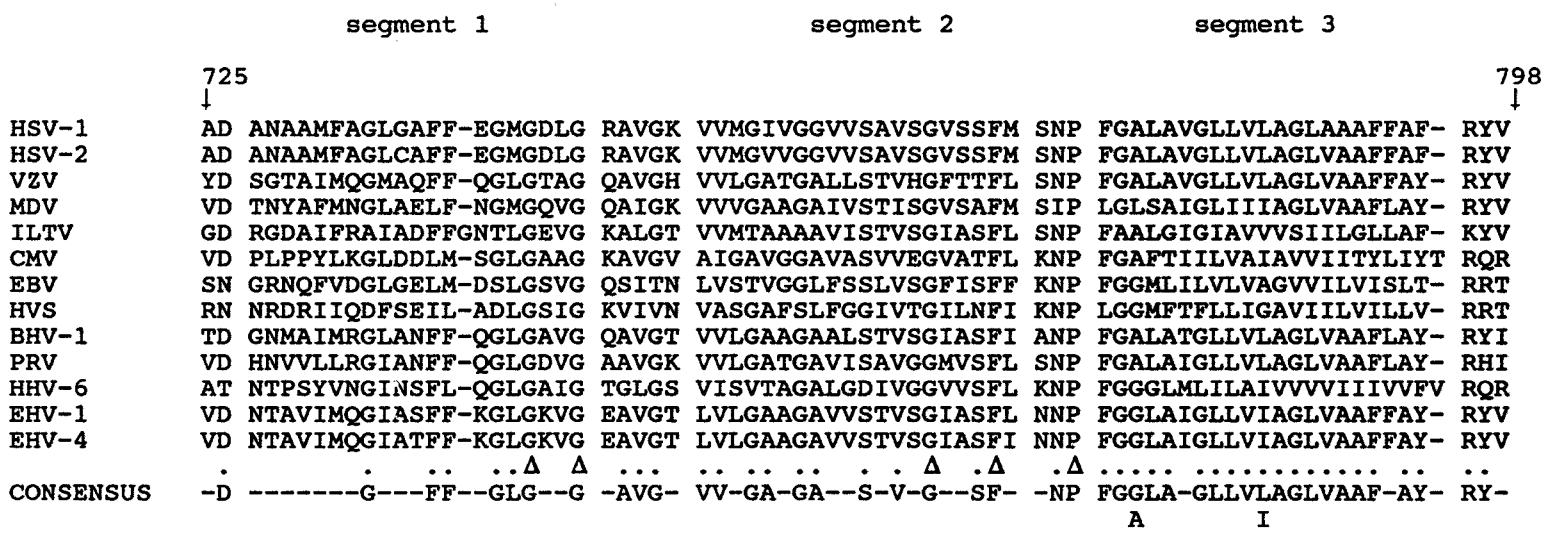
Another possible function for segments 1 and 2 of the 69 residue hydrophobic domain could be in membrane fusion activity, since gB glycoprotein is involved in this function (Manservigi *et al.*, 1977; Sarmiento *et al.*, 1979; Sarmiento and Spear, 1979; Little *et al.*, 1981; Bzik *et al.*, 1984b; Ali *et al.*, 1987; Cai *et al.*, 1988; Butcher *et al.*, 1990). A survey of myxovirus and paramyxoviruses has led to an hypothesized consensus sequence for fusion peptides which is Phe-X-Gly, where X can be any amino acid (Gallaher, 1987). An examination of the 69 residue hydrophobic domain of the gB glycoprotein reveals that there are two such sequences within segment 1. Perhaps, then, segment 3 is the membrane anchoring domain, and segment 1 and 2 are associated peripherally with the membrane, making them accessible for membrane fusion functions.

