MUTATIONAL ANALYSIS OF HERPES SIMPLEX VIRUS GLYCOPROTEIN gB

MUTATIONAL ANALYSIS OF THE HYDROPHOBIC REGION OF HERPES SIMPLEX VIRUS-1 GLYCOPROTEIN gB

By

SUSAN MICHELLE EFLER, B.Sc.

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AUTHOR: Susan Michelle Efler, B.Sc. (University of Waterloo)

SUPERVISOR: Dr. H.P. Ghosh

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ABSTRACT

The role of highly conserved amino acids within the carboxy-terminal hydrophobic domain of herpes simplex virus 1 (HSV-1) glycoprotein gB was studied by introducing point mutations using the method of site directed mutagenesis. A segment of this hydrophobic domain of glycoprotein gB contains a nuclear envelope (NE) targeting signal and the effect of these point mutations on targeting to the nuclear envelope was determined. A complementation assay was employed to determine the effect these mutations have on HSV-1 infectivity.

.The point mutations created within the transmembrane domain of glycoprotein gB had no effect on nuclear envelope targeting and localization. However, single point mutations introduced into the first and second hydrophobic domains of glycoprotein gB, $G_{743}R$ and $F_{770}S$, affected the targeting and localization of full-length glycoprotein gB at the nuclear envelope. When the transmembrane domain of HSV-1 glycoprotein gB containing the following point mutations $A_{790}Q$, $A_{791}S$, $A_{786}S$, $A_{786}Y$ and $A_{790}S$, was introduced into a chimeric protein consisting of the cytoplasmic domain and ectodomain of a plasma membrane protein, vesicular stomatitis virus glycoprotein G, NE targeting and localization were affected. These point mutations may affect the targeting of glycoprotein gB by altering the structure of the targeting signal within the protein. It can be hypothesized that the presence of the cytoplasmic domain, ectodomain domain, and the first and second transmembrane domains within full-length glycoprotein gB can compensate for the effect these point mutations have on nuclear envelope targeting, since the same point mutations had no effect on the targeting and localization of full-length glycoprotein gB.

Complementation assays showed that the glycoprotein gB mutants, $A_{786}S$, $A_{786}Y$, $A_{786}N$, $A_{790}Q$, $A_{791}S$, $F_{770}S$, or $G_{743}R$, were unable to complement a gB-null virus even though these mutant proteins are localized

at the nuclear envelope. These proteins may not have been incorporated into the viral capsid due to misfolding or due to the fact that sequences required for interaction with other viral proteins were lost. Another possibility is that the mutant proteins were incorporated into the HSV virion but were not biologically active.

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This thesis is dedicated to my family with love.

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INTRODUCTION

Herpes simplex virus type 1 (HSV-1) virion is composed of four structural elements, an electron opaque core, an icosadeltahedral capsid, an amorphous tegument and an outer envelope. The electron opaque core surrounds the viral genome which consists of approximately 150 kilobases (kb) of double stranded DNA (McGeoch et al., 1988). The icosadeltahedral capsid is composed of 162 morphological units or tubular capsomers surrounding the core which in turn is surrounded by the amorphous tegument. The tegument is sheathed by an outer envelope composed of a lipid bilayer containing a number of viral membrane proteins. (Figure 1)

The outer envelope is acquired when the viral capsids containing full length DNA attach to patches of modified inner nuclear membrane and become enveloped. Viral proteins localized at the inner nuclear membrane become incorporated into the virion envelope. Enveloped virions have been shown to contain only viral proteins within their envelopes, no detectable levels of host membrane proteins have been observed (Darlington and Moss, 1968). The mechanism by which host proteins are excluded from these membrane patches is still unknown. The localization or targeting signals required to transport viral envelope glycoproteins to the inner nuclear membrane are not fully understood.

Herpes simplex virus 1 glycoprotein gB has been used as a model to study nuclear envelope targeting and localization (Ali et al., 1987; Raviprakash et al., 1990; Gilbert et al., 1993; Rasile et al., 1993; Gilbert et al., 1994). Glycoprotein gB is the ideal protein to study because it is the most highly conserved of the herpesvirus envelope glycoproteins with an overall similarity between primary, secondary and tertiary structures and it plays an essential role in the herpesvirus life cycle.

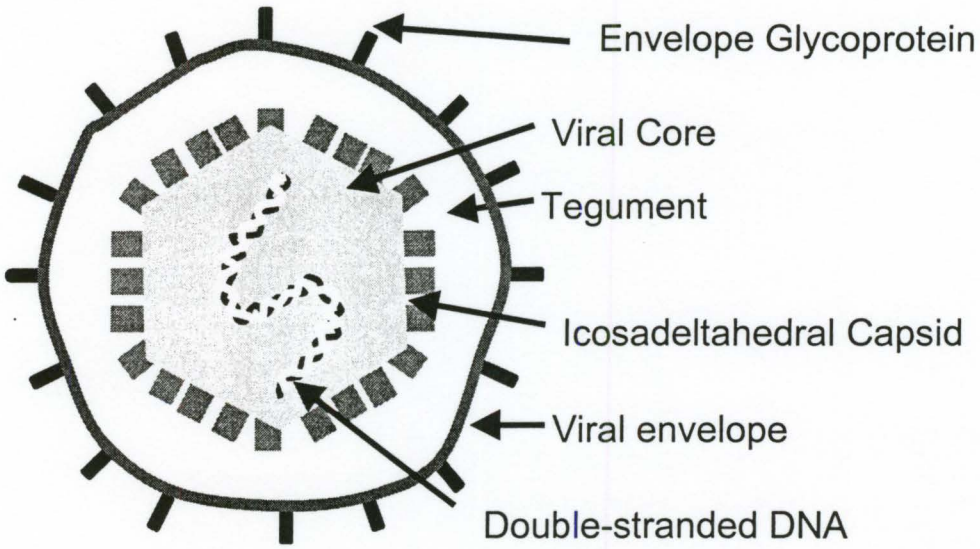


Figure 1: The herpes simplex virus (HSV-1) virion.

A schematic representation of the herpes simplex virus 1 (HSV-1) virion thrrough a cross-section of the envelope. Adapted from Café Herpé (http://www.cafeherpe.com) ©Smith Kline Beecham, 1997.

Herpes Simplex Virus 1 Glycoprotein gB

Physical mapping of temperature sensitive herpes virus mutants localized the structural peptide of glycoprotein gB to sequences between 0.345 and 0.368 map units. The nucleotide sequence of glycoprotein gB of herpes simplex virus KOS strain was determined by Bzik et al. (1984) and encoded a 904 amino acid protein (Figure 2). Sequence analysis predicted that gB was glycoprotein in character with nine putative N-linked glycosylation sites, a putative signal sequence of 44 amino acids, a hydrophobic membrane spanning sequence and a highly charged cytoplasmic sequence. The predicted size of HSV-1 KOS gB is 95.7 kD, high mannose gB is 110 kD and mature gB is 120 kD.

Highly conserved homologues of glycoprotein gB have been identified in varicella zoster virus (VZV), simian varicella zoster virus (Pumphrey and Gray, 1994), infectious laryngotracheitis virus (ILTV) (Kongsuwan et al., 1991), simian agent 8 (Borchers et al, 1991; Eberle et al., 1997), bovine herpesvirus 2 (Borchers et al, 1991), Epstein-Barr virus (EBV) (Pellett et al., 1985), human cytomegalovirus (HCMV), HSV-2, and pseudorabies virus (PrV). (Figure 3). The creation of viral recombinants and complementation of glycoprotein gB null mutants are techniques used to demonstrate functional homology between different α-herpesviruses (Eberle et al., 1997; Miethke et al., 1995; Mettenleiter and Spear, 1994; Misra and Blewett, 1991).

Pseudodiploid HSV-1 virions were constructed which express HSV-1 glycoprotein gB and also contain a gene encoding bovine herpesvirus 1 (BHV-1) gB. Both HSV-1 gB and BHV-1 gB were inserted into viral and cellular membranes but they failed to form oligomers with each other. The BHV-1 homologue was able to complement HSV-1 gB functions required for viral infection, therefore, the homologues serve similar functions in the life cycle of the herpes virus. (Misra and Blewett, 1991). A pseudorabies virus gB expressing cell line was able to complement a lethal defect in glycoprotein gB of herpes simplex virus 1, however, a cell line expressing glycoprotein gB of herpes simplex virus 1 was unable to complement defective gB of the pseudorabies virus (PrV) (Mettenleiter and Spear, 1994). BHV-

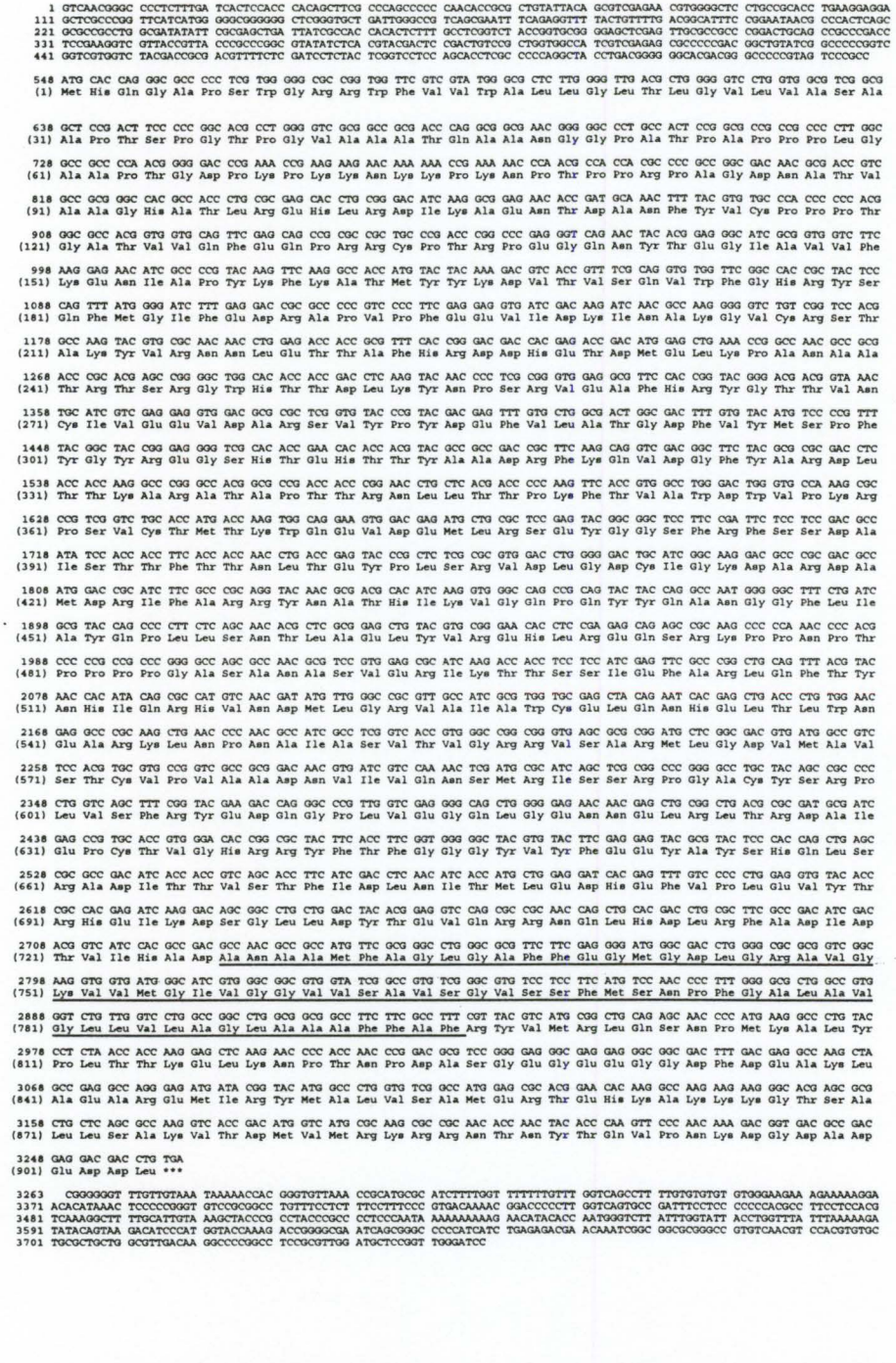


Figure 2: The nucleotide and predicted amino acid sequence of HSV-1 glycoprotein gB

The nucleotide sequence of glycoprotein gB of HSV-1 is shown along with the predicted amino acid sequence (Bzik et al., 1984; Bzik et al., 1986). The three hydrophobic domains within the carboxy-terminus are underlined, amino acids 727-795.

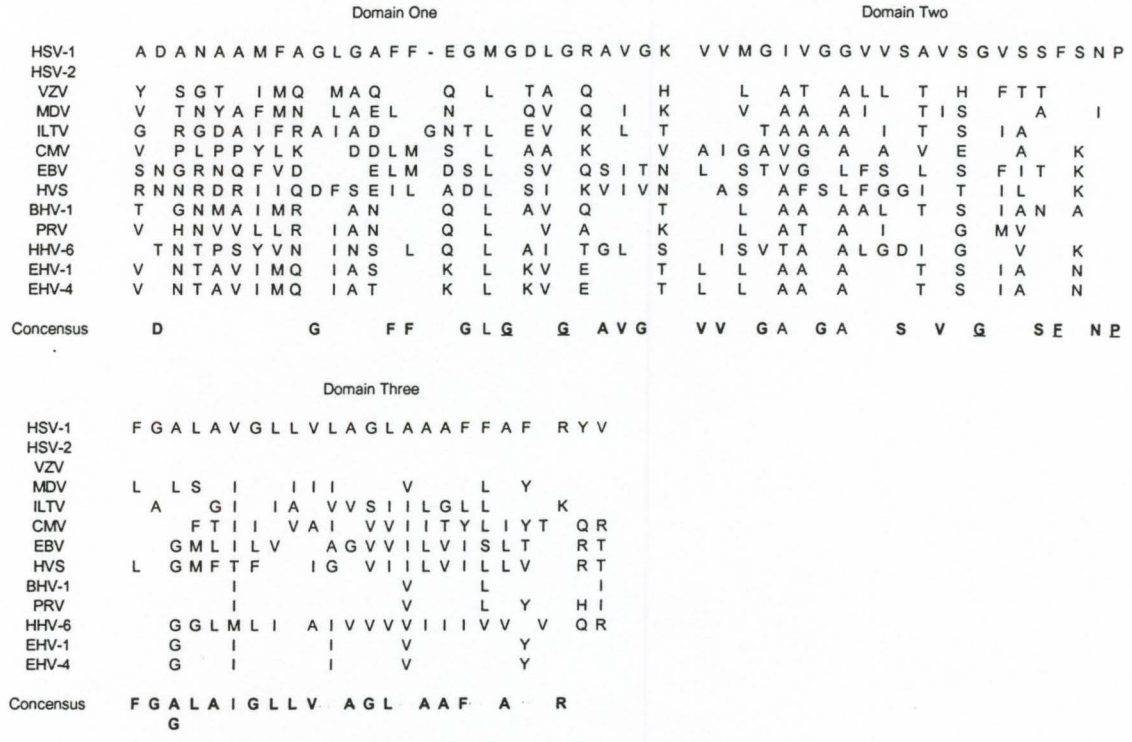


Figure 3: Highly conserved homologues of HSV-1 glycoprotein gB.

A comparison of the amino acids within the carboxy-terminal hydrophobic domain of HSV-1 glycoprotein gB with other herpesviridae: varicella zoster virus (VZV), Marek's disease virus (MDV), infectious laryngotracheitis virus (ILTV), human cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpesvirus saimiri (HVS), bovine herpesvirus 1 (BHV-1), pseudorabies virus (PRV), human herpesvirus 6 (HHV-6), equine herpesvirus 1 (EHV-1), and equine herpesvirus 4 (EHV-4). A concensus amino acid sequence was determined from the most highly conserved amino acids.

1 gB was found to functionally complement a gB-negative pRV mutant, however, PrV gB was unable to complement gB-negative BHV-1 in direct cell to cell spread or penetration even though it was incorporated into the virion envelope. The construction of chimeric proteins revealed that the carboxy-terminal of glycoprotein gB is important for function but differs between PrV gB and BHV-1 gB. (Miethke et al., 1995) As shown, functional homology is conserved amongst α–herpesviridae, however, Lee et al. (1997) showed that there is no sufficient functional homology between the different glycoprotein gBs of the β and γ herpesvirus subfamilies. The conservation of glycoprotein gB amongst α-herpesviruses may reflect essential functions in the life cycle of the herpesvirus. The conserved amino acids may therefore be essential for the structure and function of glycoprotein gB.

Biological Role of Glycoprotein gB

Glycoprotein gB plays a primary role in the infectivity of HSV-1. Glycoprotein gB is involved in viral attachment, penetration, cell-to-cell spread and syncytia formation (Bzik et al., 1984; Cai et al., 1987; Cai et al., 1988; Highlander et al., 1988; Navarro et al., 1992; Gage et al., 1993; Baghian et al., 1993; Spear, 1993; Hutchinson et al., 1993; Herold et al., 1994; Pereira, 1994).

The initial binding of HSV-1 to the host cell surface is mediated by the interaction of glycoprotein gC and or gB with cell surface glycosaminoglycans, such as heparan sulphate and dermatan sulphate (Williams and Straus, 1997). Glycoprotein gB null viruses, KΔT and K082 bound to the host cell surface but failed to produce viral proteins or form plaques, therefore, glycoprotein gB is nonessential for virus attachment (Cai et al., 1988). This finding was supported when neutralizing antibodies against glycoprotein gB failed to inhibit virus attachment (Navarro et al., 1992). However, glycoprotein gB interacts weakly with heparan sulphate at neutral pH, this interaction may assist in stabilizing virion binding and trigger subsequent events in virus entry. Herold et al. (1994) found that gC negative virions impaired for viral binding and entry, maintained significant infectivity. It was determined that glycoprotein gB mediated the attachment of gC negative virions to the cell surface. In the absence of

glycosaminoglycans, HSV-1 was able to infect sog9 cells (cells which are unable to synthesize glycosaminoglycans) because glycoprotein gB was able to interact with dextran sulphate, a glycosaminoglycan analog (Dyer et al., 1997). This suggests that HSV-1 infection can occur in the absence of glycosaminoglycans and at least one non-glycosaminoglycan receptor can initiate infection. The initial interaction of HSV with cell surface proteoglycans is followed by the interaction of glycoprotein gD with cell surface receptors such as HveA (HVEM) a lymphotoxin receptor, HveC (PRR1) and HveB (PRR2) poliovirus receptors (Whitbeck et al., 1997; Krummenacher et al., 1998).

Following attachment, penetration occurs by fusion of the viral envelope with the host cell plasma membrane. HSV does not require low-pH mediated fusion involving endosomes in order to penetrate a cell. Experimental evidence has shown that four glycoproteins are involved in virus penetration, gB, gD, gH and gL (Spear et al., 1992; Davis-Poynter et al., 1994). Glycoprotein gB plays an indispensable role in penetration. gB-negative virions attached to the host cell surface but were unable to penetrate even though the rest of the viral glycoproteins were present within the virion envelope (Cai et al., 1987). Antibodies recognizing glycoprotein gB sequences within the region of amino acids 241 to 441 inhibited penetration by the virus indicating that residues within this region are critical for virus penetration. Antibodies which had no effect on penetration localized to amino acids 596 to 737 indicating that this region of glycoprotein gB does not play are role in virus penetration (Highlander et al., 1988). Rate of entry (roe) mutations were localized to both the ectodomain and cytoplasmic domain of glycoprotein gB indicating that these regions may cooperate in virus penetration (Gage et al., 1993). Pereira (1994), proposed a model for viral membrane fusion involving a hydrophobic fusion pore formed by several gB dimers interfacing with other transmembrane glycoproteins and membrane associated proteins in the lipid bilayer.

The transfer of HSV-1 from infected cells to uninfected neighbouring cells requires cell-cell fusion. Glycoprotein gB plays an essential role in the cell to cell spread of HSV-1. The fusion of the plasma membrane of adjacent cells to form multi-nucleated giant cells is termed syncytia formation. Mutations within the cytoplasmic tail of glycoprotein gB at two independent sites, $R_{858}H$ (tsB5) and the

addition of 2 to 4 amino acid residues between 816 and 817, resulted in the syncytia phenotype of the virus (Cai et al., 1988; Gage et al., 1993) . A gB null-syncytia virus (syn082) caused extensive fusion on the gB expressing Vero cell line, D6, but failed to form syncytia on Vero cells. The fusion ability was recovered on Vero cells by transfection with pKBXX, a vector containing the gene encoding wildtype glycoprotein gB of HSV-1. Therefore, gB is essential for HSV-1 induced cell fusion. (Cai et al., 1988). Further studies showed the essential role of the cytoplasmic tail of gB in cell to cell fusion, the truncation of the cytoplasmic tail of gB by 28 amino acids (amb1511-7) resulted in extensive cell fusion. This extensive fusion may have been caused by a conformational change within the cytoplasmic tail of glycoprotein gB which may be required for cell fusion. (Baghian et al., 1993) A variant of the HSV-1 strain 17 syn+ called 17 hep syn which causes giant syncytia formation further supports the role of the cytoplasmic tail of gB in fusion. Sequence analysis of HSV-1 17 hep syn revealed that this syncytia phenotype was due to two point mutations within the cytoplasmic domain of gB, $L_{787}H$ and $A_{825}V$ (Engel et al., 1993).