As discussed in section 1.4.2, the gB glycoprotein has been sequenced for four strains of HSV-1 (Bzik *et al.*, 1984; McGeoch *et al.*, 1988; Pellet *et al.*, 1985b; Stuve *et al.*,

1987). The carboxyl terminus hydrophobic domains of the gB glycoproteins from these four strains, as well as from HSV-2, have identical amino acid sequences. Further, the gB homolog has been identified and sequenced in 12 other herpesviruses bovine herpesvirus (Albrecht and Fleckenstein, 1990; Bzik *et al.*, 1986; Cranage *et al.*, 1986; Davison and Scott, 1986; Ellinger *et al.*, 1993; Griffin, 1991; Misra *et al.*, 1988; Pellet *et al.*, 1985; Riggio *et al.*, 1989; Robbins *et al.*, 1987; Ross *et al.*, 1989; Stuve *et al.*, 1987; Whalley *et al.*, 1989). A comparison of the carboxyl terminus hydrophobic domain of these 13 herpesviruses is shown in Figure 4.2.1. Analysis of the aligned sequences yields several interesting observations. Firstly, there are 5 residues conserved in all 13 herpesviruses: three glycines, one phenylalanine, and one proline (see Figure 4.2.1). There are also 44 other residues that are conserved in at least 8 of the 13 herpesviruses. Nineteen of these consensus residues are located within segment 3. Segment 1 exhibits the greatest variation, and it is clear that it cannot serve as a membrane spanning domain in most of the other herpesvirus gB glycoproteins, due to its very low hydrophobicity. Segment 3, on the other hand, is uniformly hydrophobic throughout the gB glycoprotein homologs, and this would indicate that this segment would in fact be the membrane anchoring domain of the glycoprotein and that this function has been conserved.

Figure 4.2.1 Alignment of the amino acid sequences of the gB glycoprotein from thirteen different herpesviruses.

The sequences of the carboxyl terminus hydrophobic domain of the gB glycoproteins are aligned, with the numbers shown corresponding to the HSV-1 gB amino acid residues. The triangles indicate residues that are conserved in all thirteen sequences, while the dots indicate consensus residues (present in at least 8 of the 13 sequences). The herpesvirus from which the sequence originated is indicated to the left of each sequence, and the three segments of the HSV-1 gB glycoprotein carboxyl terminus hydrophobic domain are indicated. Spaces are used to separate the various segments and domains, while dashes indicate where the sequences were shifted in order to achieve optimum alignment. The consensus sequence obtained from the alignment is indicated. HSV-1: herpes simplex virus 1; HSV-2: herpes simplex virus 2; VZV: varicella-zoster virus; MDV: Marek's disease virus; ILTV: infectious laryngotracheitis virus; CMV: cytomegalovirus; EBV: Epstein-Barr virus; HVS: herpesvirus saimiri; BHV-1: bovine herpesvirus 1; PRV: pseudorabies virus; HHV-6: human herpesvirus 6; EHV-1: equine herpesvirus 1; EHV-4: equine herpesvirus 4.


segment 1
segment 2
segment 3
725
798
HSV-1 AD ANAAMFAGLGAFF-EGMGDLG RAVGK VVMGIVGGVVSAVSGVSSFM SNP FGALAVGLLVLAGLAAAFFAF- RYV
HSV-2 AD ANAAMFAGLCAFF-EGMGDLG RAVGK VVMGVVGGVVSAVSGVSSFM SNP FGALAVGLLVLAGLVAAFFAF- RYV
VZV YD SGTAIMQGMAQFF-QGLGTAG QAVGH VVLGATGALLSTVHGFTTFL SNP FGALAVGLLVLAGLVAAFFAY- RYV
MDV VD TNYAFMNGLAELF-NGMGQVG QAIGK VVVGAAGAIVSTISGVSAFM SIP LGLSAIGLIIIAGLVAAFLAY- RYV
ILTV GD RGDAIFRAIADFFGNTLGEVG KALGT VVMTAAAAVISTVSGIASFL SNP FAALGIGIAVVVSIILGLLAF- KYV
CMV VD PLPPYLKGLDDLM-SGLGAAG KAVGV AIGAVGGAVASVVEGVATFL KNP FGAFTIILVAIAVVIITYLIYT RQR
EBV SN GRNQFVDGLGELM-DSLGSVG QSITN LVSTVGGLFSSLVSGFISFF KNP FGGMLILVLVAGVVILVISLT- RRT
HVS RN NRDRIIQDFSEIL-ADLGSIG KVIVN VASGAFSLFGGIVTGILNFI KNP LGGMFTFLLIGAVIILVILLV- RRT
BHV-1 TD GNMAIMRGLANFF-QGLGAVG QAVGT VVLGAAGAALSTVSGIASFI ANP FGALATGLLVLAGLVAAFLAY- RYI
PRV VD HNVVLLRGIANFF-QGLGDVG AAVGK VVLGATGAVISAVGGMVSFL SNP FGALAIGLLVLAGLVAAFLAY- RHI
HHV-6 AT NTPSYVNGINSFL-QGLGAIG TGLGS VISVTAGALGDIVGGVVSFL KNP FGGGLMLILAIVVVVIIIVVFV RQR
EHV-1 VD NTAVIMQGIASFF-KGLGKVG EAVGT LVLGAAGAVVSTVSGIASFL NNP FGGLAIGLLVIAGLVAAFFAY- RYV
EHV-4 VD NTAVIMQGIATFF-KGLGKVG EAVGT LVLGAAGAVVSTVSGIASFI NNP FGGLAIGLLVIAGLVAAFFAY- RYV
. . .. ..Δ Δ ... . Δ .Δ .Δ ....
CONSENSUS -D -------G---FF--GLG--G -AVG- VV-GA-GA--S-V-G--SF- -NP FGGLA-GLLVLAGLVAAF-AY- RY-
A I

4.3 Oligomerization of the mutant HSV-1 gB glycoproteins

The HSV-1 gB glycoprotein has been shown to form homodimers in both HSV-1 infected cells and also when cloned HSV-1 gB genes are transiently expressed in the absence of other viral encoded proteins (Sarmiento and Spear, 1979; Claesson-Welsh and Spear, 1986; Zhu and Courtney, 1988; Ali, 1990; Highlander *et al.*, 1991). These dimers are detergent stable but heat sensitive (Sarmiento and Spear, 1979; Claesson-Welsh and Spear, 1986). Using transient expression of the mutant constructs, it was determined that the gB glycoprotein mutants Δ123, Δ23 and Δ13, all of which are not anchored to the cellular membranes and are secreted, do not form dimers. This would appear to indicate that stable membrane anchoring is necessary for dimerization of the glycoprotein. The mutant Δ3, however, does form dimers, even though it too is not anchored to the cellular membranes and is secreted from cells. However, the mutant Δ3 is the least secreted of the four secreted mutants, and perhaps this semi-anchoring is sufficient for oligomers to form. The presence of segment 3 alone, as is the case for the mutant Δ12, is sufficient for dimerization to occur, and this, along with the fact that the mutant Δ3, which lacks segment 3, also forms dimers indicates that this 69 residue hydrophobic region may not have a primary role in dimerization. It thus appears that the main role of this hydrophobic region in oligomerization is

to anchor the glycoprotein to the membrane, since without this anchoring, dimers cannot form, or perhaps this region is just required for proper folding of the protein.

Several other groups have attempted to identify the region(s) of the glycoprotein involved in this dimerization. Cai *et al.*, (1988b), using linker insertion mutagenesis, created a large number of addition, deletion, and chain termination mutants in glycoprotein gB. Their assay basically entailed forming hybrid dimers between the wild type gB glycoprotein and various mutant constructs that had already been shown to be complementation negative. These hybrid dimers would not be biologically functional, hence complementation is inhibited. The mutants that inhibit complementation must therefore contain the sequences necessary for dimerization, while mutants that did not inhibit complementation presumably did not form hybrid dimers with the wild type gB glycoprotein, and must therefore lack the proper interaction sequences. It was found that all the mutants that inhibited complementation, and hence formed dimers, contained residues 463 to 791 of glycoprotein gB. This region includes almost all of the 69 residue hydrophobic membrane anchoring domain (residues 727 to 795).