Mutations which reduce the fusion activity of glycoprotein gB are located within its cytoplasmic domain and ectodomain. Navarro et al. (1992) using neutralizing antibodies determined that ten antibodies mapping to three domains within glycoprotein gB blocked viral penetration. These antibodies also inhibited cell to cell spread and fusion. Three neutralizing antibodies that inhibited penetration did not inhibit plaque development and only one of these antibodies blocked fusion, therefore, the mechanism of cell to cell spread and fusion mediated by glycoprotein gB are related but not identical to penetration.

Structure and Topology

Amino acid sequence analysis of glycoprotein gB revealed the presence of a 29 amino acid membrane insertion signal sequence (Bzik et al., 1984; Bzik et al., 1986; Pellet et al., 1985). The hydropathic index was determined using the Kyte and Doolittle method (1982) and revealed three hydrophobic domains consisting of 69 amino acids. These hydrophobic domains were predicted to be three anti-parallel segments, each spanning the membrane, each segment being connected by a short turn.

The 109 amino acid carboxy-terminus was predicted to be the cytoplasmic domain since no potential glycosylation sites were present. Six potential N-linked glycosylation sites (Asn-X-Thr/Ser) were observed in the amino terminus of the predicted 696 amino acid hydrophilic ectodomain. Cai et al. (1988b) determined that only 5 of the 6 N-linked glycosylation sites were in fact glycosylated. The amino acid sequence of the ectodomain predicts 7 potential β-sheet domains, 11 major turn domains, and 17 helical domains with more than 10 amino acids.

Oligomerization

Shortly after synthesis gB forms homodimers. gB dimer formation does not require glycosylation or viral protein synthesis (Claesson-Welsh and Spear, 1986; Britt and Vugler, 1992). Glycoprotein gB has been shown to form dimers in cells transiently expressing glycoprotein gB and in HSV-1 infected cells (Sarmiento and Spear, 1979; Haffey and Spear, 1980; Claesson-Welsh and Spear, 1986; Britt and Vugler, 1992). These dimers are resistant to mercaptoethanol, as well as, mercaptoethanol and sodium dodecyl sulphate except at elevated temperatures (Sarmiento and Spear, 1979). Dimer formation is essential for glycoprotein gB function (Cai et al., 1988). In order to determine the regions within glycoprotein gB required for oligomerization, a complementation assay was employed by Cai et al. (1988). Mutations within the region, amino acids 463 to 791 of glycoprotein gB, interfered with wild-type activity of glycoprotein gB. It can be postulated that mutations within this region interfered with normal oligomer formation resulting in inactive dimers. The loss of activity indicates that oligomerization is essential for function and the region between amino acids 463 to 791 are essential for oligomerization. Highlander et al. (1991) further defined the region required for oligomerization to amino acids 596 to 711. They also identified a second oligomer binding site upstream between residues 93 and 282. This was later determined to provide only weak interaction. The transmembrane domain and the carboxy-terminus of gB are not required for oligomerization. This observation defined an even smaller region responsible for oligomer formation, amino acids 600 to 690 (Qadri et al., 1991). Laquerre et al. (1996) has narrowed

down the region of oligomerization to amino acids 626 to 675, this region was able to form dimers independently. Cysteine residues 596 and 633 were found to be non-essential in oligomerization but are required for proper folding, processing and incroporation of glycoprotein gB into mature virion particles (Laquerre et al., 1998).

Membrane Anchoring Domain

A Kyte and Doolittle (1982) hydropathic profile of HSV-1 glycoprotein gB revealed three hydrophobic segments consisting of 69 amino acids. These hydrophobic segments are highly conserved amongst herpesviridae and the third hydrophobic segment is almost completely conserved (Figure 3). Segment one (amino acids 727-746), segment two (amino acids 752-772) and segment three (amino acids 775-795) of glycoprotein gB have hydrophobicities of 0.8, 1.7 and 2.4 respectively. These hydrophobic regions were predicted to be three anti-parallel segments which span the membrane connected by a short turn (Pellett et al., 1985; Cai et al., 1988; Claesson-Welsh and Spear, 1986). A membrane spanning domain must be at least 19 amino acid residues in length with a hydrophobicity of 1.65 or greater (Kyte and Doolittle, 1982; Adams and Rose, 1985), segment one does not possess these characteristics of a membrane spanning domain. Rasile et al. (1993) created and analyzed several mutant gB glycoproteins in order to determine the minimal sequence required for membrane anchoring. Deletion mutants were created that contained only the first (Δ2,3), second (Δ1,3) or third (Δ1,2) segments of the hydrophobic region. Also mutants without the first hydrophobic segment (Δ1), without the second segment (Δ2) or without the third segment of the hydrophobic region (Δ3). Another mutant had all three hydrophobic segments of glycoprotein gB deleted (Δ1,2,3). The ability of the deletion mutants to stably anchor the protein within the membrane was determined by three types of assays; secretion of the glycoproteins from transfected cells into the medium, susceptibility to protease digestion, and alkaline extraction of non-integral membrane proteins. The following deletion mutants were secreted into the medium following transfection, Δ1,3; Δ2,3; Δ3 and Δ1,2,3. These results indicate that the third hydrophobic domain is essential for

membrane anchoring. Results from the protease sensitivity assay showed that the deletion mutants Δ1, Δ2, and Δ1,2 were anchored within the membrane with the same orientation as wild-type gB. The following deletion mutants were protected from protease digestion, Δ1,3; Δ2,3 and Δ1,2,3, therefore, they must have been translocated to the lumen of the microsomal vesicles. Results for the gB Δ3 mutant indicated that a small fraction of the protein was anchored to the membrane while the majority was translocated to the lumen. This demonstrates that the Δ3 mutant was not stably associated with the membrane. The alkali extraction assay involves extracting microsomal vesicles containing labelled proteins with alkali. Non-integral membrane proteins will be found in the supernatant while membrane bound proteins will be retained within the pellet. The deletion mutants found within the supernatant were Δ2,3; Δ1,3 and Δ1,2,3, while Δ1, Δ2, and Δ1,2 were present in the pellet fraction. The deletion mutant Δ3 was present in both the pellet and supernatant fraction indicating that deleting the third hydrophobic domain decreased the ability of the protein to remain membrane anchored. The above data indicate that the third hydrophobic domain is sufficient for membrane anchoring glycoprotein gB. The first domain is not required for membrane anchoring but in the absence of the third hydrophobic domain, the first and second domains may exhibit weak membrane anchoring activity. The second domain may help to stabilize the protein within the membrane. Its hydrophobic index indicates that it may lie along the surface of the membrane exerting its stabilizing ability. Figure 4 shows the orientation of glycoprotein gB within the membrane proposed by Rasile et al. (1993).

Processing and Transport

Viral membrane proteins are synthesized on membrane-bound ribosomes and inserted into the endoplasmic reticulum (ER) (Doms, 1993). Glycoprotein gB of HSV-1 contains an N-terminal cleavable signal sequence which is removed by a signal peptidase. Shortly after translocation, protein folding occurs.

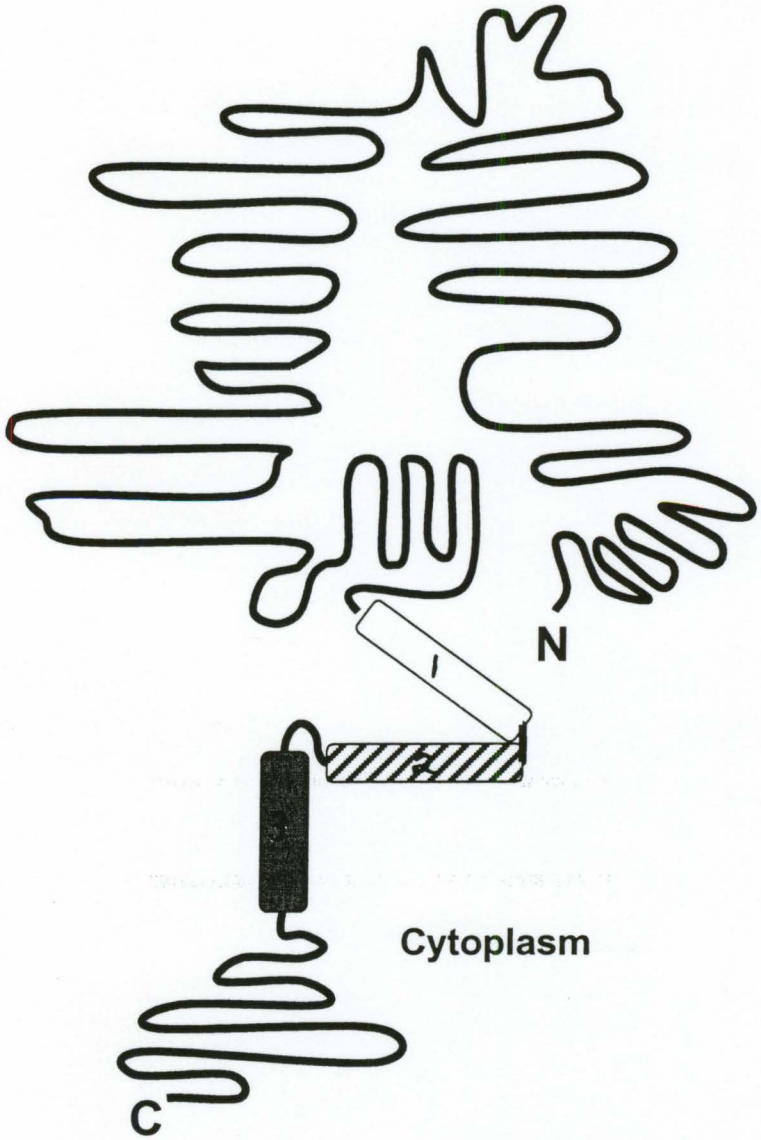


Figure 4: Predicted topology of glycoprotein gB of HSV-1.

A diagrammatic representation of the topology of HSV-1 glycoprotein gB predicted by Rasile et al. (1993). The predicted positions of the hydrophobic domains one, two and three relative to the cellular membrane are indicated.

Oligomerization is a late folding event which occurs in the ER and is essential for efficient transport of the full length protein. Glycoprotein gB contains six potential N-linked glycosylation sites (Asn-X-Thr/Ser) within its amino terminus. Cai et al. (1988b) determined that only 5 of the 6 N-linked glycosylation sites were in fact glycosylated following the same pathway as that of eukaryotic cell glycoproteins. The rate of transport of glycoprotein gB through the exocytic pathway can be determined by monitoring the glycans.

Glycoprotein gB is synthesized 4 hours post infection, is converted to its mature form (t½, 120 min.) and then appears at the plasma membrane (t½, 270 min.). Glycoprotein gB is processed and transported much more slowly than other viral glycoproteins. The rate limiting step was determined to be the conversion of the high-mannose form of glycoprotein gB to the complex form (Sommer and Courtney, 1991).

The cytoplasmic tail, hydrophobic domain, and the oligomerization of glycoprotein gB play a role in the processing and transport of the protein (Cai et al., 1988a; Cai et al., 1988b; Raviprakash et al., 1990; Navarro et al., 1993; Rasile et al., 1993; Zheng et al., 1996). Proper folding is a strict requirement for the transport of membrane-anchored forms of glycoprotein gB through the exocytic pathway.

Deletions within the carboxy-terminal hydrophobic domain of glycoprotein gB resulted in impaired intracellular transport of the molecule. The mutant proteins were glycosylated but at a slower rate than wild-type gB (Rasile et al., 1993). Navarro et al. (1993) created several glycoprotein gB deletion mutants and studied the effect of these deletions on dimer formation and transport. Two mutants, gB (1-690) and gB (1-690Δ849) have the transmembrane domain of glycoprotein gB deleted as well as all (1-690) or part of the cytoplasmic domains (1-690Δ849). These mutants were able to form dimers but were incompletely transported and were retained in the Golgi. These results demonstrate that the transmembrane domain and the cytoplasmic tail are required for proper transport and the formation of dimers alone is not sufficient for efficient transport. The mutants gB (1-600) and gB (1-475), both lacking a cytoplasmic tail and transmembrane domain failed to form dimers. gB (1-600) however, was retained within the ER, while gB (1-475) was transported efficiently and secreted. Dimerization was not necessary

for short soluble forms of gB to be transported efficiently provided the conformational regions were properly assembled enabling correct folding of the protein. The cytoplasmic tail and transmembrane domains were not required for processing but they may regulate the translocation of the protein to the cell surface (Cai et al., 1988). Glycoproteins lacking the cytoplasmic tail or comprising 3 to 6 amino acids within the cytoplasmic tail had reduced rates of transport and their appearance at the cell surface was reduced. Cai et al., (1988) found that mutations throughout glycoprotein gB had an effect on processing, therefore, proper folding is a prerequisite for efficient transport.

Immature forms of the viral glycoproteins are found within the inner nuclear membrane of infected cells and the envelopes of virions located within the perinuclear space. Extracellular virions and some of the virions in transit to the extracellular space have glycoproteins with complex N- and O-linked oligosaccharides indicative of Golgi processing.

There are two proposed models for the processing of glycoproteins on the surface of the virion. For the first model, the viral capsid buds through the inner nuclear membrane acquiring its viral envelope containing immature glycoproteins. Post-translational glycosylation of the envelope proteins occurs in a stepwise manner along the exocytic pathway, after the virus contained within a membraned vesicle fuses with the Golgi apparatus (Johnson and Spear, 1982). The second theory was first introduced by Stackpole (1969), virions acquire an envelope by budding through the inner nuclear membrane, are de-enveloped at the outer nuclear membrane, and re-enveloped at the trans-Golgi acquiring mature glycoproteins.

Van Genderen et al. (1994) found that the phospholipid composition of mature virions was similar to that of Golgi membranes and differed from nuclear membranes, indicating that the viral envelope was acquired from the Golgi membrane. DiLazzaro et al. (1995) postulated that if the envelopment/de-envelopment model was correct, intermediate forms of the glycoproteins should not be observed. Helix pomatia lectin reacts specifically with unsubstituted N-acetylgalactosamine, an intermediate sugar added in the cis-Golgi. Virions in the perinuclear space did not react with helix pomatia lectin, while virions within vesicles close to the Golgi were labelled. Wheat germ agglutinin, a lectin specific for mature

glycoproteins, reacted with extracellular virions and variably with virions within vesicles near the Golgi. Their results rule out re-envelopment at the trans or post-Golgi compartment but do not rule out re-envelopment earlier in the exocytic pathway at the cis-Golgi. Browne et al., (1996) created recombinant herpesviruses in which glycoprotein gH was retained in the endoplasmic reticulum due to an ER retention signal KKXX. If de-envelopment, re-envelopment occurs, then retaining gH within the endoplasmic reticulum will result in a reduction in the number of infectious virus. They found that the virus yield was reduced by 100-fold, indicating that the virion envelope was acquired in a post-endoplasmic reticulum compartment such as the Golgi. The precise model of virion envelopment has not yet been elucidated and experimental results confirming and refuting both theories are still being published.

Protein Sorting and Targeting

Newly synthesized polypeptides must be sorted to the correct organelle or membrane of the cell in order to carry out their unique function. The endoplasmic reticulum (ER) consists of a network of interconnected tubules and cisternae extending throughout the cytoplasm. Proteins which are synthesized on cytoplasmic ribosomes bound to the endoplasmic reticulum (ER) membrane include secreted proteins, integral membrane glycoproteins, lysosome enzymes, rough ER enzymes and Golgi complex proteins. Rough ER enzymes and structural proteins remain in the ER after synthesis while other proteins leave the ER in transport vesicles. These transport vesicles bud from ribosome free areas of the ER membrane adjacent to the cis Golgi. Repeated cycles of vesicular budding and fusion transports proteins through the Golgi complex. A few proteins will remain within the Golgi complex while the rest are transported to their functional site or secreted. (Pelham, 1989; Darnell et al., 1990) Mechanisms for sorting of these proteins and the signal sequences involved have been studied extensively. In theory, movement of a protein through the secretory pathway could be mediated by a specific transport signal and the final destination of a protein determined by its lack of a signal for further transport. Alternatively, the retention of proteins within a specific compartment may be mediated by a signal or targeting sequence within the primary

structure of the protein and secretion of proteins occurs by a default pathway. Studies have shown that proteins are non-selectively transported to the cell surface and several signal or targeting sequences have been identified that divert these proteins to their specific functional site. (Pelham, 1989)

Plasma Membrane

The transport of proteins to the plasma membrane occurs by a default pathway, however, these membrane proteins are sorted between apical and basolateral domains. The creation and maintenance of plasma membrane polarity is achieved by proper sorting of membrane elements (Matlin, 1986). The mechanism of sorting proteins within the plasma membrane has not yet been resolved.

Endoplasmic Reticulum (ER)

The amino acid sequences of a number of unrelated luminal ER proteins were compared and found to contain similar C-terminal residues, KDEL, HDEL, RDEL and KEEL (Pelham, 1989). This C-terminal sequence is recognized by a specific receptor within the ER and causes retention of the protein (Munro and Pelham, 1987, Pelham, 1988). Putative receptors have been identified for this sequence and are thought to retrieve escaped resident proteins that leave the ER in the general bulk flow (Pelham, 1990). The integral membrane proteins, prostaglandin endoperoxide H synthase-1 and -2 (PGHSs), are associated with the luminal surface of the ER and the nuclear envelope. Within their C-termini they contain a sequence similar to KDEL, PTEL. Deletion and mutagenesis experiments determined that this PTEL sequence targets the enzymes to the ER. (Song and Smith, 1996)

A carboxy-terminal retention sequence KKXX, KXKXX, RKXX or RXKXX, where X is any amino acid, was determined for ER membrane bound proteins such as adenovirus E3/19k (Gabathuler and Kvist, 1990) and CD4 (Shin et al.,1991). The mechanism by which these proteins are retained has not yet been elucidated.

Golgi Complex

Within the Golgi complex, post-translational processing of newly synthesized membrane bound and secreted proteins occurs, as well as, protein sorting. The Golgi complex consists of flattened stacks of cisternal membranes which are divided into four subcompartments, cis, medial, trans and trans Golgi network (TGN). These stacks are polarized with respect to the distribution of resident enzymes and proteins move through the complex in a cis to trans direction. Golgi proteins must be retained within the appropriate subcompartment of the Golgi complex while substantial traffic flows through the organelle. (Machamer et al., 1993)

Avian coronavirus, (infectious bronchitis virus) glycoprotein M (IBV M) is targeted to the cis Golgi membrane. The first membrane spanning domain of IBV M is required for targeting to the cis Golgi. Single amino acid substitutions within the membrane domain revealed that 4 amino acids, N_{465}, T_{469}, T_{476} and Q_{480} which line one face of the predicted α helix are essential for retention within the cis Golgi. (Machamer et al., 1993)

The transmembrane domain of β-1,4-galactosyltransferase (β-1,4-GT) and α-1,3-galactosyltransferase plays a role in targeting these proteins to the trans Golgi cisternae. It was shown that increasing the size of the transmembrane domain inhibited Golgi retention and localized the enzymes within the plasma membrane (Masibay et al., 1993). Ten amino acids within the transmembrane domain of β-1,4-GT (VYYLAGRDLS) are sufficient for proper retention of the protein (Nilsson et al., 1991). α-2,6-sialyltransferase (α-2,6-ST) is a type II membrane protein located within the trans Golgi and the trans Golgi network (TGN). The transmembrane domain of α-2,6-ST specifies Golgi retention, however the retention is augmented by the presence of the ectodomain and cytoplasmic domain (Munro, 1991; Masibay et al., 1993). If retention is due to an interaction between transmembrane domains then it can be inferred that sequences within the ectodomain and cytoplasmic domain would also interact and therefore play a role in retention. Munro (1991) showed that when the ectodomain and cytoplasmic domain of α-2,6-ST are separated by a transmembrane domain with the same number of amino acids as α-2,6-ST, the protein is

retained within the Golgi.

Cholesterol is not uniformly distributed within cellular membranes, high levels are found within the plasma membrane while low levels are observed in the ER. Since cholesterol is synthesized in the ER, the cell requires a method of removing this cholesterol in order to maintain the required low level. Cells can progressively concentrate cholesterol as it moves along the secretory pathway subsequently forming a gradient within the Golgi. The amount of cholesterol found within the membrane affects the thickness and stability of the membrane bilayer. A high concentration of cholesterol within the plasma membrane creates a thickened bilayer. The length of the hydrophobic transmembrane domain of a protein affects its distribution within cellular membranes. A short transmembrane domain makes it energetically unfavourable for a protein to enter a thick bilayer such as the plasma membrane. Also, bulky side chains, such as phenylalanine, within the transmembrane domain would also be energetically unfavourable in a cholesterol rich, thicker bilayer. Structural analysis of Golgi proteins reveals that they contain short transmembrane domains as compared to plasma membrane proteins. (Bretscher and Munro, 1993)

More than one mechanism operates to ensure the correct localization of proteins within the Golgi, Nilsson and Warren (1994) revealed the presence of both a retrieval signal and a retention signal within a trans Golgi network protein. The cytoplasmic domain of the protein contains the retrieval signal and the retention signal resides within the transmembrane domain.

Nucleus

The nuclear envelope consists of two membranes, the inner and the outer membranes, which act as a selective barrier between the nucleus and the cytoplasm. These membranes are connected with each other by nuclear pores which are occupied by the nuclear pore complex. Targeting of proteins to the cell nucleus is initiated by the interaction of nuclear localization signals (NLSs) found within the primary structure of the protein to the cytosolic receptor, importin (Lee and Melese, 1989; Adam et al., 1989; Benditt et al., 1989; Pandey and Parnaik, 1991; Gorlich et al., 1994; Jans and Hubner, 1996). The importin

complex of alpha and beta subunits binds the nuclear substrate in the cytosol and the resulting trimeric complex moves through the nuclear pores. The α-subunit of importin binds the nuclear localization signal and the β-subunit docks at the nuclear pore complex (Gorlich et al., 1995; Gorlich et al., 1996). Makkerh et al. (1996) has studied the sequences involved in nuclear localization signals. The NLS of SV40 large T antigen is a single cluster of basic amino acids (PKKKRKV), while the NLS of nucleoplasmin is a bipartite requiring two clusters of basic amino acids separated by a mutation-tolerant spacer (KRPAATKKAGQAKKKK). It was discovered that more than 50% of nuclear proteins contain this consensus sequence. The oncoprotein c-Myc has an NLS sequence in which only three of nine residues are basic and one residue is acidic (PAAKRVKLD). Nuclear targeting of constructs containing the single cluster of KKKK was dependent on it being preceded by PAA and was stimulated if it was followed by the dipeptide LD. The relative positions of these elements was found to be crucial to the function of the NLSs and neutral and acidic amino acids play a critical role.

A second receptor-mediated nuclear import pathway has been identified. A novel 38 amino acid transport signal, termed M9, was discovered in the hnRNP (heterogeneous-nuclear ribonucleoproteins) A1 protein. This transport signal mediates bidirectional transport across the nuclear envelope. A 90 kDa protein termed transportin interacts with the M9 sequence and mediates the nuclear import of M9 carrying proteins. (Pollard et al., 1996)

Nuclear Envelope

The nuclear envelope consists of three separate domains, the inner nuclear membrane, the outer nuclear membrane and the pore nuclear membrane. Integral membrane proteins which are synthesized on the ER can be transported to all of the nuclear membrane domains by simple diffusion through the proteolipid bilayer (Soullam and Worman, 1993). Several proteins have been studied to determine the precise signal sequence required to transport and retain these membrane proteins in the desired membrane domain of the nuclear envelope. These proteins include the lamin B receptor (LBR) and lamina-associated

polypeptide 2 (LAP2) which are localized in the inner nuclear membrane and gp210 which is localized in the pore membrane domain. Results indicate that the transmembrane segment of LBR (Smith and Blobel, 1993) and its amino-terminal domain (Soullam and Worman, 1993) contain independent sorting signals which are sufficient for targeting to and localization in the inner nuclear membrane. LAP2 has been shown to contain multiple domains within its nucleoplasmic region which promote localization to the inner nuclear membrane (Furukawa et al., 1995). In the case of gp210, it was found that its single transmembrane segment was sufficient for its localization (Wozniak and Blobel, 1992)

Immunoelectron microscopy, immunofluorescence, and cell fractionation were used to determine the cellular localization of glycoprotein gB (Compton and Courtney, 1984; Ali et al., 1987; Raviprakash et al., 1990; Rasile et al., 1993; Gilbert et al., 1994; Veit et al., 1996) Gilbert et al. (1994) determined that glycoprotein gB was localized to both the inner and outer nuclear membrane, Golgi complex, endoplasmic reticulum, and the cell surface. Immunoelectron microscopy revealed that HSV-1 nucleocapsids were labelled when they became associated with the nuclear envelope indicating that glycoprotein gB became incorporated into the viral envelope at this site. Localization of glycoprotein gB to the inner nuclear membrane of the infected cell is therefore essential for incorporation into the viral envelope and ultimately for virus infectivity.

Two pathways have been proposed for intracellular transport of gB. Mature HSV-1 glycoprotein gB localizes to the cell surface by default through the exocytic pathway, while immature gB is localized to the inner nuclear membrane by passive diffusion from the ER. Several insertions, deletions, truncations, point mutations and chimeric proteins have been produced in order to determine the targeting sequence required for transport and localization of glycoprotein gB to the inner nuclear membrane (Cai, et al., 1987; Cai et al., 1988; Raviprakash et al., 1990; Navarro et al., 1993; Rasile et al., 1993; Gilbert et al., 1994; Desai et al., 1994). Deletion mutations within the carboxy-terminal, hydrophobic domain of glycoprotein gB revealed that the third hydrophobic domain, aa 774-795, was sufficient for nuclear envelope localization (Rasile et al., 1993). Chimeric proteins consisting of both gB and vesicular stomatitis virus

glycoprotein G (VSV G) were constructed in order to determine the minimum sequence requirements for transport and localization of glycoprotein gB to the nuclear envelope. The three topological domains, the ectodomain, transmembrane domain and cytoplasmic domain, fold under different conditions and comprise independent folding domains within the viral protein, therefore, different domains may be exchanged resulting in a stable chimeric protein (Doms et al., 1993). VSV G was chosen as a reporter protein because it is localized in the plasma membrane of the cell (Gilbert et al., 1994). Replacing the hydrophobic domains and the cytoplasmic tail of gB with VSV G (gB-G) inhibited nuclear envelope localization of the protein, however, when the hydrophobic domain and cytoplasmic tail of G were replaced with the corresponding domains of glycoprotein gB, the protein was localized to the nuclear envelope (Gilbert et al., 1994). Further substitutions showed that the hydrophobic domain of gB was sufficient for nuclear envelope localization of the chimeric protein (Gilbert et al., 1994). A chimeric protein consisting of the ectodomain and cytoplasmic tail of G and only the third hydrophobic, membrane anchoring segment of gB, (Gtm3gBG) was efficiently transported and localized in the nuclear envelope (Gilbert et al., 1994). Since the third hydrophobic, membrane anchoring region of HSV-1 gB was sufficient for efficient localization of the chimeric protein to the nuclear envelope, it can be hypothesized that the signal sequence required for nuclear envelope localization of glycoprotein gB resides within this third hydrophobic segment. The third hydrophobic, membrane anchoring domain of gB has a high degree of sequence conservation amongst herpesviruses and is almost entirely conserved amongst the α-herpesviruses (Figure 3).

Several mechanisms have been proposed in order to explain how sequences within the primary structure of proteins can determine the localization of these proteins within their specific functional domains in the nuclear envelope. The lamin B receptor (LBR) is localized to the inner nuclear membrane due to sequences within its transmembrane and amino-terminal domains (Smith and Blobel, 1993; Soullam and Worman, 1993). When the size of the amino-terminal domain was increased from 22.5 to 70 kD, the protein was unable to reach the inner nuclear membrane. Since the nuclear pore complex resides at the

junctions between the nuclear envelope membranes, it may play a role in targeting proteins to specific membrane and the increased size of the amino-terminal domain of LBR may have inhibited its movement to the inner nuclear membrane due to size constraints (Soullam and Worman, 1993). It has been proposed that integral membrane proteins are localized to the inner nuclear membrane by lateral diffusion through the ER by way of the nuclear pore complexes and are retained within the inner nuclear membrane due to interactions with nuclear ligands and other components associated with this membrane (Soullam and Worman, 1993; Furukawa et al., 1995). Other models include the existence of sorting receptors or transport vesicles, the formation of hetero-oligomers which would be retained within the inner nuclear membrane due to the size of the aggregate and signal sequences such as the nuclear localization signal and ER retention signal (Pelham, 1989) Cai et al. (1988) proposed the existence of both positive and negative sorting signals within HSV-1 glycoprotein gB. The positive signal would be a part of the native protein and would be recognized by the cellular transport machinery, while the negative signal would be exposed upon mutagenesis due to protein misfolding leading to the protein's retention in the ER and degradation.

A mechanism proposed for retention of proteins in the Golgi complex could also be true for glycoprotein gB within the inner nuclear membrane. The transmembrane domain of glycoprotein gB may bind to membrane spanning domains of identical or related molecules already present within the inner nuclear membrane creating large complexes which are retained within this membrane (Machamer, 1991).

Studies have shown that the transmembrane segment of a protein may contain the signal sequence required for membrane targeting. The transmembrane domain of both β-1,4-galactosyltransferase and protein M from the infectious bronchitis virus (IBV) is sufficient for retention in the Golgi complex and the transmembrane of LBR and gp210 is sufficient for sorting to the nuclear membrane (Wozniak and Blobel, 1992; Masibay, 1993; Machamer et al., 1993; Smith and Blobel, 1993). Since the third hydrophobic, membrane anchoring domain of HSV-1 gB is sufficient for efficient localization of the protein to the nuclear envelope and proper dimerization, it can be hypothesized that the primary sequence required for nuclear envelope localization of glycoprotein gB resides within this third hydrophobic domain. In order to

determine the specific amino acids within the third transmembrane segment which are essential for localization of HSV-1 gB to the NE, point mutations within this highly conserved sequence were created by site-directed mutagenesis. In order to determine the appropriate amino acids to study, the highly conserved third transmembrane domain of HSV-1 glycoprotein gB was compared with that of other proteins localized to the nuclear envelope, HSV-1 glycoprotein gD, gp210, LBR, LAP1 and 2 (Furukawa et al., 1995) and Adenovirus AdE3 10.5K (Chroboczek et al., 1992), as well as, the amino acids conserved within the transmembrane of VSV G, a plasma membrane protein (Figure 5). Doped mutagenesis was used to create more than one mutation in a single mutagenesis reaction. A single amino acid was mutated to four different amino acids using a mixture of oligonucleotides. Point mutations within the third transmembrane domain of full-length gB, as well as the chimeric protein Gtm3gBG, were used to study the role specific amino acids play in nuclear envelope targeting and localization, as well as, viral infectivity. Indirect immunofluorescence was employed to determine the effect of point mutations on intracellular localization of both wild-type and chimeric glycoproteins and further define the nuclear envelope localization signal of HSV-1 gB. A complementation assay, consisting of an HSV-1 gB-expressing cell line (VB38) that allows the growth of gB-defective viruses and a gB-null virus (K082) that provides all of the regulatory factors required for gB expression, was used in order to determine the effect these point mutations have on viral infectivity.

HSV-1 gB	NPFGALAVGLLVLAGLAAFFAF
HSV-1 gD	NMGLIAGAVGGSLLAALVICGIVYWM
gp210	QVMFFTFFALLAGTAVTAVTIIAYH
LBR	RFGTFMLMFFLPATVLYLVLMCK
AdE3 10.5k	NLGMWWFSIALMFVCIIMWLICCLK
LAP2	RLTGNFKHASSILPITEFSDITRRTPK KKPLTRAEVGEKTEERRVERDILKEM FPYEASTPTGISASCRRPIKGAAGRP

Figure 5: Comparison of putative nuclear envelope localization signals

The amino acid sequence of the third hydrophobic domain of glycoprotein gB of HSV-1 was compared to the nuclear membrane localization signals identified within glycoprotein gD of HSV-1 (gD), gp210, lamin B receptor (LBR), adenovirus AdE3 10.5k (AdE3 10.5K) and lamin associated protein (LAP2).

MATERIALS AND METHODS

Chemicals and Reagents

Acetic acid	Fisher
Acrylamide	Research Organics Inc
Adenosine 5'-triphosphate (ATP)	Pharmacia
Agar	Difco, BDH
Agarose	Gibco/BRL
Ammonium sulfate	Gibco/BRL, BDH
Ammonium acetate	BDH
Ammonium persulfate	Gibco/BRL
Ampicillin	Sigma
Aprotinin (Trasylol)	Miles
Bactotryptone	Difco
Bovine serum albumin	Sigma
Bromophenol blue	Sigma
Butanol	Fisher
Calf serum	Gibco/BRL
Calcium chloride	BDH
Cesium chloride	Interscience
Chloramphenicol	Sigma
Chloroform	Caledon
Coomassie blue	Gibco/BRL
Crystal violet	J.T. Baker
Deoxycholic acid (sodium salt)	Sigma
Deoxynucleotides (dNTPs)	Pharmacia
Dextrose	J.T. Baker
Diethylaminoethyl-dextran (DEAE-dextran)	Sigma
Dimethyl sulphoxide (DMSO)	BDH
Dithiothreitol (DTT)	Calbiochem
Ethidium bromide	Sigma
Ethylene-diamine tetra-acetic acid (EDTA)	BDH
Glucose	BDH
Glycerol	BDH
Glycine	Sigma, BioRad
Histidinol	Sigma
8-Hydroxyquinoline	Sigma
L-glutamine	Gibco/BRL
Isoamyl alcohol	Fisher
Isopropanol	Caledon
Magnesium sulphate	BDH, J.T. Baker
Magnesium chloride	BDH
β-Mercaptoethanol	BDH
Methanol	Caledon
3-[N-morpholino] propanesulfonic acid (MOPS)	Sigma

N-, N-methylene bisacrylamide	Gibco/BRL
N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)	Calbiochem, Boehringer Mannheim
N,N,N',N'-tetramethylethylene-diamine (TEMED)	Gibco/BRL
Nonidet P-40	BDH
Paraformaldehyde	Sigma
Penicillin/Streptomycin	Gibco/BRL
Phenol	BDH
Phenylmethylsulfonide fluoride (PMSF)	Sigma
Polyethylene-glycol 8000 (PEG)	Fisher, BDH
Potassium acetate	BDH
Potassium hydroxide	BDH
Potassium phosphate	Fisher, J.T. Baker
Protein A Sepharose	Pharmacia
Rubidium chloride	Sigma
Salicylic acid	Sigma
Sodium chloride	BDH
Sodium hydroxide	BDH
Sodium dodecyl sulphate	BDH
Sodium phosphate	Caledon
Sucrose	BDH, Gibco/BRL
T4 Gene 32	BioRad
Thiamine hydrochloride	Sigma
Tricine	Sigma
Tris (hydroxymethyl) aminomethane (Tris)	Gibco/BRL, Boehringer Mannheim
Triton-X 100	Sigma
Urea	Research Organics Inc.
Uridine	Sigma
Xylene cyanol FF	J.T. Baker
Yeast extract	Difco

Enzymes

Calf intestinal alkaline phosphatase (CIP)	Pharmacia
Restriction endonucleases	Pharmacia, Gibco/BRL, NEB
Ribonuclease A	Sigma
T4 polynucleotide kinase	BioRad
T4 DNA ligase	Gibco/BRL
T7 DNA polymerase (Sequenase)	United States Biochemical
Taq DNA polymerase	Perkin Elmer Cetus
Trypsin/EDTA	Gibco/BRL

Radiochemicals

[α-^{35}S]dATP (10 μCi/μL)	DuPont-New England Nuclear
[^{35}S]methionine (8 μCi/μL)	DuPont-New England Nuclear

Antibodies

Polyclonal antibody to glycoprotein gB of herpes simplex virus type 1 was prepared by Renald Gilbert. Essam Wanas prepared the polyclonal antibody to glycoprotein G of vesicular stomatitis virus. Fluorescein isothiocyanate-conjugated (FITC) goat anti-rabbit IgG was purchased from Organon Teknika Inc.

Multicomponent Kits

Geneclean II®Kit	BIO 101 Inc
Sequenase® Version 2.0, DNA Sequencing Kit	United States Biochemical
Muta-Gene® M13 In Vitro Mutagenesis Kit, Version 2	BioRad

Molecular Weight Markers

The 1 kilobase (kb) DNA ladder was purchased from Gibco/BRL and was suitable for sizing linear double-stranded DNA fragments from 500 to 12, 000 base pairs (bp) and contains vector DNA fragments ranging from 75 to 1,636 bp.

The high molecular weight protein marker (30,000 to 200,000 D) was purchased from Sigma and contains the following six proteins, carbonic anhydrase (29,000), egg albumin (45,000), bovine albumin (66,000), phosphorylase b (97,400), β-galactosidase (116,000) and myosin (205,000). The radioactive vesicular stomatitis virus (VSV) protein marker was prepared by Essam Wanas. The proteins are VSV L (190 kD), G (69 kD), N/P (50/49 kD) and M (29 kD).

Oligonucleotides

The oligonucleotides used for M13 mutagenesis, doped mutagenesis, sequencing, and polymerase chain reaction (PCR) are listed in Table 1.

Oligonucleotides were synthesized by The Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University. The amount of each oligonucleotide prepared was 40 nmoles and the samples were desalted.

Table 1 A summary of the oligonucleotides used for site directed mutagenesis, PCR and DNA sequencing reactions.

Letters in **bold** represent the new nucleotides introduced, ⟨brackets⟩ represent a mixture of nucleotides introduced at this site and *italics* represent the unique restriction enzyme site introduced.

Code	Description of the Oligonucleotides Function	Mutagenic Oligonucleotide Sequence (5'→3')
Oligonucleotides used for M13 mutagenesis of glycoprotein gB of HSV-1		
AB 5311	Mutagenesis of gB $A_{791}S$ creating a *HindIII site*	GCC GGC CTG GCG GC***A AGC TT***C TTC GCC TTT (nt 2903-2932)
AB 7798	Mutagenesis of gB $F_{770}S$ creating a *Xho1 site*	G GGC GTG TC***C TCG AG***C ATG TCC AAC CCC TT (nt 2839-2868)
AB 7799	Mutagenesis of gB $G_{743}R$ creating a *BglII site*	C TTC GAG GGG ATG **AG***A GAT CT*G GGG CGC GCG GT (nt 2761-2793)
Oligonucleotides used for doped mutagenesis of glycoprotein gB of HSV-1		
AB 5058	Mutagenesis of gB A_{790} to S, Q, P and STOP	GCC GGC CTG GCG ⟨**CA**G **TC**⟩ GCC TTC TTC GCC TT (nt 2903-2931)
AB 5059	Mutagenesis of gB A_{786} to S, N, Y and T	CTG TTG GTC CTG ⟨**AA**C **TC**⟩ GGC CTG GCG GCG GCC TT (nt 2891-2922)
Oligonucleotides used as PCR pairs for cloning the 3rd hydrophobic domain of HSV-1 gB into pXMG(AXB)		
AB 7779	Created a unique *Xho1* site at the 5' end of the third hydrophobic domain of gB	*CTC GAG CTC GAG* C AAC CCC TTT GGG GCG CT (nt 2863-2880)
AB 7780	Created a unique *BssHII* site at the 3' end of the third hydrophobic domain of gB	*GCG CGC GCG CGC* ACG AAA GGC GAA GAA G (nt 2923-2938)
Oligonucleotides used for sequencing		
AB 5797	Sequencing gB mutants	C GCG TTC TTC GAG GGG ATG (nt 2755-2733)
AB 1885	Sequencing pXMG tm3gB mutants	GAT ACT GGG CTA TCC AAA (nt 1356-1373)

Plasmids, Bacteriophages and Bacterial Strains

The replicative form of M13mp18 was included in the Muta-Gene M13 *in vitro* Mutagenesis Kit purchased from BioRad. The eukaryotic expression vector, pXM, was provided by Dr. G.G. Wong, Genetics Institute Inc., Cambridge, MA. pGem-4Z was purchased from Promega. The vector, pKBXX (Cai et al., 1987), was a gift from Dr. S. Person and contains the DNA for wild-type glycoprotein gB of herpes simplex virus type one.

Escherichia coli (*E. coli*) DH5α were used to prepare competent cells for transformation of plasmid DNA. CJ236 *E. coli*, (dut^{-1}, ung^{-1}) and MV1190 *E. coli* were provided in the Muta-Gene M13 *in vitro* Mutagenesis Kit and used for M13 mutagenesis reactions.

Growth Media and Buffers

Frequently used growth media and buffers and their components are listed in Table 2.