Ali (1990) used a series of truncation mutants in an attempt to identify the regions required for oligomerization of the glycoprotein. Truncating the gB glycoprotein after

residue 802 (that is, removing the last 102 cytoplasmic domain residues), did not affect dimerization, but truncation mutants at residues 507 or 616 (which are upstream of the membrane anchoring domain and within the putative interaction region of residues 463 to 791) resulted in the formation of monomer gB molecules of 507 or 616 residues in length that were secreted from the cells. Further mapping of the interaction region was done using more linker insertion mutants (Qadri *et al.*, 1991; Navarro *et al.*, 1993). It was shown that a mutant gB glycoprotein (designated pTR690) that had residues 720 to 879 deleted, which includes the entire 69 residue hydrophobic anchoring domain (residues 727 to 795) as well as the first 67 residues of the cytoplasmic tail, could form dimers. As well, gB molecules truncated at residue 720 (just upstream of the membrane anchoring domain), were secreted from cells and formed dimers, suggesting that membrane anchoring is not required for dimer formation, and that the 69 residue hydrophobic domain is not required for dimerization of the gB molecule. These results appear to contradict the results presented here. However, both the mutant pTR690 and the truncation at residue 720 do not contain all or part of the cytoplasmic tail of glycoprotein gB. The presence of the entire cytoplasmic tail in the deletion mutants used in this study could affect the stability of the dimer, or could interfere with protein folding, resulting in the absence of

dimerization in the case of the mutants not anchored to the cellular membranes.

Highlander *et al*. (1991), mapped the dimerization domain of glycoprotein gB to two separate regions on glycoprotein gB using a co-precipitation assay. They were able to map out two non-contiguous regions: an upstream site between residues 93 and 282; and a downstream site between residues 596 and 711. Glycoprotein gB dimer formation can results from interactions between two upstream sites, two downstream sites or one upstream and one downstream site. This result is somewhat contradictory to those obtained in this study, and with those of Ali (1990), Qadri *et al*. (1991) and Navarro *et al*. (1993). According to the model of Highlander *et al*. (1991), all of the deletion mutants used in these studies should form dimers, since they all contain the upstream interaction site between residues 93 and 282. However, the truncation mutants at residues 507, 616, and 630 of Ali (1990) and Navarro *et al*. (1993), also did not form dimers, even though they contained the upstream site. This would indicate that perhaps more than just primary sequence information is needed for oligomerization, and that perhaps some minimal folding information may also be required.

4.4 Processing and transport of the mutant HSV-1 gB glycoproteins

The gB glycoprotein of HSV-1 is synthesized on membrane bound ribosomes and translocated across the endoplasmic reticulum (ER). Several modifications and processing reactions occur during the synthesis of the glycoprotein. The first of these is the cleavage of the signal sequence which directs the protein to the rough ER. The next processing reaction is the co-translational addition of N-linked high mannose oligosaccharides (Honess and Roizman, 1975; Spear, 1976; Pizer *et al.*, 1980; Kornfeld and Kornfeld, 1985). There are 6 N-linked glycosylation sites in the gB glycoprotein, but it appears that only 5 of these sites are actually glycosylated (Cai *et al.*, 1988b). The glycoprotein is then transported from the ER to the nuclear envelope and the Golgi complex. The high mannose oligosaccharides that were added in the ER are eventually processed to complex oligosaccharide moieties in the Golgi apparatus (Serafini-Cessi and Campadelli-Fiume, 1981; Person *et al.*, 1982; Wenske *et al.*, 1982). This processing reaction can be monitored with endoglycosidase H (endo H) digestion. The high mannose moieties are sensitive to cleavage with endo H, while the processed, complex oligosaccahirides are resistant to endo H digestion (Tarentino and Maley, 1974; Kornfeld and Kornfeld, 1985).