Table 2 Growth Media and Buffers

Media/Buffer	Composition
Luria-Bertani media (LB)	1% bactotryptone, 0.5% yeast extract, 171 mM NaCl; pH 7
H media	1% bactotryptone, 86 mM NaCl
2xYT media	1.6% bactotryptone, 1.0% yeast extract, 86 mM NaCl; pH 7
Glucose Minimal Media	26 mM K_2HPO_4, 77 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl, 1 mM $MgSO_4$ $7H_2O$, 0.001% thiamine HCl, 0.2% glucose; pH 7
TBE	0.045M Tris-borate, 0.001M EDTA
TBS	0.14 M NaCl, 5 mM KCl, 0.5 mM $MgCl_2$ $6H_2O$, 0.7 mM $CaCl_2$ $2H_2O$, 25 mM Tricine; pH adjusted to 7.3 with NaOH
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
TE	10 mM Tris-HCl, 1mM EDTA

Preparation of Competent Cells

Competent cells were prepared by the rubidium chloride method which is a modification of the method developed by Hanahan (1983). The expected transformation efficiency with this method was $>10^7$ colony forming units (cfu) per μg of plasmid DNA. A single colony of DH5α *E. coli* was grown with shaking overnight at 37°C in LB media (Luria-Bertani media) containing 1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 86 mM sodium chloride and 0.002 N NaOH. After overnight growth, it was subcultured 1:100 in LB media containing 20 mM magnesium sulphate. The culture was grown at 37°C with shaking to an OD_{590} between 0.4 and 0.6. When the appropriate OD was reached (approximately 3 hours), the sample was spun at 5,000 rpm for 5 minutes at 4°C in a Sorvall GSA rotor. All subsequent steps were performed on ice, with the pipettes and glassware pre-chilled and the solutions ice-cold. The cell pellet was resuspended with 0.4 times the original volume of TFB1 (30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM manganese chloride, and 15% (w/v) glycerol; pH adjusted to 5.8 with acetic acid) and incubated on ice. After 5 minutes, the sample was spun at 5, 000 rpm for 5 minutes at 4°C in a Sorvall GSA rotor. The pellet was resuspended in 1/25 the original volume of cold TFB 2 (10 mM 3-[N-Morpholino] propanesulfonic acid (MOPS), 75 mM calcium chloride, 10 mM rubidium chloride and 15% (w/v) glycerol; pH adjusted to 6.5 with potassium hydroxide). The cell suspension was incubated on ice for 1 hour and then aliquoted into pre-chilled microcentrifuge tubes, 150 μl per tube. Prior to storage at -70°C, the competent cells were flash frozen in liquid nitrogen .

Transformation of Competent Bacteria

An aliquot of competent DH5α cells was thawed on ice and 5 μl of ligation mixture or approximately 10 ng of plasmid DNA was added. The cells were incubated on ice for 1 hour, followed by heat shock in a heating block for 2 minutes at 42°C. After heat shock, the cells were placed on ice for 2 minutes. The cells were then diluted with 800 μl of LB media and incubated at 37°C with shaking for 20

minutes. Following the incubation, the cells were plated on selection medium containing the appropriate antibiotic, typically LB agar plates containing ampicillin (80 μg/ml). Normally, aliquots of 50 and 100 μl of transformed cells were plated and the remaining cells pelleted by centrifugation. The media was removed from the pelleted cells and they were resuspended in 100 μl of fresh LB media and then plated.

Phenol Neutralization

A phenol solution containing 0.1% hydroxyquinoline was neutralized by the addition of an equal volume of 1 M Tris-Cl, pH 8.0. The mixture was stirred for 15 minutes, allowed to settle, and then stirred again for a few minutes. After settling, the bottom phenol layer was recovered using a separatory funnel. The pH of the aqueous layer was checked. An equal volume of 0.1 M Tris-Cl, pH 8.0 was added and the above steps repeated until the pH of the aqueous layer was approximately 7.8.

Small Scale Preparation of Plasmid DNA

Plasmid DNA was isolated using the lysis by alkali method described in Sambrook, et al (1989) which was a modification of the methods of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981). A single bacterial colony was grown overnight at 37°C in 5 ml of LB media containing the appropriate antibiotic. After incubation, 1.5 ml of culture was spun in a microcentrifuge in order to pellet the bacteria and the media was removed by aspiration. The bacterial pellet was resuspended in 100 μl of ice-cold solution I containing glucose (50 mM) , Tris-Cl (25 mM, pH 8) and ethylene-diamine tetra-acetic acid (EDTA, 10 mM, pH 8). Once the pellet was completely resuspended, 200 μl of a freshly prepared solution of 0.2 N NaOH and 1% (w/v) sodium dodecyl sulphate (SDS) was then added to the mixture and the contents inverted several times. The sample was placed on ice and 150 μl of an ice-cold solution of 3 M potassium acetate and 11.5% (v/v) glacial acetic acid was added. The sample was then mixed in an inverted position. After five minutes on ice, the sample was spun and the supernatant transferred to a new

tube. Following this step, phenol:chloroform extractions were performed. An equal volume of phenol:chloroform:isoamyl alcohol (1:1:1/48) was added to the supernatant. The sample was mixed, then spun to separate the two phases and the bottom phenol:chloroform:isoamyl alcohol phase was discarded. Two volumes of ethanol were then added to the extracted supernatant to precipitate the double-stranded DNA. After a five minute incubation at room temperature, the sample was spun at 4°C and the supernatant removed by gentle aspiration. Once all of the fluid was removed the pellet was rinsed with ice-cold 70% ethanol. The supernatant was again removed by aspiration and the pellet allowed to air dry. The double-stranded DNA was then resuspended in TE buffer containing 20 μg/ml DNase-free RNase and then stored at -20°C.

Large Scale Preparation of Plasmid DNA

After screening the small scale plasmid DNA preparation by restriction digest, 1 ml of the overnight culture was added to 500 ml of LB media containing the appropriate antibiotic. After incubating overnight at 37°C with vigorous shaking, the sample was spun at 5, 000 rpm for 15 minutes in a Sorvall GSA rotor in a Sorvall centrifuge at 4°C. The supernatant was removed by aspiration and the pellet allowed to dry by inverting the tube. By vigorous mixing, the bacterial pellet was resuspended in 10 ml of ice-cold Solution I (50 mM glucose, 25 mM Tris-Cl (pH 8), 10 mM EDTA (pH 8)), 20 ml of freshly prepared Solution II (0.2 N NaOH and 1% (w/v) sodium dodecyl sulphate (SDS)) was then added and the tube inverted several times. After 10 minutes on ice, 15 ml of ice-cold Solution III (3 M potassium acetate, 11.5% (v/v) glacial acetic acid) was added and the contents mixed by shaking. The sample was then stored on ice for 10 minutes prior to centrifugation at 12,000 rpm for 15 minutes at 4°C in a Sorvall SS-34 rotor in a Sorvall centrifuge. After centrifugation, the supernatant was filtered through four layers of cheesecloth into a fresh tube and 0.6 volumes of isopropanol was added. The sample was mixed using a vortex and stored at room temperature for at least 30 minutes. After the incubation, the sample was spun at 12, 000

rpm for 15 minutes in a Sorvall SS-34 rotor at 20°C. The supernatant was removed by aspiration and the pellet washed with 70% ethanol. The ethanol was removed and the pellet allowed to air dry for a few minutes. The pellet was then dissolved in 8 ml of TE buffer, pH 8 and 1.1 g/ml cesium chloride was added. The solution was mixed gently in order to dissolve the salt and then ethidium bromide (0.8 mg/ml) was added. Using a 5 cc syringe and 22G 1½ needle, the solution was transferred to two Beckman 13 x 51 mm polyallomer Quick-Seal™ centrifuge tubes. The tubes were balanced and then sealed. The sealed tubes were placed in a vertical Beckman type VTi 65 rotor and spun at 40, 000 rpm for 16 to 20 hours at 20°C. After centrifugation, a 2 cc syringe and 18G 1½ needle was used to remove the lower band of closed circular plasmid DNA. When the DNA bands were difficult to visualize, longwave ultra violet (UV) light was used. After removing the lower band of plasmid DNA, the solution was extracted with an equal volume of 1-butanol saturated with water. The two phases were mixed by vortex and then allowed to settle so that the top phase containing ethidium bromide in butanol could be removed and discarded. This was repeated several times until the pink colour was no longer visible. The plasmid DNA solution was then diluted with three volumes of distilled water and eight volumes of 100% ethanol added. After vigorous mixing, the solution was stored at -20°C for several hours (usually overnight). After this incubation, the DNA solution was spun at 12,000 rpm for 30 minutes at 4°C in a Sorvall centrifuge using a Sorvall SS-34 rotor. The supernatant was removed and the plasmid DNA pellet washed with 70% ethanol. The pellet was allowed to air dry and was then resuspended in 1 ml of TE buffer, pH 8. The OD_{260} and OD_{280} were measured in order to estimate the final DNA concentration and purity. An OD_{260} of 1 is equal to 50 μg/ml of double-stranded DNA. The ratio of OD_{260}/ OD_{280} indicates the amount of contaminating proteins and phenol, a ratio of 1.8 to 2.0 is acceptable.

Restriction Digests

Restriction digests were performed as outlined in the manufacturer's protocols. The recommended

buffers, as well as, incubation and heat inactivation temperatures were utilized. When multiple enzymes were required One-Phor-All Buffer PLUS from Pharmacia was typically used.

Agarose Gel DNA Electrophoresis

Plasmid DNA, restriction endonuclease digested DNA, PCR products and single stranded DNA were analyzed on 0.7 to 2% agarose gels. An agarose gel was prepared by dissolving the desired amount of agarose in Tris-borate/EDTA electrophoresis buffer (TBE; 0.045M Tris-borate, 0.001M EDTA (pH8.0)) with heating. After the solution had cooled, 0.4 μg/ml ethidium bromide was added. The agarose gel was poured and allowed to solidify at 4°C. One-sixth volume of 6X loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (w/v) glycerol) was added to each DNA sample and this was loaded on the agarose gel along with 5 l of 1 kb DNA ladder (Gibco/BRL). The gels were typically run at 100 volts. After the dye had travelled the appropriate distance, the DNA was visualized under short wave ultraviolet light. Photographs of the agarose gel were taken using a Polaroid MP-4 Land Camera (Fotodyne) and Polaroid 667 Professional Black and White Instant Pack film (8.5 x 10.8 cm).

Purification of DNA Fragments

The Geneclean II® Kit from BIO 101 Inc. was used to remove and purify DNA from agarose. The protocol followed was as outlined in the kit and the reagents used were supplied with the kit.

DNA was separated on an agarose gel prepared with TBE. The DNA was visualized using long wave ultraviolet light and the appropriate DNA band excised using a sterile razor blade. The excised agarose was transferred to a tared 1.5 ml microcentrifuge tube, the tube was weighed and the volume of agarose estimated. Four and a half volumes of sodium iodide and 0.5 volumes of TBE modifier were added and the tube placed in a 55°C heating block. After two minutes the sample was mixed and then returned to the waterbath until the agarose was completely dissolved. After the agarose had melted, 5 μl of

Glassmilk® suspension was added, the solution mixed and placed on ice for 5 minutes to allow binding of the DNA to the silica matrix. The solution was mixed every 1 to 2 minutes in order to ensure that the Glassmilk® remained in suspension. After a five minute incubation, the sample was spun for five seconds in a microcentrifuge and the supernatant removed. The pellet was washed with 500 1 of NEW Wash (sodium chloride, Tris, EDTA, ethanol and water). The pellet was resuspended in NEW Wash, spun for 5 seconds and the supernatant discarded. The wash procedure was repeated two more times. After the third supernatant had been completely removed, the pellet was resuspended in 10 µl of distilled water. The sample was incubated in a 55°C heating block for 3 minutes in order to elute the DNA from the silica matrix. After 3 minutes, the sample was spun for 30 seconds and the supernatant containing the eluted DNA was transferred to a microcentrifuge tube. A second elution was normally done by resuspending the pellet in 5 µl of distilled water and repeating the above incubation and centrifugation. An aliquot of the eluted DNA was run on an agarose gel to determine the purity and estimate the concentration of the recovered DNA.

Dephosphorylation of DNA

During ligation reactions, plasmid DNA with cohesive ends has the ability to recircularize. Therefore, alkaline phosphatase (calf intestinal mucosa) is employed to remove the 5'-phosphate residues and 3' mono-phosphate groups from DNA and inhibit recircularization of plasmid DNA. The alkaline phosphatase used was from Pharmacia and required the use of One-Phor-All PLUS Buffer. The desired plasmid DNA was digested with the appropriate restriction enzyme and diluted to a final volume of 50 µl using distilled water and One-Phor-All PLUS Buffer to a final concentration of 1X One-Phor-All PLUS. The digested plasmid DNA was dephosphorylated by the addition of 0.1 units of alkaline phosphatase and incubation at 37°C for 30 minutes. The alkaline phosphatase was then heat inactivated by incubation at 85°C for 15 minutes. After this treatment less than 1% of the DNA should retain its terminal phosphate

groups. The heat inactivation of the alkaline phosphatase (calf intestinal mucosa) eliminated the need for phenol extractions.

DNA Ligation

Once the desired vector and insert had been isolated and purified, the amount of DNA recovered was estimated by agarose gel electrophoresis. The vector DNA and the fragment(s) of foreign insert DNA were mixed at a molar ratio of 1:3 (vector:insert) in a volume of 8 µl. To this reaction mixture, 2 µl of 5X ligation buffer (250 mM Tris-Cl (pH 7.6), 50 mM $MgCl_2$, 5 mM adenosine 5'-triphosphate (ATP), 5 mM dithiothreitol (DTT), 25% (w/v) polyethylene glycol (PEG) 8000) (Gibco/BRL) and 0.5 Weiss units of bacteriophage T4 DNA ligase (Gibco/BRL) were added. A control reaction containing digested vector DNA only was also prepared as above. The reaction mixtures were spun, incubated at 16°C overnight and then used to transform competent bacteria.

Polymerase Chain Reaction

The reaction was performed as outlined in Current Protocols in Molecular Biology (Ausubel, 1995). An oligonucleotide containing new unique restriction sites was used to amplify a region of DNA that was to be subcloned into a vector containing compatible restriction sites. Cesium chloride purified pXMgB mutant DNA was used for the amplification reaction and the PCR product was subcloned into pGemG(AXB) to produce pGemGtm3gBG containing the desired mutations.

Amplifying the Target DNA

In a 0.5 ml sterile microcentrifuge tube, 10 µl of 10X amplification buffer (100 mM Tris-Cl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin) (Perkin Elmer Cetus), 10 µl of 2 mM four dNTP mix (2 mM each of dATP, dCTP, dGTP, dTTP), 1 µl of oligonucleotide A (50 pmol), 1 µl of

oligonucleotide B (50 pmol), 1 ng template DNA and 2.5 units of *Taq* polymerase (Perkin Elmer Cetus) were mixed. Two negative controls, minus oligonucleotides A and B and minus *Taq* polymerase were also included in the procedure. The reaction mixtures were overlayed with mineral oil and placed in a Techne PHC-3 automated thermal cycler. The cycle was set to denature samples by heating at 94°C for 90 seconds, then anneal at 50°C for 2 minutes, followed by extension at 72°C for 30 seconds. The denaturation, annealing, and extension cycle was repeated 30 times.

Recovering the Amplified Fragment

An aliquot of the reaction mixture was analyzed on a 1.5% agarose gel in order to ensure that the desired reaction product was recovered. The mineral oil was removed from the reaction mixture and the mixture extracted once with an equal volume of chloroform:isoamyl alcohol (1:1/48) and once with neutralized phenol. The DNA was then precipitated with 2.5 volumes of 100% ethanol and recovered by centrifugation. The DNA pellet was dissolved in 20 µl of TE buffer and 15 µl of the DNA solution was run on a 2% agarose gel. The DNA fragment was observed under longwave ultra violet light and excised with a sterile razor blade. The desired PCR products was then purified by an agarose spin column.

DNA Purification by an Agarose Spin Column

The polymerase chain reaction (PCR) product was recovered from the agarose gel by using GenElute™ Agarose spin columns (Supelco). Prior to use, the GenElute™ Agarose spin column was washed by adding 100 µl of TE buffer. The spin column was placed on top of a 1.5 ml microcentrifuge tube and spun for 5 seconds at maximum speed in a microcentrifuge. The TE buffer collected in the microcentrifuge tube was discarded. The excised agarose gel slice was placed in the washed GenElute™ Agarose spin column. The spin column was placed on top of a 1.5 ml microcentrifuge tube and spun for 10 minutes at maximum speed in a microcentrifuge. The DNA collected in the microcentrifuge tube was

precipitated with 0.1 volumes of 3 M sodium acetate (pH5.3) and 2 volumes of ethanol.

Ligation of PCR Product

The primers used in the reaction mixture created unique restriction enzyme sites. Half of the amplified DNA was digested in a volume of 20 µl with the appropriate restriction enzymes. An excess of enzyme was used and digestion was carried out for several hours. The vector DNA was prepared for cloning by digesting an excess of DNA with the appropriate enzymes. The digested vector was isolated on an agarose gel and the desired fragment purified by the Geneclean II® Kit from BIO 101 Inc.. The purified PCR fragment was ligated into the digested vector following the procedure outlined in DNA Ligation. The ligation reaction product was transformed into competent cells. Putative clones were isolated, DNA was prepared, digested with the appropriate restriction enzymes and analyzed by agarose gel electrophoresis. The clones were then analyzed by sequencing to confirm the desired mutation.

Site Directed Mutagenesis

The point mutations within the hydrophobic domains of HSV-1 glycoprotein gB were created using site directed mutagenesis and doped mutagenesis (Hermes et al., 1989) following the method of Kunkel et al. (1987) and the Muta-Gene® M13 In Vitro Mutagenesis Kit, Version 2. A 2.1 kb fragment (SalI/EcoRI) of HSV-1 gB containing the transmembrane domain, the entire cytoplasmic domain and 404 bases of the ectodomain was cloned into the SalI/EcoRI site of M13mp18. The recombinant M13mp18-gB was introduced into a dut^{-1}, ung^{-1} strain of E.coli, CJ236, which yields single stranded DNA containing uracil. The single-stranded uracil-containing DNA was isolated and purified and used as a template for the mutagenesis reaction. The mutagenic primer was annealed to the single-stranded DNA, the second strand was synthesized with T7 DNA polymerase and ligated with T4 DNA ligase. Wild-type *E. coli* with functional uracil N-glycosylase, MV1190, were transformed with the reaction mixture

resulting in selection against the parental strand. The resulting phage were screened by restriction enzyme digests and sequencing to identify the desired mutants.

Transformation of the Recombinant Phage DNA

Competent MV1190 were transformed with an aliquot of the ligation reaction mixture containing glycoprotein gB (Sal1/EcoR1) and M13mp18 (Sal1/EcoR1). For each transformation 0.3 ml of MV1190 competent cells were thawed on ice, 1 to 10 ng of the ligation reaction was added and mixed gently. The cells were held on ice for 90 minutes, heat shocked at 42°C for 3 minutes and then returned to the ice. The transformed cells were added in 50 and 100 µl aliquots to 0.3 ml of an MV1190 overnight culture and 2.5 ml of molten H top agar. The mixture was mixed and poured onto H agar plates. After the top agar solidified, the plates were inverted and placed at 37°C overnight. The next morning, isolated plaques were picked by inserting a sterile Pasteur pipet through the plaque and blowing the entire plug into 1 ml of TE buffer. The plaques containing approximately 10^7 phage were kept at 4°C until screening for putative clones and titration. To ensure that the proper insert was present, a small DNA preparation from phage-infected cells was performed.

Small Scale Preparation of the Replicative Form of Phage DNA

A stock of presumptive mutant phage was grown up by adding 1/10 of the plaque suspension to 10 ml of LB media containing 250 µl of an MV1190 overnight culture and incubating with shaking at 37°C. After a five hour incubation, 5 ml of the culture was pelleted by centrifugation. The supernatant was removed for isolation of phage and the bacterial pellet containing the replicative form of phage DNA was resuspended in 200 µl of ice cold Solution I (50 mM glucose, 25 mM Tris-Cl (pH 8) and 10 mM ethylene-diamine tetra-acetic acid (EDTA, pH 8)). After the pellet was resuspended, 400 µl of a freshly prepared solution of 0.2 N NaOH and 1% (w/v) sodium dodecyl sulphate (SDS) was added and the

contents inverted several times. The sample was placed on ice and 300 µl of an ice-cold solution of 3 M potassium acetate and 11.5% (v/v) glacial acetic acid was added. The sample was then mixed in an inverted position. After five minutes on ice, the sample was spun and 600 µl of the supernatant was transferred to a new tube. The supernatant was extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (1:1:1/48). After the extractions, the DNA was precipitated by the addition of 600 µl of isopropanol. The sample was mixed vigorously and maintained at room temperature for 10 minutes. The DNA was pelleted by centrifuging for 5 minutes. The supernatant was removed by gentle aspiration and the DNA pellet was allowed to air dry. Once all of the fluid was removed the pellet was rinsed with ice-cold 70% ethanol. The ethanol was removed by aspiration and the pellet allowed to air dry. The replicative form of the DNA was then resuspended in 30 µl TE buffer containing 20 µg/ml DNAase-free RNAase and stored at -20 C. To ensure that the proper insert was present and that the desired unique restriction site was created, a restriction digest was performed.

Titration of Recombinant Phage Stock

An isolated colony of MV1190 was grown in LB media overnight at 37°C with shaking . Four tubes of LB top agar were prepared by melting the agar and pipetting 2.5 ml into sterile tubes which were maintained in a 55°C waterbath until needed. Serial dilutions of the phage stock were prepared 10^2, 10^4, 10^6 and 10^8-fold in sterile tubes of LB media. In sterile test tubes, 0.2 ml of the MV1190 overnight culture was added along with 100 µl of the phage dilutions 10^4, 10^6 and 10^8, and a tube which contained MV1190 alone served as a negative control. After incubating the culture and phage at room temperature for five minutes, a tube of top agar was added, the solution mixed thoroughly and poured onto an LB agar plate. This was repeated for all of the tubes. The top agar was allowed to harden for 15 minutes and then the plates were inverted and placed at 37°C overnight. After the overnight incubation, the number of plaques on each plate were quantified and the pfu/ml calculated (titer (plaque forming units/ml) = # of plaques x 10

x dilution factor). The recombinant phage stock was used to prepare uracil-containing phage DNA.

Growth of Uracil-Containing Phage

A frozen stock of CJ236, dut^{-1}, ung^{-1} *E. coli*, was streaked onto an LB plate containing chloramphenicol (30 μg/ml) and grown at 37°C until distinct colonies appeared. An isolated colony was placed in 20 ml of LB containing chloramphenicol (30 μg/ml) and incubated overnight with shaking at 37°C. After the incubation, 50 ml of 2xYT media was inoculated with 1 ml of the overnight culture and incubated with shaking at 37°C until an OD_{600} of 0.3 was reached (approximately 2 hours). The recombinant phage, M13mp18-gB, was then added in order to obtain a multiplicity of infection of 0.2 or less (i.e. 0.2 phage/cell). Uridine was also added at a concentration of 0.5 μg/ml and the culture was incubated at 37°C with shaking. After five hours, 30 ml of the culture was transferred to a 50 ml polypropylene centrifuge tube and spun at 12,000 rpm in a Sorvall SS-34 rotor for 15 minutes at 4°C. The supernatant containing the phage particles was transferred to a fresh centrifuge tube and respun. The pellet containing the double-stranded replicative form of the recombinant DNA was stored at -20°C for use in restriction enzyme digests and cloning. The second supernatant was transferred to another centrifuge tube containing 150 μg of DNase-free RNase A and incubated at room temperature for 30 minutes. After the incubation, a fresh solution of 3.5 M ammonium acetate and 20% (w/v) polyethylene glycol (PEG) 8000 was added to the supernatant at ¼ the volume. The solution was mixed by vortex and placed on ice for at least 30 minutes. The sample was then spun at 12,000 rpm for 15 minutes at 4°C. After centrifugation, the supernatant was removed by aspiration and the liquid allowed to drain thoroughly. It was important to ensure that all of the fluid had been removed. The phage pellet was resuspended in 200 μl of high salt buffer (300 mM NaCl, 100 mM Tris, pH 8.0, 1 mM EDTA), transferred to a microcentrifuge tube and placed on ice for 30 minutes. The sample was spun in order to remove any insoluble material and the supernatant transferred to a new tube and stored at 4°C. The phage stock was titered on CJ236 and

MV1190 in order to determine whether the phage DNA contained sufficient uracil for future mutagenesis reactions.

Titering Uracil-Containing Phage

Productive infection should result in a titer of at least 5 x 10^{11} pfu/ml on CJ236. The efficiency of the titre on MV1190 should be at least 10^4- fold lower than on CJ236 if the DNA contains sufficient uracil.

In order to titrate uracil-containing phage, an isolated colony of CJ236 was transferred from an LB plate to 20 ml of LB containing 30 μg/ml of chloramphenicol. Non-uracil containing phage were titered using an isolated colony of MV1190 grown in LB media. Both cultures were grown overnight with shaking at 37°C. Eight tubes of LB top agar were prepared by melting the agar and pipetting 2.5 ml into sterile tubes which were maintained in a 55°C waterbath until needed. Serial dilutions of the phage stock were prepared 10^2, 10^4, 10^6, 10^8, 10^{10}, and 10^{12}-fold in sterile tubes of LB media. In a sterile test tube, 0.2 ml of overnight culture (either MV1190 or CJ236) was added along with 100 μl of the phage dilutions. The three lowest dilutions were added for titering the phage on MV1190 and the three highest dilutions of the phage stock were added for titering on CJ236, the extra tubes served as negative controls for each cell culture. After incubating the culture and phage at room temperature for five minutes, a tube of top agar was added, mixed thoroughly and poured onto LB agar plates. This was repeated for all the dilutions. The top agar was allowed to harden for 15 minutes and then the plates were inverted and placed at 37°C overnight. After the overnight incubation, the number of plaques on each plate were quantified and the pfu/ml calculated (titer (pfu/ml) = # of plaques x 10 x dilution factor). If the infection was productive and sufficient uracil-containing phage DNA was obtained, the DNA was purified by extraction as outlined below.

Extraction of Single-Stranded Uracil-Containing DNA

The entire phage stock was extracted twice with an equal volume of neutralized phenol, once with phenol:chloroform:isoamyl alcohol (1:1:1/48), and several times with chloroform:isoamyl alcohol (24:1). In order to increase the yield, each step was back-extracted with 100 µl of TE (10 mM Tris, pH 8.0, 1 mM EDTA). After the extractions, the aqueous phases were pooled, 1/10 volumes 7.8 M ammonium acetate and 2.5 volumes 100% ethanol were added in order to precipitate the DNA. The sample was mixed and stored at -70°C for at least 30 minutes. The DNA was pelleted by centrifugation at 4°C for 15 minutes followed by washing with 90% ice-cold ethanol. After drying, the pellet was resuspended in 20 µl of TE buffer. In order to estimate the concentration of uracil-containing single-stranded DNA, a small aliquot was run on an agarose gel along with a known amount of single-stranded DNA.

Phosphorylation of the Mutagenic Primer

The lyophilized oligonucleotide was resuspended in distilled water to a concentration of 20 pmol/µl and phosphorylated using T4 polynucleotide kinase before use in the synthesis of a mutagenic strand. The following reaction mixture was prepared in a 1.5 ml microcentrifuge tube, 200 pmol of the mutagenic primer, 100 mM Tris (pH 8.0), 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.4 mM adenosine triphosphate (ATP) and distilled water to a volume of 30 µL. After mixing, 4.5 units of T4 polynucleotide kinase (BioRad) was added and the reaction mixture incubated at 37°C for 45 minutes. The reaction was stopped by heating at 65°C for 10 minutes. The mutagenic oligonucleotide was then diluted to 6 pmol/µl with distilled water and stored at -20°C.

Annealing of the Primer to the Template

In order to anneal the primer to the single-stranded DNA template the following reaction mixture

was prepared in a 1.5 ml microcentrifuge tube, 200 ng of previously isolated uracil-containing single-stranded DNA, 3 pmol of mutagenic oligonucleotide, 1 µl of 10X annealing buffer (20 mM Tris-Cl (pH 7.4), 2 mM $MgCl_2$, 50 mM NaCl) and distilled water to a total volume of 10 µl. A control reaction which tested for non-specific endogenous priming was prepared with all of the above ingredients except for the mutagenic primer. The reaction mixtures were placed in a 70°C heating block and allowed to cool to 30°C over a period of 40 minutes. Once 30°C had been reached, the samples were removed from the heating block, spun and placed on ice for the synthesis reaction.

Synthesis of the Complementary DNA Strand

With the reaction mixture on ice, 10X synthesis buffer (0.4 mM each deoxynucleotide triphosphate (dNTP), 0.75 mM ATP, 17.5 mM Tris-Cl (pH 7.4), 3.75 mM $MgCl_2$, 21.5 mM DTT), 3 units T4 DNA ligase, 0.5 units T7 DNA polymerase, and 1.6 µg T4 Gene 32 (BioRad) were added. The reaction mixture was spun and incubated on ice for 5 minutes, at 25°C for 5 minutes and then at 37°C for 90 minutes. After the 90 minute incubation, 90 µl of stop buffer containing 10 mM Tris, pH 8.0 and 10 mM EDTA was added. The synthesis reaction mixture was stored at -20°C until competent cells were transformed.

Transformation of the Synthesis Reaction Mixture

Competent MV1190 cells were transformed with an aliquot of the synthesis reaction mixture after synthesis of the mutagenized strand on the uracil-containing template. A 10 µl aliquot of the synthesis reaction mixture was added to 0.3 ml of MV1190 competent cells, mixed gently and held on ice for 90 minutes. After the 90 minute incubation, the cells were heat shocked at 42°C for 3 minutes and then placed on ice. The transformed cells were added in 50 and 100 µl aliquots to 0.3 ml of an MV1190 overnight culture and 2.5 ml of molten H top agar. The mixture was mixed and poured onto H agar plates. After the

top agar solidified, the plates were inverted and placed at 37°C overnight. The next morning, isolated plaques were picked by inserting a sterile Pasteur pipet through the plaque and blowing the entire plug into 1 ml of TE buffer. The plaques containing approximately 10^7 phage were kept at 4°C until screening for putative mutants.

Screening Putative Mutants

An overnight culture of MV1190 was grown at 37°C with shaking. The next day, 1 ml of the overnight culture was added along with 100 µl of the phage suspension to 30 ml of LB media. The cultures were grown for 5 hours at 37°C with shaking. After the incubation, the sample was spun at 12,000 rpm in a Sorvall SS-34 rotor for 15 minutes at 4°C. The supernatant containing the phage particles was transferred to a fresh centrifuge tube and respun. The pellet containing the double-stranded replicative form of the recombinant DNA was saved at -20°C for restriction enzyme digests and cloning reactions. The alkaline lysis method was used to isolate the double-stranded replicative form of the recombinant DNA and the appropriate restriction enzymes were used when unique restriction sites were introduced along with the desired mutation. The second supernatant was transferred to another centrifuge tube containing 150 µg of DNase-free RNase A and incubated at room temperature for 30 minutes. After the incubation, ¼ the volume of a fresh solution of 3.5 M ammonium acetate and 20% (w/v) PEG 8000 was added to the supernatant, mixed vigorously, and placed on ice for at least 30 minutes. The sample was spun at 12,000 rpm for 15 minutes at 4°C, the supernatant was removed by aspiration and the liquid allowed to drain thoroughly. It was important to ensure that all of the fluid had been removed. The phage pellet was resuspended in 200 µl of high salt buffer (300 mM NaCl, 100 mM Tris, pH 8.0, 1 mM EDTA), transferred to a microcentrifuge tube and placed on ice for 30 minutes. The sample was spun in order to remove any insoluble material and the supernatant transferred to a new tube. The entire phage stock was extracted twice with an equal volume of neutralized phenol, once with phenol:chloroform:isoamyl alcohol

(1:1:1/48), and several times with chloroform:isoamyl alcohol (24:1). In order to increase the yield, each step was back-extracted with 100 µl of TE (10 mM Tris, pH 8.0, 1 mM EDTA). After the extractions, the aqueous phases were pooled, 1/10 volumes 7.8 M ammonium acetate and 2.5 volumes ethanol were added in order to precipitate the DNA. The sample was mixed and stored at -70°C for at least 30 minutes. The DNA was pelleted by centrifugation at 4°C for 15 minutes followed by washing with 90% ice-cold ethanol. After drying, the pellet was resuspended in 20 µl of TE buffer. In order to estimate the concentration of single-stranded DNA, a small aliquot was run on an agarose gel along with a known amount of single-stranded DNA. After sufficient purified single-stranded DNA was obtained it was sequenced by the chain termination method using the Sequenase® Version 2.0, DNA Sequencing Kit.

DNA Sequencing

The Sequenase® Version 2.0, DNA Sequencing Kit was used for all DNA sequencing. The kit employs the chain termination-method of sequencing (Sanger et al., 1977) with T7 DNA polymerase.

Preparation of Double Stranded DNA

Double-stranded DNA was denatured by the alkaline-denaturation method (Lim, 1988). Excess DNA template, 5 µg, was denatured by the addition of 0.1 volumes of 2 M NaOH and 2 mM EDTA. The reaction mixture was incubated at 37°C for 30 minutes and then neutralized by the addition of 0.1 volumes of 3 M sodium acetate (pH 4.5-5.5). The DNA was precipitated by the addition of 2.5 volumes of 95% ethanol and incubation at -70°C for 15 minutes. After the incubation, the DNA was pelleted by centrifugation and washed with 70% ethanol. The DNA pellet was allowed to dry and was then dissolved in 7 µl of distilled water.

Sequencing Reaction

The annealing mixture was prepared in a 1.5 ml microcentrifuge tube, 1-2 μg of single-stranded DNA or 5 μg of denatured double stranded DNA was mixed with 2 μl of reaction buffer (200 mM Tris-Cl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl), 50 ng of the appropriate sequencing primer and distilled water to a final volume of 10 μl. The primer was annealed by heating at 65°C for 2 minutes and then the mixture was cooled to less than 35°C over 30 minutes. During the annealing reaction, 1.5 ml microcentrifuge tubes were labeled and filled with 2.5 μl of each Termination Mixture (8 μM ddATP, ddGTP, ddTTP or ddCTP, 80 μM dGTP, 80 μM dATP, 80 μM dCTP, 80 μM dTTP, 50 mM NaCl), covered and kept at room temperature. The Labeling Mix (7.5 μM dGTP, 7.5 μM dCTP, 7.5 μM dTTP) was diluted 5-fold with distilled water and maintained on ice. After the 30 minute annealing reaction, the samples were spun briefly and placed on ice. The termination tubes were pre-warmed in a 37°C waterbath. To the annealed template and primer mixture, 1 μl of 0.1 M DTT, 2 μl of dilute labeling mix, 0.5 μl of ^{35}S-dATP (10 μCi/μL) and 2 μl of Sequenase® polymerase (1.6 units/μl of T7 DNA polymerase) were added, mixed and incubated at room temperature for 5 minutes. When the sequence to be read was close to the primer, 1 μl of manganese buffer (0.15 M sodium isocitrate, 0.1 M $MnCl_2$) was added to the labeling mixture. After the 5 minute incubation, 3.5 μl of the labeling reaction was added to each termination tube (G, A, T, C), mixed and incubated at 37°C for 5 minutes. The reaction was stopped after five minutes by the addition of 4 μl of stop solution (95% (w/v) formamide, 20 mM EDTA, 0.05% (w/v) Bromophenol blue, 0.05% (w/v) Xylene cyanol FF).

Denaturing Gel Electrophoresis

A 6% gel was prepared by mixing 5.7 g acrylamide, 0.3 g bisacrylamide and 42 g urea in 20 ml of 5X TBE (Tris-borate/EDTA electrophoresis buffer). The volume was adjusted to 100 ml with distilled water and 1 ml of freshly prepared 10% (w/v) ammonium persulfate and 25 μl of N, N, N', N'

tetramethylethylenediamine (TEMED) was added immediately prior to pouring the gel. The gel was left at room temperature to solidify overnight and pre-run for at least 15 minutes prior to use.

The samples were denatured by heating to 75°C for 2 minutes immediately before loading 2 to 3 µl onto the sequencing gel. The gel was run using a Fotodyne DNA Sequencing System Model 4200 Constant Power Supply set at 2500 volts for 2 to 5 hours depending on the number of nucleotides to be read. After electrophoresis, the gel was removed and dried at 80°C for 1 to 2 hours on a BioRad Model 483 slab dryer. The gels were then exposed to Kodak X-OMAT AR film at -70°C.

Mammalian Cells Lines and Culture Conditions

Vero (African green monkey kidney cells) and COS-1 cells (Gluzman, 1981) were grown in high glucose Dulbecco's modified Eagle's medium (HGD) (Gibco/BRL) supplemented with 7% (v/v) calf serum. The following components were added to all media, 100 µg/ml penicillin-streptomycin (Gibco/BRL), 4 mM glutamine (Gibco/BRL), 45 mM $NaHCO_3$ (Gibco/BRL), 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (Gibco/BRL) and 1x MEM non-essential amino acids (Gibco/BRL). VB38 cells, a stably transformed Vero cell line expressing gB-1 were obtained from Dr. David Johnson. VB38 cells were grown in Dulbecco's Minimal Media minus histidine, supplemented with 7% (v/v) calf serum and 0.3 mM histidinol.

Monolayer cultures of Vero, COS-1, and VB38 cells were grown in plastic culture dishes at 37°C under an atmosphere of 5% CO_2. The cultures were routinely passaged every three to four days at ratios ranging from 1:5 to 1:20 using trypsin-EDTA (Gibco/BRL). Prior to its use in experiments, VB38 cells were passaged once in Dulbecco's modified Eagle's medium (HGD) supplemented with 7% calf serum.

Frozen stocks were prepared for long term storage of each cell line. The cells were stored in liquid nitrogen or at -70°C in HGD media supplemented with 7% (v/v) calf serum and 10% (v/v) dimethyl sulphoxide (DMSO).

Transfection

In order to express the wild-type and mutant proteins, subconfluent monolayers of COS-1 cells were transfected by the calcium phosphate method (Graham and Van der Eb, 1973) and Vero cells were transfected by the DEAE dextran method (Sompayrac and Danna, 1981) for the complementation assays.

Calcium Phosphate Transfection

COS-1 cells were transfected following the calcium phosphate method of Graham and Van der Eb (1973). Briefly, 20 μg of DNA was added to 0.25 M $CaCl_2$ to a final volume of 250 μl with distilled water. While bubbling air slowly into 250 μl of HeBS (1% (w/v) HEPES, 1.6% (w/v) NaCl, 0.075% (w/v) KCl, 0.025% (w/v) Na_2HPO_4, 0.2% (w/v) glucose; pH adjusted to 7.1), the above mixture was added. The mixture was allowed to stand at room temperature for 20 to 30 minutes. The DNA-$Ca_3(PO_4)_2$ co-precipitate was added to 60 mm plates containing 60% confluent COS-1 cells and fresh HGD media. Following a 4 hour incubation at 37°C, the media was removed and 1 ml of HGD containing 15% (v/v) glycerol was added for exactly 2 minutes. The glycerol was diluted out with 5 ml HGD, the cells were washed and fresh medium added. The cells were then incubated for 24-48 h.

DEAE Dextran Transfection

The complementation assay utilized diethylaminoethyl-dextran (DEAE dextran) transfection (Sompayrac and Danna, 1981) of 60% confluent Vero cells in 60 mm plates with pKBXX mutant plasmids. A stock solution of DEAE dextran (Sigma, M.W. 500,000, lot # 9885) was prepared as follows, 30 mg of DEAE dextran was dissolved in 95 ml of serum free HGD and 5 ml of 1 M Tris pH 7.4. The solution was allowed to stir for 1 hour. After 1 hour, the solution was filter sterilized and stored at 4°C.

Warm DEAE dextran solution (0.3 mg/ml) was diluted in serum-free HGD media to a concentration of 100 μg/ml and 1 ml was added to 10 μg of the plasmid DNA to be transfected. The

solution was incubated at room temperature for 30 minutes. During the 30 minute incubation, the Vero cells to be transfected were washed twice with serum free HGD media. The DEAE dextran solution was then added to the cells and incubated for 3 hours at 37°C. After the incubation period, the media was removed and the transfected cells washed twice with serum free HGD media followed by the addition of 5 ml of HGD supplemented with 7% (v/v) calf serum. The plates were incubated overnight at 37°C.

Indirect Immunofluorescence

COS-1 cells were grown on glass slides placed in 60 mm plates and the calcium phosphate transfection protocol of Graham and Van der Eb (1973). Twenty-four hours after transfection, the media was removed and the cells 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). The glass slides were transferred to six well plates and the cells fixed with 2% (w/v) paraformaldehyde at 4°C for 20 minutes. For internal immunofluorescence, the cells were subsequently permeabilized with 1.5% (v/v) Triton-X 100 for 15 minutes at room temperature. Cells were washed with PBS, followed by PBS with 1% (w/v) bovine serum albumin (BSA) for 15 minutes. After air drying the slides, 40 µl of a 1:50 dilution of the appropriate antibody (anti-G or anti-gB-1 antiserum) was added to the cells. After a 20 minute incubation at 37°C the cells were washed with PBS for 15 minutes followed by PBS containing 1% (w/v) BSA for 15 minutes. The secondary antibody, fluorescein isothiocyanate-conjugated (FITC) goat anti-rabbit IgG, diluted 1:75 in PBS containing 1% (w/v) BSA was added and the cells incubated at 37°C. After 20 minutes, the cells were washed with PBS containing 1% (w/v) BSA for 15 minutes followed by PBS for 15 minutes. The slides were allowed to air dry and were subsequently mounted on microscope slides using 85% (v/v) glycerol in PBS. The slides were then examined under a Zeiss epi-fluorescence microscope equipped with a high pressure mercury lamp and photographs of both fluorescence and phase contrast were taken using Kodak black and white T-max 400 film.