The mutants constructed were tested for their transport to the Golgi complex using endo H digestion as an indication of transport. Only the mutant Δ3, along with the wild type glycoprotein, exhibited maturation of the oligosaccharide. The rate of transport for the mutant Δ3 was however much slower than that of the wild type glycoprotein. It is quite apparent from this that the deletion of amino acids within this 69 residue hydrophobic domain drastically affects the rate of intracellular transport. One explanation for this lack of transport could be that either membrane anchoring or dimerization of the glycoprotein is necessary for transport. This would only explain the mutants Δ13, Δ23 and Δ123. Another possible explanation for this lack of transport could be that the mutations result in misfolding of the protein. These misfolded proteins could be recognized by the BIP protein in the ER (Bole *et al.*, 1986), resulting in ER retention. These results are consistent with those obtained in other studies with various mutant gB glycoproteins, which are discussed below.

The structural requirements of the gB glycoprotein needed for proper transport and processing have been examined by several researchers (Cai *et al.*, 1988b; Huff *et al.*, 1988; Raviprakash *et al.*, 1990; Navarro *et al.*, 1991; Qadri *et al.*, 1991; Navarro *et al.*, 1993). A number of linker-insertion, chain termination, and deletion mutants were constructed and

tested for their transport and processing. Most mutations introduced into the ectodomain, which still allowed the gB protein to remain anchored in the membrane, generally resulted in drastically affecting, or abolishing transport and processing, suggesting that proper folding of the glycoprotein is necessary for transport.

Chain termination mutations upstream of the membrane anchoring domain, which resulted in secretion of the protein, generally did not affect transport and processing as drastically, indicating that membrane anchoring is not required for transport and processing. Mutations in the cytoplasmic domain generally had the least affect on transport and processing.

Interestingly, it was found that in the case of some truncation mutants that are not anchored to the membranes, and thus secreted, oligomerization of the gB protein was not necessary for transport and processing (Ali, 1990; Navarro *et al.*, 1993). Dimerization of the gB glycoprotein is not necessarily sufficient for proper transport and processing neither, as shown by the fact that insertion of 4 amino acids in the ectodomain of gB results in a protein that forms stable dimers, but is not transported out of the ER (Qadri *et al.*, 1991; Navarro *et al.*, 1991). This mutant glycoprotein was shown to be associated with the ER resident proteins GRP78 and GRP94, which apparently act as chaperones in the assembly of

wild type gB glycoproteins (Navarro *et al.*, 1991). These results indicate that proper folding of the gB glycoprotein is essential for transport out of the ER, and agree with the results obtained with the deletion mutants in this present study.

4.5 Subcellular localization of the mutant HSV-1 gB glycoproteins

The HSV-1 glycoprotein gB has been found localized in both the plasma membrane and the nuclear envelope in both HSV-1 infected cells and in cells transfected with the gB gene. This localization was determined using a variety of techniques, including immunofluorescence, cellular fractionation and immunoelectron microscopy (Heine *et al.*, 1972; Spear and Roizman, 1972; Spear, 1976; Eberle and Courtney, 1980; Compton and Courtney, 1984; Koga *et al.*, 1986; Ali *et al.*, 1987; Pachl *et al.*, 1987; Cai *et al.*, 1988; Raviprakash *et al.*, 1990; Torrisi *et al.*, 1992; Gilbert and Ghosh, 1993).

The subcellular localization of the deletion mutants in the 69 residue hydrophobic domain was established using cell surface iodination, indirect immunofluorescence and by cellular fractionation. It was found that of all the mutants, only Δ3 was localized in the plasma membrane. This is not surprising, since all the other mutants fail to move from the

ER to the Golgi complex. In contrast, almost all the deletion mutants, with the exception of the mutants Δ13, Δ23 and Δ123, are localized in the nuclear membrane. It is interesting to note that these same mutants that are not localized to the nuclear envelope are the same mutants that do not form dimers, indicating that the same minimum folding requirement necessary for oligomerization may also be necessary for nuclear envelope localization.

The nuclear envelope localization is not all that surprising, even though these same mutants do not move to the Golgi complex or the cell surface. The reason is that it has been shown that the gB glycoprotein associated with the nuclear envelope is endo H sensitive, indicating that these molecules do not pass through the Golgi complex on the way to the nuclear envelope (Compton and Courtney, 1984; Torrisi *et al.*, 1992).