Cell Labelling and Immunoprecipitation

Following calcium phosphate transfection, cells were washed with PBS and 1 ml of medium lacking methionine was added. After one hour at 37°C, 50 μCi/ml of ^{35}S methionine was added. The cells were labelled for 2 hours at 37°C, the media was then removed and the cells washed with PBS. Cold lysis buffer (1% (v/v) Nonidet P-40, 0.4% (w/v) sodium deoxycholate, 66 mM EDTA, 10 mM Tris (pH 7.4), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% (v/v) Aprotinin (Trasylol)) was added and the plates placed on ice for a few minutes. The cells were scrapped and transferred to microcentrifuge tubes. Cell debris was removed by centrifugation at 4°C for 5 minutes. To the lysate, 1 to 5 μl of the appropriate antibody (anti-gB-1 or anti-G antiserum) was added and the tubes were rotated for 2 hours at 4°C. After the addition of 50 μl of 10% (v/v) protein A Sepharose, the sample was incubated for an hour at 4°C with rotation. The protein A Sepharose beads were pelleted by centrifugation and then washed with lysis buffer containing 0.3% (w/v) sodium dodecyl sulphate (SDS). The washing was repeated four times and then 20 μl of sample buffer containing 0.125 M Tris-Cl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 0.05% (w/v) bromophenol blue and 10% (v/v) β-mercaptoethanol was added. The sample was analyzed by SDS-PAGE followed by fluorography.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) by the method developed by Laemmli (1970) and outlined by Sambrook, et al. (1989). The 10% separating gel was prepared by mixing 0.375 M Tris-Cl, pH 8.0, 10% (w/v) acrylamide mix (29% (w/v) acrylamide/1% (w/v) methylene bisacrylamide), 0.1% (w/v) sodium dodecyl sulphate (SDS), 0.1% (w/v) fresh ammonium persulfate, and 0.04% (v/v) TEMED. The separating gel was poured and allowed to polymerize at room temperature. After polymerization was complete the overlay was removed and the stacking gel prepared by mixing 0.125 M Tris-Cl, pH 6.8, 5% (v/v) acrylamide mix, 0.1% (w/v) sodium

dodecyl sulphate (SDS), 0.1% (w/v) fresh ammonium persulfate, and 0.1% (v/v) TEMED. The comb was inserted and the stacking gel was poured. After polymerization was complete, the comb was removed and the wells washed with distilled water. The samples to be analyzed were then boiled for 3 minutes in order to denature the proteins. The gel was mounted in the electrophoresis apparatus and 10 µl of the sample diluted in sample buffer was added to each well. A high molecular weight marker and/or a radioactive vesicular stomatitis virus marker were included with each run. The wells were filled with reservoir buffer (0.02 M Tris base, 0.192 M glycine, 0.1% (w/v) SDS) and the electrophoresis apparatus filled with reservoir buffer. The apparatus was attached to a power supply and the gel ran at 120 volts until the first dye reached the bottom of the gel.

After electrophoresis, the gel was stained for 15 minutes with Coomassie Blue stain (0.125% (w/v) Coomassie Blue, 50% (v/v) methanol, 10% (v/v) acetic acid). The gel was then destained with destaining solution containing 30% (v/v) ethanol and 12% (v/v) acetic acid for 30 minutes. Following destaining, the gels were processed for fluorography.

Fluorography of Polyacrylamide Gels

Fluorography was used to impregnate the gel with sodium salicylate and enhance the detection of radioactivity in the polyacrylamide gel. After destaining, the gels were rinsed with water for 15 minutes and then placed in a solution of 1 M salicylic acid and 1 M sodium hydroxide for 35 minutes. The gels were then placed on filter papers and dried on a BioRad Model 483 slab dryer for 1 hour at 60°C. After drying the gels were exposed to Kodak X-OMAT AR film at -70°C.

Viruses

Stocks of HSV-1 (KOS) were prepared by low-multiplicity passage on Vero cell lines. K082, a gB-1 null virus (Cai, 1987; Cai, 1988b), was plaque purified and passaged on VB38 cells, titers were

determined on both Vero and VB38 cell lines as outlined below. Stocks of K082 were prepared by low-multiplicity (0.01 moi) passage on VB38 cell lines.

Virus stocks were prepared by growing up multiple plates of the appropriate cells to 80% confluency. Virus was added to each plate at a multiplicity of infection (m.o.i.) of 0.01 in 1 ml of serum free HGD. The plates were incubated at 37°C for two hours and then 10 ml of HGD media supplemented with 7% calf serum was added. The plates were returned to the 37°C incubator until CPE (cytopathic effect) was visible. The cells and virus were harvested by adding 1 ml of serum free HGD media to each plate, scraping the cells with a rubber policeman, resuspending the cells in the media with a sterile pipette and pooling the cells and virus in a falcon tube. The tube was placed on ice, mixed briefly and the virus stock solution aliquoted into 1.5 ml cryovials. The samples were freeze (-80°C)/thawed (37°C) three times, titered on the appropriate cell lines and stored at -80°C.

Plaque Purification of Virus

The K082 gB null virus was plaque purified in order to reduce contaminating wild-type HSV-1 virus.

VB38 cells were grown to 80% confluency in 60 mm plates containing HGD supplemented with 7% (v/v) calf serum. The media was removed and K082 virus was added at an moi of 0.01 in 2 ml of serum-free HGD media. The plates were incubated for 2 hours at 37°C with rocking. After 2 hours, the media was removed and 5 ml of HGD supplemented with 2% (v/v) calf serum and 0.2% (v/v) human immune serum was added. The plates were incubated at 37°C until plaques were visible, usually one to two days. Sterile pasteur pipettes were used to isolate virus from secluded plaques and transfer them to cryovials containing 1 ml of serum-free HGD media. The virus stocks were freeze/thawed three times and then titered on both VB38 and Vero cell lines.

Virus Titration

HSV-1 was titered on Vero cells, while K082 was titered on both Vero and VB38 cell lines. Both Vero and VB38 cells were grown to 80% confluency in 6 well plates. Serial dilutions of the virus were prepared and added in duplicate to both Vero and VB38 cells. After 2 hours at 37°C with rocking, the media was removed and HGD supplemented with 2% (v/v) calf serum and 0.2% (v/v) human immune serum was added. The plates were incubated at 37°C until plaques were visible, the media was removed and 1 ml of fixative containing 1% (w/v) crystal violet, 60% (v/v) ethanol, 0.3% (v/v) formaldehyde and 5% glacial acetic acid was added to each well for a few minutes at room temperature. The fixative was removed, the wells were rinsed with water and the plaques counted.

Complementation

The complementation assay was a modified version of that outlined in Cai, et al. (1988b). Transfection of 60% confluent Vero cells in a 60 mm plate was performed using the DEAE dextran method. The cells were washed twice with serum free media, then 10 μg of mutant plasmid DNA (pKBXX) was added in 1 ml of warm serum free HGD containing 100 ug/ml DEAE Dextran. After a 3 hour incubation at 37°C, the media was removed and the cells washed with serum free HGD twice. After washing, 5 ml of HGD supplemented with 7% (v/v) calf serum was added and the plate incubated at 37°C. After 17 hours, 10^6 pfu of the gB-1 null virus, K082, was added in 0.2 ml of tricine buffered saline (0.14 M NaCl, 5 mM KCl, 0.5 mM $MgCl_2 \bullet 6H_2O$, 0.7 mM $CaCl_2$ $2H_2O$, 25mM Tricine; pH adjusted to 7.3 with NaOH). The cells were incubated at room temperature for 1 hour and then 5 ml of HGD supplemented with 7% (v/v) calf serum was added. The plates were placed at 37°C for another hour. After the one hour incubation, the media was removed and 2 ml of glycine buffer (0.14 M NaCl, 5 mM KCl, 0.5 mM $MgCl_2$ $6H_2O$, 0.7mM $CaCl_2$ $6H_2O$, 0.1 M glycine; pH adjusted to 3) was added for 5 minutes in order to remove any unabsorbed viruses. The glycine buffer was removed, the cells washed twice with PBS and 2

ml of HGD supplemented with 7% (v/v) calf serum was added. After 24 hours at 37°C, the virus was harvested and titered on both VB38 and Vero cell lines.

RESULTS

Plasmid Construction and Mutagenesis

Previous experiments have shown that the third hydrophobic segment of HSV-1 gB is sufficient for both membrane anchoring and nuclear envelope localization (Gilbert et al., 1994). To determine the role of highly conserved amino acids within this segment, point mutations were made using the techniques of site-directed and doped mutagenesis. Eight point mutations were introduced into glycoprotein gB by the method of doped mutagenesis. The primers AB5058 and AB5059, outlined in Table 1, were used to mutagenize alanine 786 (A_{786}) of glycoprotein gB to serine (S), asparagine (N), tyrosine (Y) and threonine (T) and alanine 790 (A_{790}) to serine (S), proline (P), glutamine (Q) and a premature stop codon (STOP) in one mutagenesis reaction (Figure 6). Putative mutants were screened by sequencing single-stranded DNA (Appendix 2). The replicative form of the positive mutants was digested with SalI/EcoRI and subcloned into the eukaryotic expression vector pXM creating pXMgB A_{786}S, pXMgB A_{786}N, pXMgB A_{786}Y, pXMgB A_{786}T, pXMgB A_{790}S, and pXMgB A_{790}Q (Appendix 1). Sequencing revealed the introduction of another mutation within glycoprotein gB upon doped mutagenesis, A_{786}S;L_{788}M (Appendix 2, Figure 6). The resulting gB mutant was also subcloned into pXM creating pXMgB A_{786}S;L_{788}M. Site-directed mutagenesis using the primer AB5311 (Table 1) was employed in mutating alanine 791 (A_{791}) to serine (S) while creating a unique HinDIII restriction enzyme site (Figure 6). The unique restriction enzyme site assists in screening the replicative form of the putative mutants by restriction enzyme digest. Clones containing this newly introduced restriction enzyme site were sequenced (Appendix 2) and positive mutants subcloned into pXM creating pXMgB A_{791}S.

The first and second hydrophobic segments of HSV-1 gB also contain amino acids which are highly conserved amongst herpesviridae (Figure 3). The role of these regions has not yet been elucidated. Site-

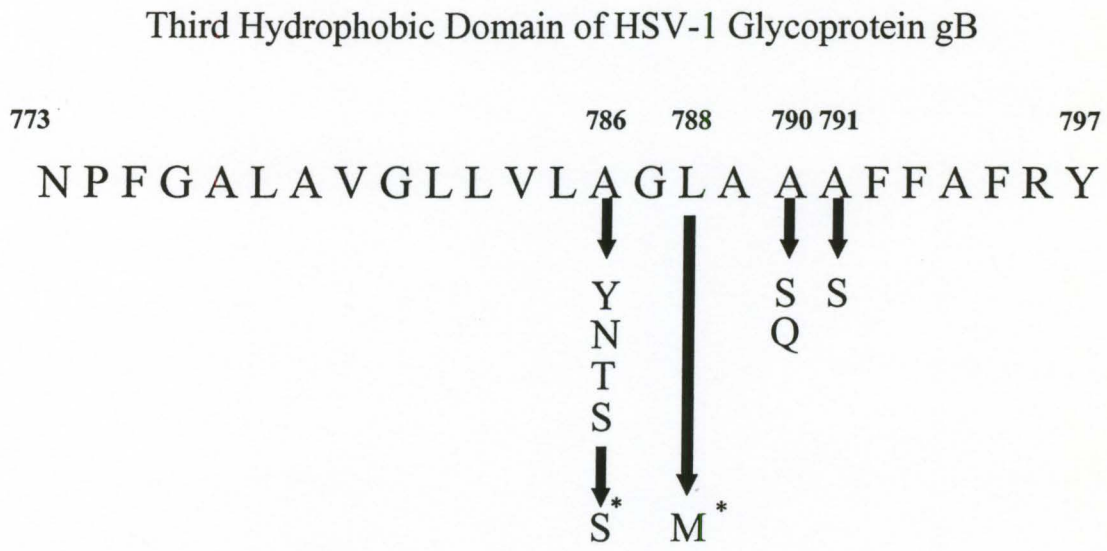


Figure 6: Point mutations introduced into the transmembrane domain of HSV-1 glycoprotein gB.

The amino acids that were mutated are indicated by their amino acid number and the new amino acids created are indicated by arrows. The double mutation is indicated by asterisk (*).

directed mutagenesis was employed to create point mutations in both the first ($G_{743}R$) and second ($F_{770}S$) hydrophobic regions in order to determine a function for these conserved amino acids. Upon creating these mutations, unique restriction enzyme sites were introduced into glycoprotein gB in order to facilitate the screening of putative mutants. After putative mutants had been identified, they were sequenced (Appendix 2) and the positive mutants cloned into pXM creating, pXMgB $F_{770}S$ and pXMgB $G_{743}R$.

Gilbert et al. (1994) showed that a mutant glycoprotein lacking the third hydrophobic segment of gB was localized to the nuclear envelope but the labelling of the nuclear envelope was less than that observed in the presence of the third hydrophobic segment. Although the third hydrophobic region is sufficient for nuclear envelope localization, hydrophobic segments one and two may assist in the nuclear envelope localization of gB. The chimeric construct pXM Gtm3gBG, made up of the ectodomain and cytoplasmic tail of vesicular stomatitis virus glycoprotein G, a membrane protein, and the third hydrophobic region of HSV-1 gB, has been shown to localize to the nuclear envelope and the plasma membrane as efficiently as wild-type HSV-1 glycoprotein gB (Gilbert et al., 1994). The mutant transmembrane domains were subcloned into pXMG in order to determine the effect of these point mutations within the third hydrophobic region of gB in the absence of the possible synergistic effects of hydrophobic segments one and two. The transmembrane region of VSV G was replaced by PCR generating the pXM Gtm3gBG mutants (Appendix 1). The fidelity of the PCR reaction was determined by sequencing the resulting pXM Gtm3gBG mutants (Appendix 2).

Hydrophobicity

The hydrophobicity of each of the third hydrophobic domain mutant glycoproteins was determined by using the Kyte Doolittle hydrophobic indices (1982). The effect of point mutations on the hydrophobicity of the transmembrane domain of glycoprotein gB is outlined in Figure 7. The point mutations did not reduce the average hydrophobicity of this domain below 1.65 suggesting that the point mutations will not affect the membrane anchoring ability of the third hydrophobic domain.

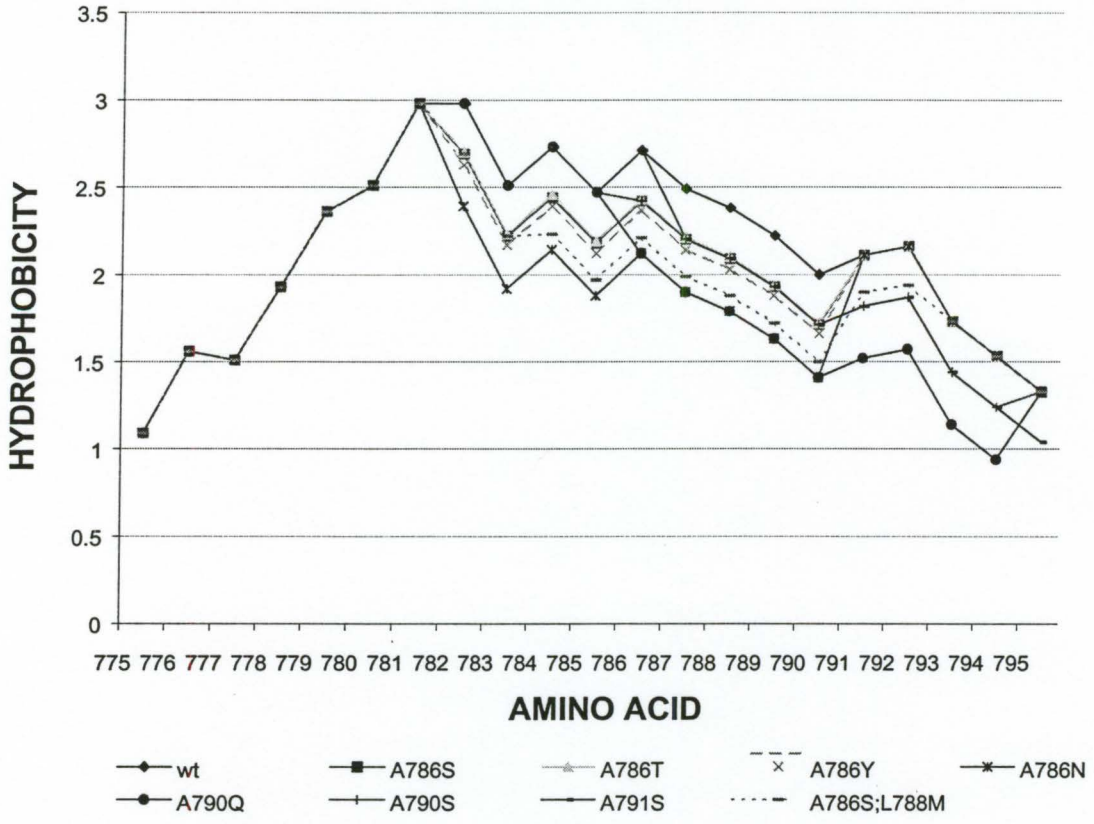


Figure 7: Hydrophobicity of the transmembrane domain of HSV-1 glycoprotein gB

The Kyte-Doolittle hydrophobic index (1982) was determined for each of the HSV-1 gB mutants and the hydrophobicities compared graphically.

Expression of mutant gB and Gtm3gBG proteins

In order to determine whether the mutant glycoproteins were synthesized, COS-1 cells were transfected with pXM encoding the mutants, as well as, wild-type gB and G glycoproteins and the chimera Gtm3gBG containing both mutant and wild-type gB. The transfected cells were labelled with ^{35}S Methionine and the proteins from the cell lysate were immunoprecipitated with the appropriate rabbit polyclonal antibody, anti-gB or anti-G antiserum and analyzed on sodium dodecyl sulphate polyacrylamide gels. The predicted molecular weight of HSV-1 glycoprotein gB is 116 kD. The pXMgB constructs with mutations within the hydrophobic domain of gB, $A_{786}S$, $A_{786}Y$, $A_{786}T$, $A_{786}N$, $A_{790}Q$, $A_{790}S$, $A_{791}S$, $A_{786}S;L_{788}M$, $F_{770}S$ and $G_{743}R$, expressed proteins which were recognized specifically by the rabbit polyclonal anti-gB antibody and had proteins migrating to the same position as wild-type glycoprotein gB indicating that the proteins were of a molecular size similar to wild-type HSV-1 gB (Figure 8). It can be seen in Figure 9 that VSV G, the chimeric protein Gtm3gBG and the mutant chimeric proteins were immunoprecipitated specifically with anti-G antibody. This indicates that the Gtm3gBG mutants were expressed in COS-1 cells and recognized specifically by anti-G antibody. Although all of the chimeric proteins containing point mutations were expressed in COS-1 cells, two of the mutants Gtm3gBG $A_{790}S$ and $A_{790}Q$ were not expressed as well as wild-type Gtm3gBG and the other mutants. The following mutants, Gtm3gBG $A_{786}S;L_{788}M$ and Gtm3gBG $A_{786}Y$, appeared to migrate further than wild-type Gtm3gBG and the rest of the mutant glycoproteins.

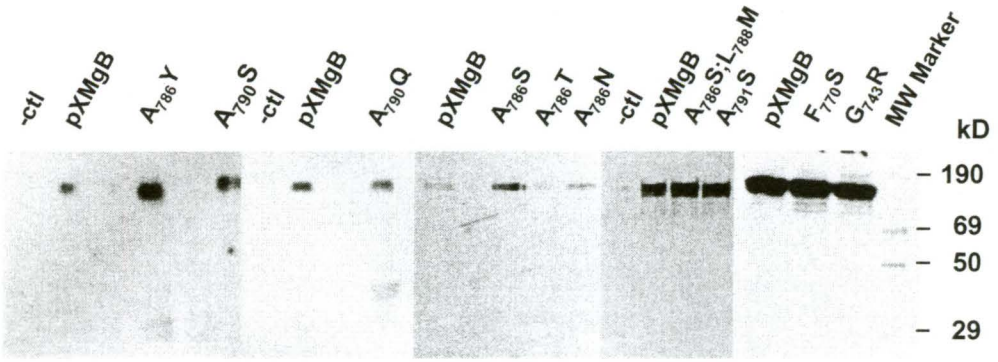


Figure 8: Expression of mutant HSV-1 gB glycoproteins.

COS-1 cells were transfected with the pXM expression vectors encoding the gB mutants. The transfected cells were labelled with [^{35}S] methionine and the proteins recovered from the cell lysate were immunoprecipitated with anti-gB antiserum. The immunoprecipitates were subjected to electrophoresis on sodium dodecyl sulphate polyacrylamide gels. The gels were fluorographed, dried and exposed overnight at -70°C. The -ctl represents COS-1 cells transfected with the pXM vector alone and the +ctl pXMgB represents COS-1 cells transfected with the pXM vector expressing wild-type glycoprotein gB.

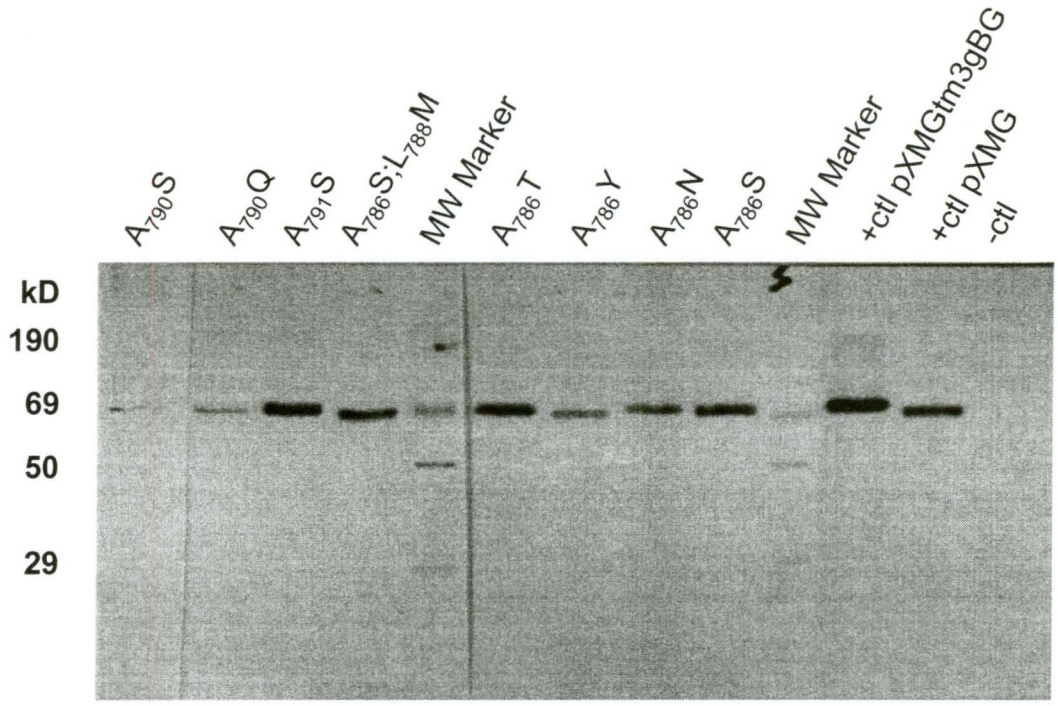


Figure 9: Expression of mutant hybrid glycoproteins, Gtm3gBG.

COS-1 cells were transfected with the pXM expression vectors encoding the Gtm3gBG mutants. The transfected cells were labelled with [^{35}S] methionine and the proteins recovered from the cell lysate were immunoprecipitated with anti-G antiserum. The immunoprecipitates were subjected to electrophoresis on sodium dodecyl sulphate polyacrylamide gels. The gels were fluorographed, dried and exposed overnight at -70°C. The -ctl represents COS-1 cells transfected with the pXM vector alone. The +ctl pXMG represents COS-1 cells transfected with the pXM vector expressing wild-type vesicular stomatitis virus glycoprotein G. +ctl pXMGtm3gB represents COS-1 cells transfected with the pXM vector expressing the hybrid protein containing the transmembrane domain of wild-type HSV-1 glycoprotein gB.

Localization of Mutants

Glycoprotein gB has been shown by indirect immunofluoresence and immunoelectron microscopy to be localized in the nuclear envelope, endoplasmic reticulum (ER), Golgi complex, as well as the plasma membrane (Compton and Courtney, 1984; Ali et al., 1987; Raviprakash et al., 1990; Rasile et al., 1993; Gilbert et al., 1994; Veit et al., 1996). However, glycoprotein G of VSV a plasma membrane protein, localizes at the cell surface, ER, and Golgi complex (Gilbert et al., 1994). The chimeric protein, Gtm3gBG, with the transmembrane of VSV G replaced with the transmembrane region of HSV-1 glycoprotein gB, localizes to the nuclear envelope, ER, Golgi complex and the cell surface (Gilbert et al., 1994).

Indirect immunofluorescence was used to determine the localization patterns of wild-type gB and G glycoproteins in transfected COS-1 cells. The mutant gB proteins and the chimeric protein, Gtm3gBG containing the transmembrane region of both wild-type and mutant glycoprotein gB were also analyzed by indirect immunofluorescence. Transfected COS-1 cells were fixed with 2% paraformaldehyde and treated with Triton X-100 for internal immunofluorescence. Sequential treatment with anti-gB or anti-G rabbit IgG and FITC-conjugated goat anti-rabbit IgG was followed by fluorescence microscopic examination. In the absence of transfected DNA, no labelling was observed (Figure 10). Fluorescence microscopic examination of cells expressing wild-type gB revealed internal perinuclear staining indicating localization in the nuclear envelope, ER and Golgi complex as well as surface labelling (Figure 10). Glycoprotein G localized to a compact nuclear region consistent with the endoplasmic reticulum and the Golgi complex as well as the cell surface. Labelling was not observed at the nuclear envelope (Figure 10). This is consistent with others findings (Rasile et al., 1993; Gilbert et al., 1994). The chimeric protein Gtm3gBG had labelling patterns similar to wild-type gB (Figure 10). Internal perinuclear and surface labelling was observed, however, the degree of labelling was less than that of wild-type glycoprotein gB. All eight of the glycoproteins with mutations within the third hydrophobic domain showed similar staining patterns as that observed for wild-type HSV-1 glycoprotein gB, internal perinuclear staining indicative of localization in the nuclear envelope, ER and Golgi complex as well as

surface labelling (Figure 11 and Figure 12). The mutants $F_{770}S$ and $G_{743}R$ showed similar staining patterns as that of wild-type gB, however, the degree of labelling was less than that observed for wild-type gB (Figure 13).

The chimeric Gtm3gBG proteins containing the following mutations $A_{786}N$, $A_{786}T$, $A_{791}S$, $A_{790}Q$ and $A_{786}S;L_{788}M$ showed localization patterns similar to that of Gtm3gBG containing wild-type gB. Labelling was observed at the cell surface, nuclear envelope, ER and Golgi complex (Figure 14 and 15). The mutants Gtm3gBG $A_{791}S$ and $A_{790}Q$ showed less labelling at the cell surface, nuclear envelope, ER and Golgi complex than Gtm3gBG containing wild-type gB. Mutants Gtm3gBG $A_{786}S$, $A_{786}Y$ and $A_{790}S$ showed staining patterns similar to wild-type VSV G, the compact juxtanuclear region consistent with the endoplasmic reticulum and the Golgi complex and the cell surface were stained while no staining was observed in the nuclear rim (Figure 14 and 15).

Figure 10: Cellular localization of wild-type HSV-1 glycoprotein gB, wild-type VSV glycoprotein G and the chimeric protein Gtm3gBG containing the transmembrane domain of wild-type gB.

COS-1 cells were transfected with the pXM vector expressing wild-type HSV-1 glycoprotein gB (pXMgB), wild-type VSV glycoprotein G (pXMG) and the chimeric protein Gtm3gBG containing the transmembrane domain of wild-type gB (pXMGtm3gB). The cells were fixed with 2% paraformaldehyde for surface immunofluorescence (SURFACE) or fixed with 2% paraformaldehyde and treated with Triton X-100 for internal immunofluorescence (INTERNAL). Sequential treatment with ant-gB or anti-G rabbit IgG and FITC-conjugated goat anti-rabbit IgG was followed by fluorescence microscopic examination. -CTL represents COS-1 cells transfected with the pXM expression vector alone.

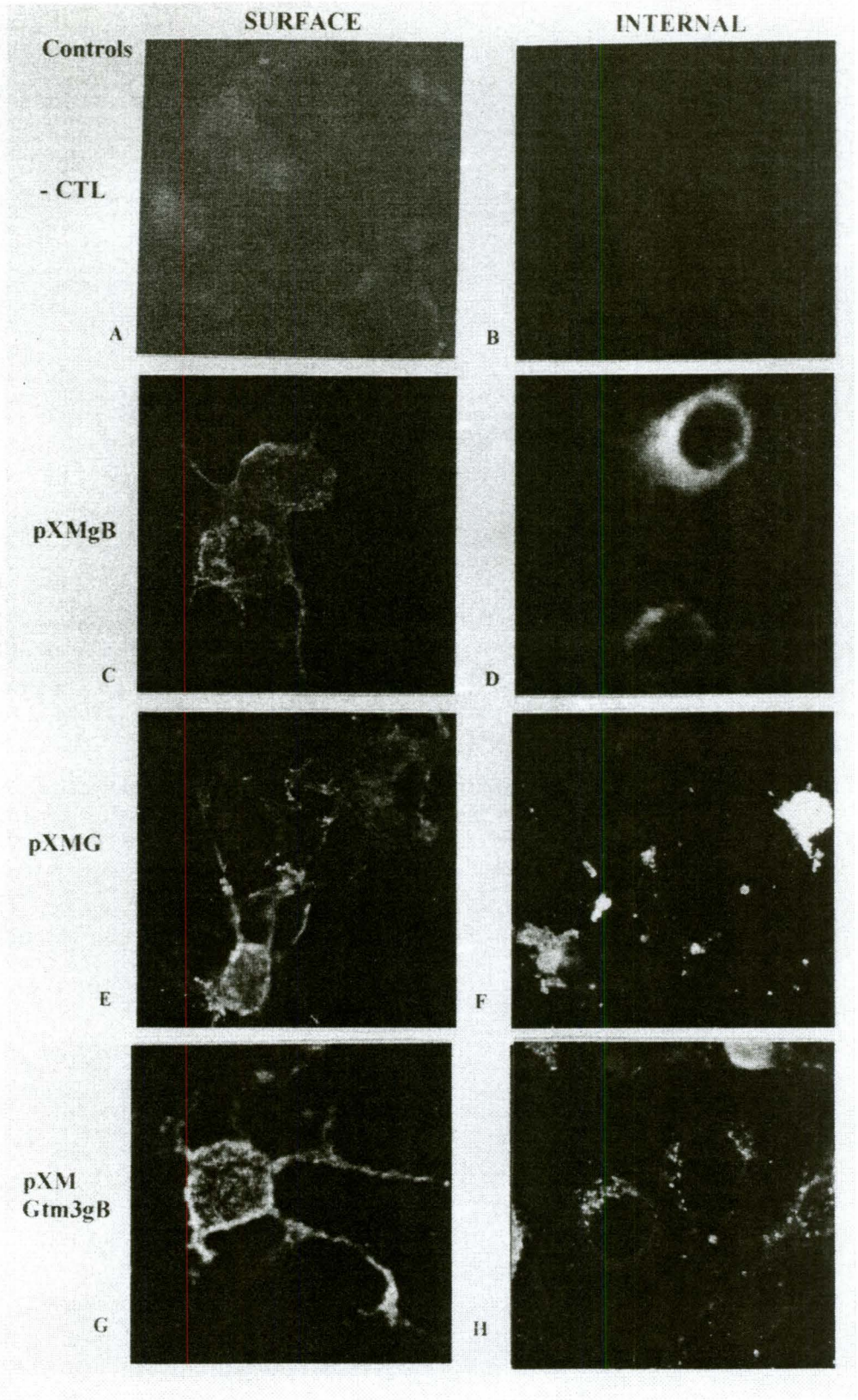

SURFACE
INTERNAL
Controls
- CTL
A
B
pXMgB
C
D
pXMG
E
F
pXM
Gtm3gB
G
H

Figure 11: Cellular localization of HSV-1 glycoprotein gB mutants

COS-1 cells were transfected with the pXM vector expressing HSV-1 glycoprotein gB mutants pXMgB $A_{786}S$, $A_{786}N$, $A_{786}T$ and $A_{786}Y$. The cells were fixed with 2% paraformaldehyde for surface immunofluorescence (SURFACE) or fixed with 2% paraformaldehyde and treated with Triton X-100 for internal immunofluorescence (INTERNAL). Sequential treatment with ant-gB IgG and FITC-conjugated goat anti-rabbit IgG was followed by fluorescence microscopic examination.

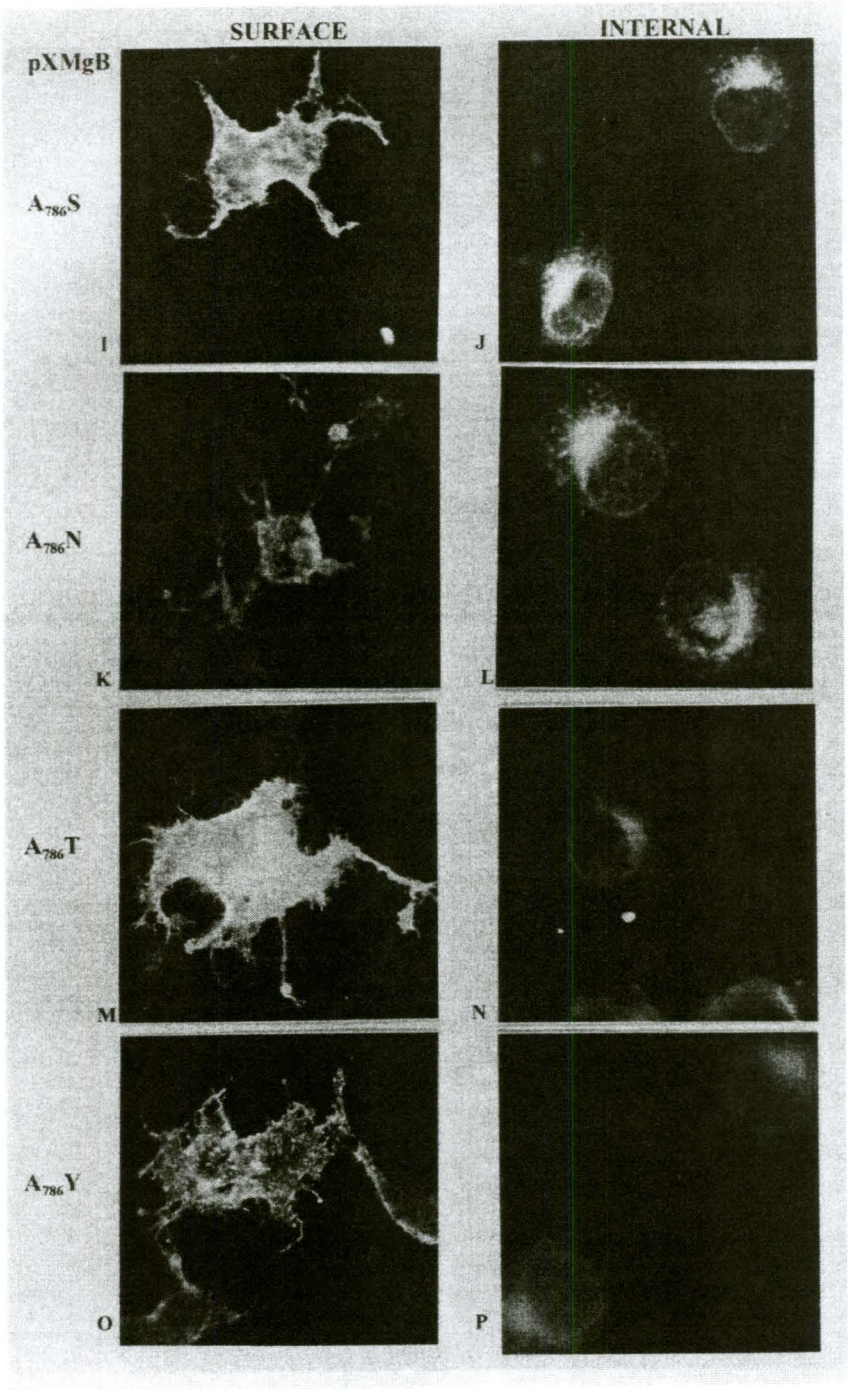
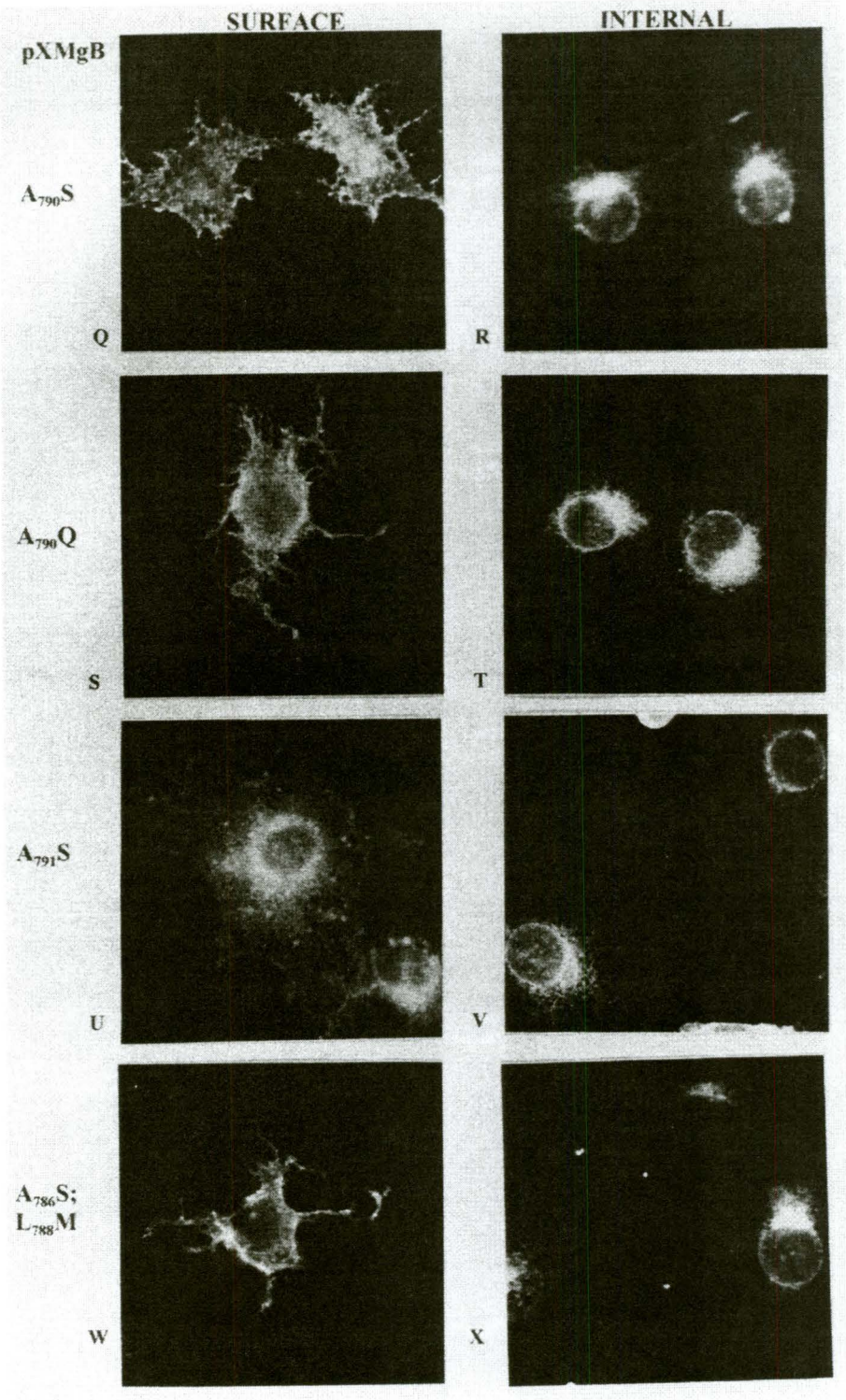

SURFACE
INTERNAL
pXMgB
$A_{786}S$
I
J
$A_{786}N$
K
L
$A_{786}T$
M
N
$A_{786}Y$
O
P

Figure 12: Cellular localization of HSV-1 glycoprotein gB mutants

COS-1 cells were transfected with the pXM vector expressing HSV-1 glycoprotein gB mutants pXMgB $A_{790}S$, $A_{790}Q$, $A_{791}S$ and $A_{786}S;L_{788}M$. The cells were fixed with 2% paraformaldehyde for surface immunofluorescence (SURFACE) or fixed with 2% paraformaldehyde and treated with Triton X-100 for internal immunofluorescence (INTERNAL). Sequential treatment with ant-gB IgG and FITC-conjugated goat anti-rabbit IgG was followed by fluorescence microscopic examination.