Since all the mutants that contain segment 3 of the hydrophobic domain are localized in the nuclear envelope, it may be possible that this particular segment carries the information necessary for nuclear envelope targeting. Several recent reports indicate that hydrophobic transmembrane domains are capable of targeting proteins to the nuclear membranes. Wozniak and Blobel (1992) have shown that the single transmembrane segment of gp210 is sufficient for targeting to the pore membrane domain of the nuclear envelope. Smith and

Blobel (1993) have shown that the first of eight membrane spanning segments of the lamin B receptor is sufficient for targeting the protein to the inner nuclear membrane. Further, Raviprakash *et al.*, (1990), created a series of chain termination mutants in the cytoplasmic domain of glycoprotein gB. These constructs contained cytoplasmic tails of only 43, 22, 6, 3, or 0 residues, compared to the wild type 109 residue cytoplasmic tail. All these mutant constructs were localized to the nuclear envelope. These results suggest that the cytoplasmic domain of glycoprotein gB is not required for nuclear envelope localization, and that the nuclear localization signals must lie in the ectodomain or in the 69 residue hydrophobic membrane anchoring domain of the gB glycoprotein.

4.6 Complementation ability of the mutant glycoproteins

The biological activity of the mutant glycoproteins constructed was determined by looking at the ability of these mutants to complement a gB null virus. As described above, all the mutants but Δ3 and the wild type glycoprotein are defective in intracellular transport to the Golgi complex and plasma membrane. However, most of the mutants, with the exception of Δ13, Δ23 and Δ123, form dimers, and are transported to the nuclear envelope, where the virion particles are assembled. It would thus be possible for these

mutants to be incorporated into the virion particle. The results of the complementation experiments, however, showed that none of the mutant constructs could complement the gB null virus.

These results are in agreement with those obtained by Cai *et al.* (1988b). They found that addition mutations at residue 734, which is within segment 1 of the hydrophobic domain, abolished complementation activity of the glycoprotein. Chain termination mutations located within the 69 residue hydrophobic domain also abolished complementation activity. There are several possible explanations for this inability of the mutant glycoproteins to complement the gB null virus. Firstly, the mutant glycoproteins may not be incorporated into the virion, perhaps because they are not folded properly or have lost sequences necessary for interacting with other viral proteins. This can be verified by simply purifying the virus obtained in the complementation assay, and checking for the presence of the mutant gB glycoproteins in these particles. Secondly, the mutant glycoproteins may in fact be incorporated in the virion particle, but the mutant glycoproteins are, for some reason, biologically inactive.

4.8 Future directions of study

There are several directions of research that can be pursued with these deletion mutants in the hydrophobic domain of HSV-1 glycoprotein gB. Firstly, the biological activity of the mutants needs to be further analyzed. Although none of these mutants exhibit complementation activity, it remains to be determined exactly why this is so. Specifically, the prescence of the mutant glycoproteins in the virion particle needs to be established.

Perhaps the most exciting future work is the determination of the signals required for nuclear envelope localization. In fact, several hybrid mutants have already been constructed. The vesicular stomatitis virus glycoprotein G (VSV G) has been used as a reporter protein, and several hybrids have been constructed using VSV G and HSV-1 gB. One series of hybrids basically exchanges various domains between VSV G and HSV-1 gB. In one construct, the cytoplasmic tail and transmembrane domain of VSV G have been replaced with the 69 residue hydrophobic domain and cytoplasmic tail of HSV-1 gB. A second construct has the transmembrane domain of VSV G replaced with the 69 residue hydrophobic domain of HSV-1 gB. A third construct has the ectodomain of VSV G replaced with that of HSV-1 gB.

A second series of hybrid constructs has the transmembrane domain of VSV G replaced with segment 3, segment

1 and 3 as well as with segments 2 and 3 of the hydrophobic domain of the HSV-1 gB glycoprotein. Experiments to localize these hybrid constructs are currently in progress, and should lead to the determination of the nuclear targeting domain of HSV-1 glycoprotein gB.

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