SURFACE
INTERNAL
pXMgB
A790S
Q
R
A790Q
S
T
A791S
U
V
A786S;
L788M
W
X

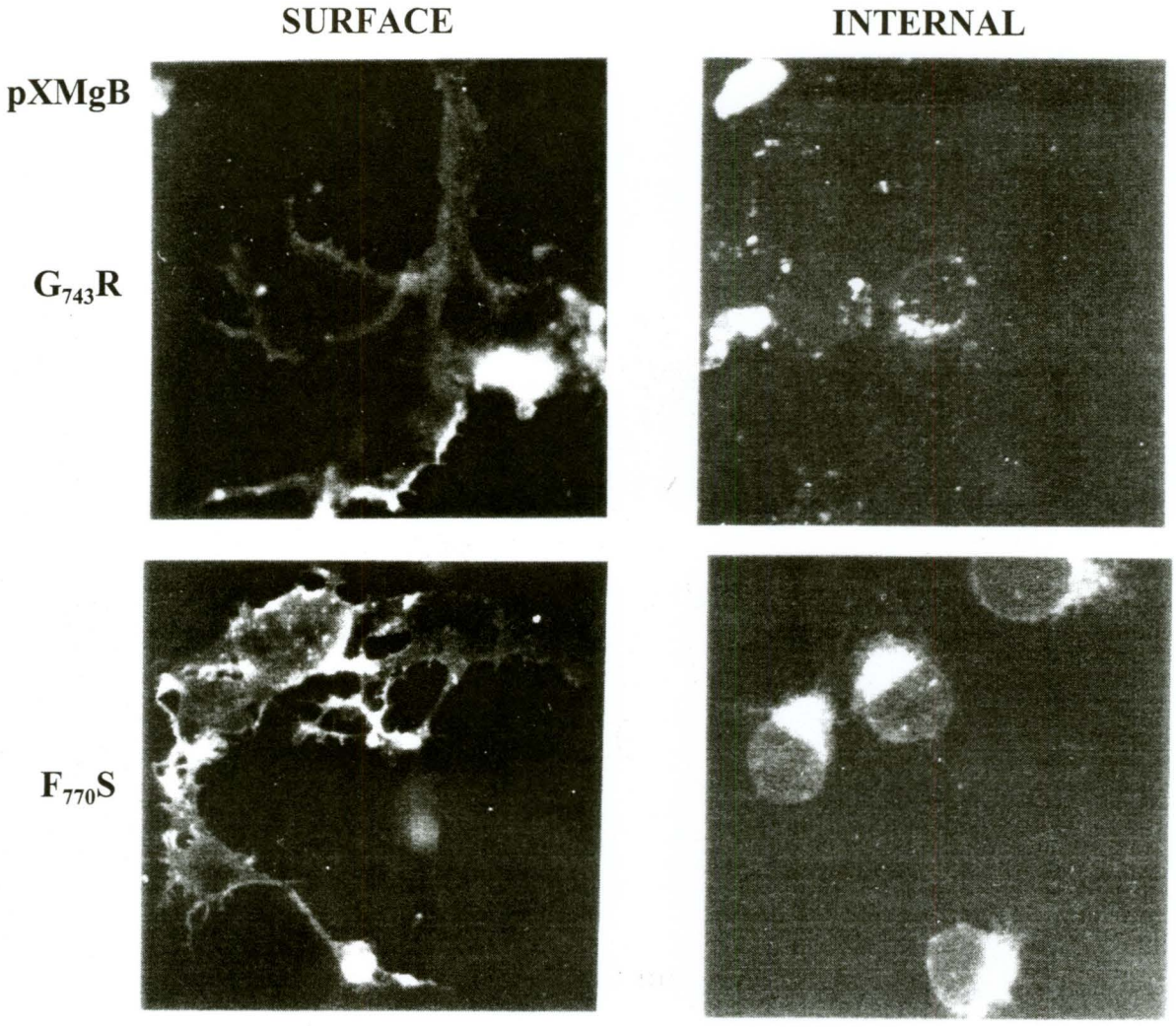


Figure 13: Cellular localization of HSV-1 glycoprotein gB mutants

COS-1 cells were transfected with the pXM vector expressing HSV-1 glycoprotein gB mutants pXMgB $G_{743}R$, and $F_{770}S$. The cells were fixed with 2% paraformaldehyde for surface immunofluorescence (SURFACE) or fixed with 2% paraformaldehyde and treated with Triton X-100 for internal immunofluorescence (INTERNAL). Sequential treatment with ant-gB IgG and FITC-conjugated goat anti-rabbit IgG was followed by fluorescence microscopic examination.

Figure 14: Cellular localization of the chimeric proteins Gtm3gBG containing point mutations within the transmembrane domain of HSV-1 glycoprotein gB.

COS-1 cells were transfected with the pXM vector expressing the chimeric protein Gtm3gBG containing the following point mutations within the transmembrane domain of HSV-1 glycoprotein gB: $A_{786}S$, $A_{786}N$, $A_{786}T$ and $A_{786}Y$. The cells were fixed with 2% paraformaldehyde for surface immunofluorescence (SURFACE) or fixed with 2% paraformaldehyde and treated with Triton X-100 for internal immunofluorescence (INTERNAL). Sequential treatment with anti-G rabbit IgG and FITC-conjugated goat anti-rabbit IgG was followed by fluorescence microscopic examination.

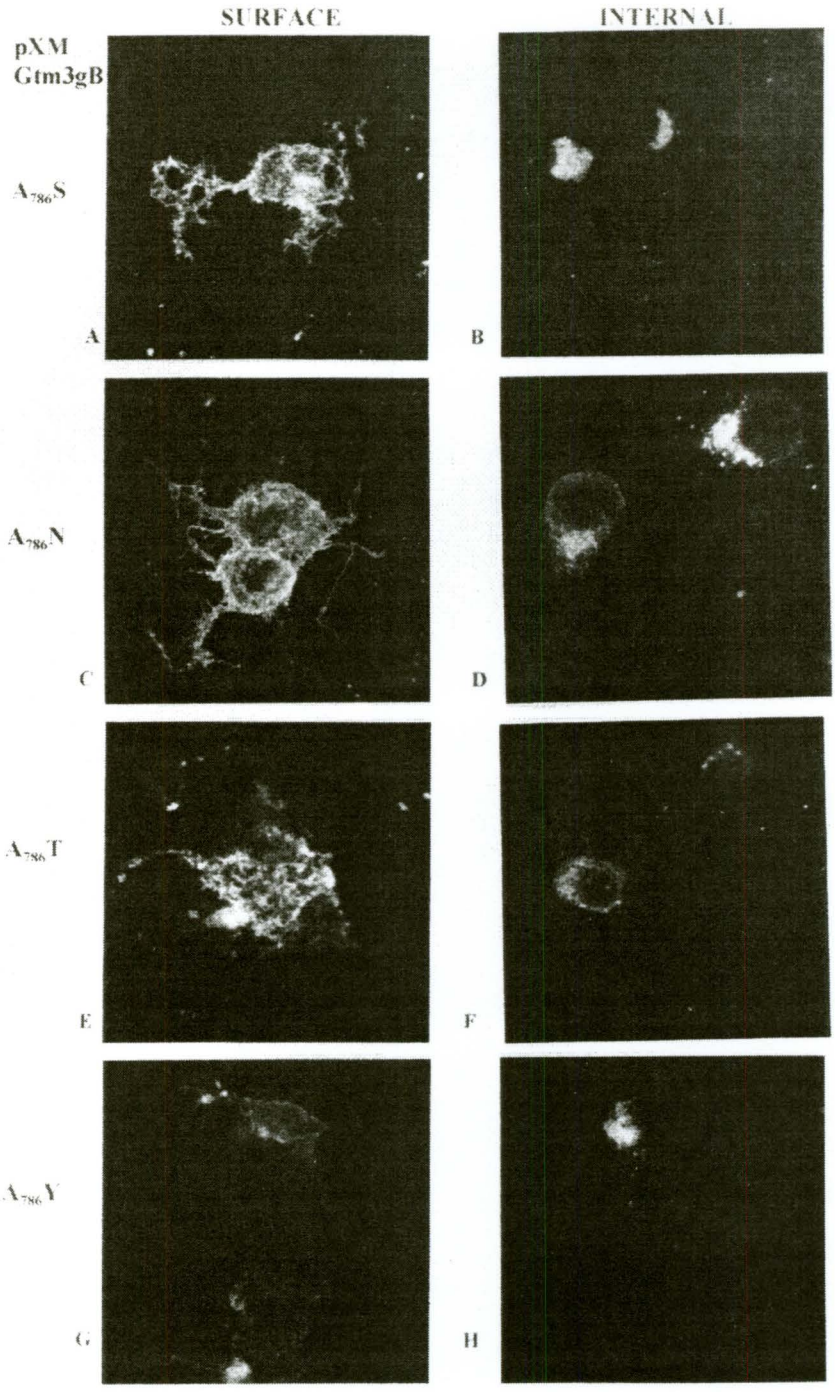
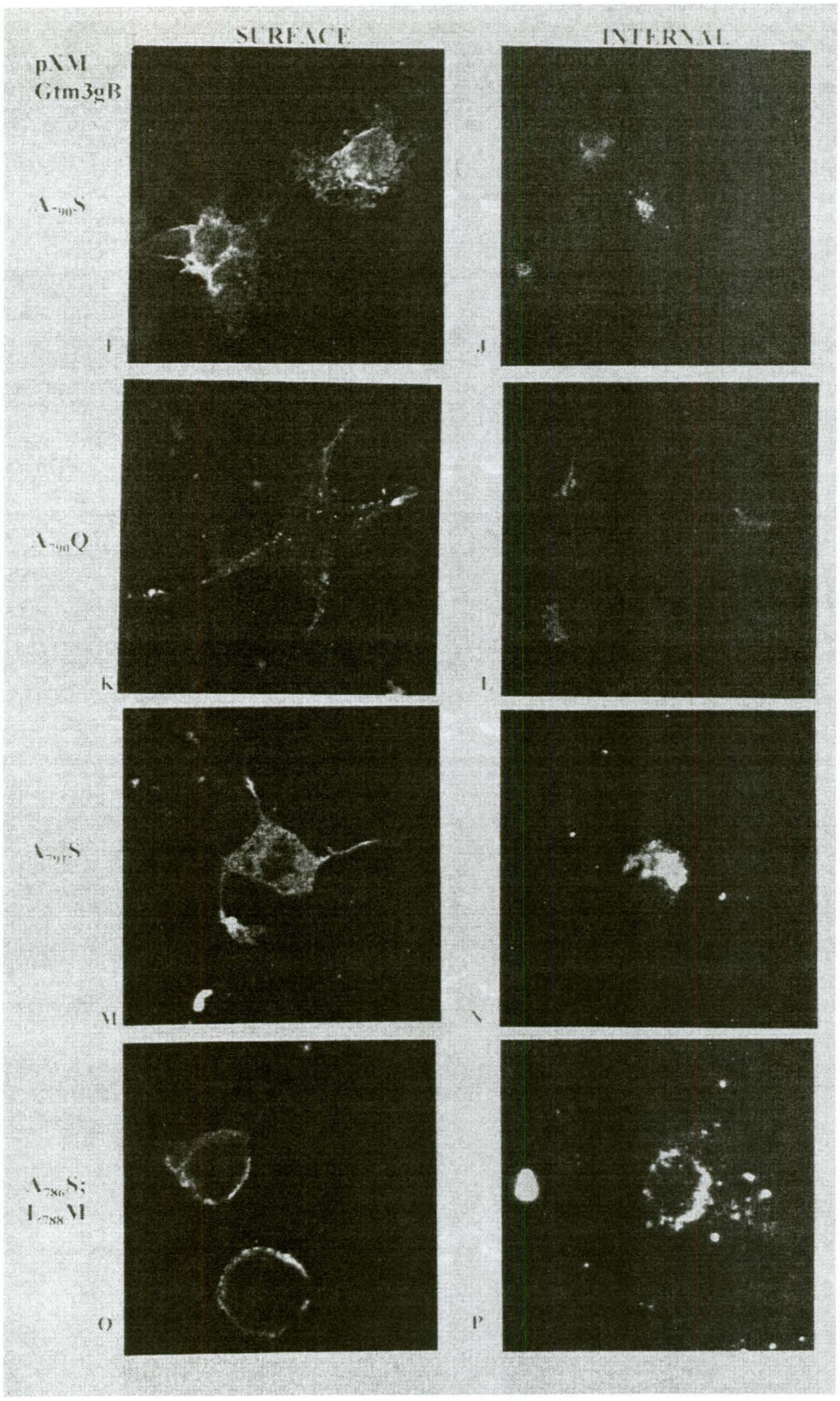

SURFACE
INTERNAL
pXM
Gtm3gB
A786S
A
B
A786N
C
D
A786T
E
F
A786Y
G
H

Figure 15: Cellular localization of the chimeric proteins Gtm3gBG containing point mutations within the transmembrane domain of HSV-1 glycoprotein gB.

COS-1 cells were transfected with the pXM vector expressing the chimeric protein Gtm3gBG containing the following point mutations within the transmembrane domain of HSV-1 glycoprotein gB: $A_{790}S$, $A_{790}Q$, $A_{791}S$ and $A_{786}S;L_{788}M$.. The cells were fixed with 2% paraformaldehyde for surface immunofluorescence (SURFACE) or fixed with 2% paraformaldehyde and treated with Triton X-100 for internal immunofluorescence (INTERNAL). Sequential treatment with anti-G rabbit IgG and FITC-conjugated goat anti-rabbit IgG was followed by fluorescence microscopic examination.


SURFACE
INTERNAL
pXM
Gtm3gB
A-90S
I
J
A-90Q
K
L
A-91S
M
N
A-86S;
L-88M
O
P

Complementation Assay

The ability of the HSV-1 gB mutants to complement a gB null virus K082 (Cai et al., 1987; Cai et al., 1988) was determined. All of the pXM-gB mutants were subcloned into the vector pKBXX, which contains full-length wild-type glycoprotein gB of HSV-1 as well as a 790 bp 5' and a 498 bp 3' flanking sequences of HSV-1. Within the pKBXX plasmid the expression of glycoprotein gB is under the control of the HSV-1 promoter. Vero cells were transfected with 10 μg of the mutant plasmid by the DEAE Dextran transfection method. After 17 hours, the cells were infected with the gB-1 null mutant virus K082 at a concentration of 10^6 pfu. K082, contains the pK082 nonsense mutation at codon 43, therefore, it produces no detectable gB-specific polypeptide (Cai et al., 1988). K082 can only grow when a transformed cell line expressing gB is employed such as D6 or VB38. The K082 gB null virus was initially plaque purified in order to reduce contaminating wild-type HSV-1 virus. After 24 hours at 37°C, the virus was harvested and titered on both VB38 and Vero cell lines. The percent complementation efficiency was calculated as the plaquing efficiency of the virus produced by transfection with mutant HSV-1 gB, as a percentage of the plaquing efficiency of the virus produced by transfection with the wild-type gB plasmid (pKBXX). Plaquing efficiency of complemented virus is expressed as the ratio of the titre on Vero cells to the titre on gB expressing cells, VB38. The complementation assay results for each gB mutant are outlined in Table 3 and Figure 16.

The HSV-1 gB mutants, $A_{786}S$, $A_{786}N$, $A_{786}Y$, $A_{790}Q$, $A_{791}S$, $G_{743}R$ and $F_{770}S$ were unable to efficiently complement the gB null virus, K082 with complementation efficiencies of less than 50%. However, the HSV-1 gB mutants $A_{786}T$, $A_{786}S;L_{788}M$ and $A_{790}S$ were able to complement the gB null virus as well as or better than wild-type gB.

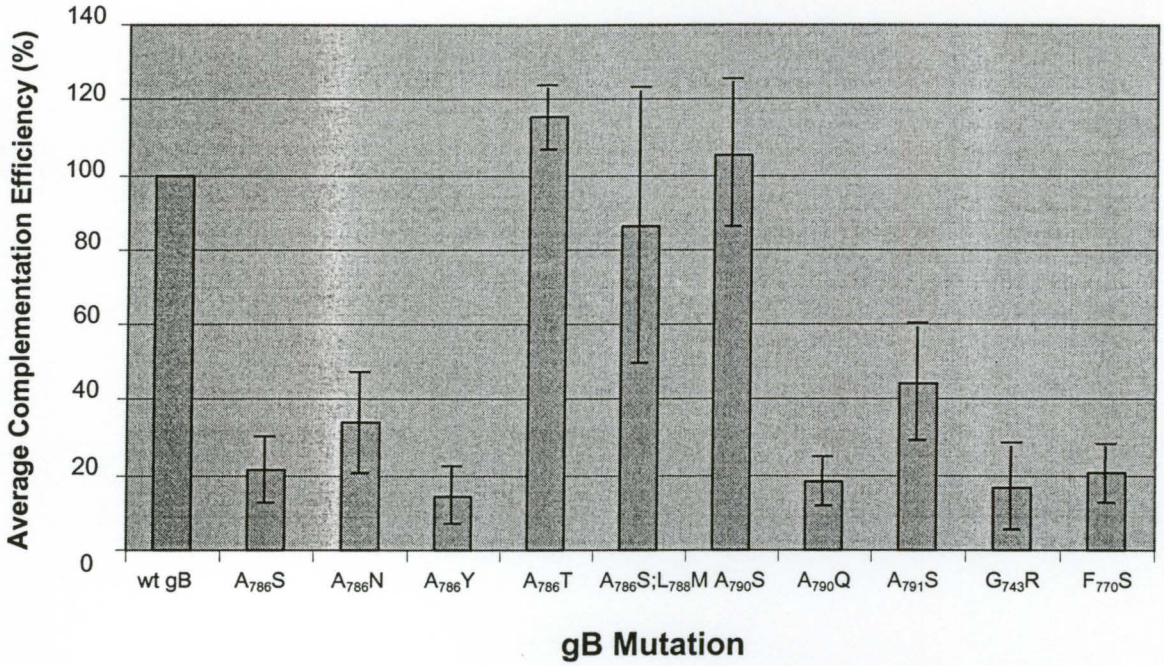


Figure 16: Complementation efficiency of the HSV-1 glycoprotein gB mutants.

Vero cells were transfected with the pKBXX vector containing each of the HSV-1 transmembrane domain mutants. After 17 hours, the cells were infected with the gB-1 null virus K082 at a concentration of 10^6 pfu. After 24 hours at 37 C, the virus was harvested and titered on both VB38 and Vero cell lines. Plaquing efficiency of complemented virus is expressed as the ratio of the titre on Vero cells to the titre on gB-expressing cells, VB38. The average percent complementation efficiency was calculated as the plaquing efficiency of the virus produced by transfection with the mutant HSV-1 gB vectors as a percentage of the plaquing efficiency of the virus produced by transfection with the vector expressing wild-type gB , pKBXX.

Table 3: A summary of protein expression, cellular localization, and complementation efficiency for each of the mutant constructs as well as the wild-type controls.

Backbone	Mutation	Protein Expression	Cellular Localization			Complementation Efficiency (%)
			Plasma Membrane	ER/Golgi	Nuclear Envelope	
pXMgB	CTL	+	+	+	+	100
	A786S	+	+	+	+	21.4
	A786T	+	+	+	+	115.1
	A786Y	+	+	+	+	14.5
	A786N	+	+	+	+	34.3
	L788M;A786S	+	+	+	+	86.5
	A790Q	+	+	+	+	18.3
	A790S	+	+	+	+	105.4
	A791S	+	+	+	+	44.5
	F770S	+	+	+	+/-	20.3
	G743R	+	+	+	+/-	16.6
pXMG	CTL	+	+	+	-	
pXMGtm3gB	CTL	+	+	+	+	
	A786S	+	+	+	-	
	A786T	+	+	+	+	
	A786Y	+	+	+	-	
	A786N	+	+	+	+	
	A786S;L788M	+	+	+	+	
	A790Q	+	+	+	+/-	
	A790S	+	+	+	-	
	A791S	+	+	+	+/-	

DISCUSSION

Glycoprotein gB of herpes simplex virus 1 plays a primary role in the infectivity of the herpes simplex virus. The specific localization of glycoprotein gB to the nuclear envelope is essential for its incorporation into the viral envelope where it is required for viral attachment and penetration. Localization of glycoprotein gB at the cell surface is essential for cell to cell spread of the virus and syncytia formation (Bzik et al., 1984; Cai et al., 1987; Cai et al., 1988; Highlander et al., 1988; Navarro et al., 1992; Gage et al., 1993; Baghian et al., 1993; Spear, 1993; Hutchinson et al., 1993; Herold et al., 1994; Pereira, 1994). It has been shown that the primary structure of proteins contain targeting or signal sequences which are essential for the sorting and localization of proteins to their functional site (Pelham, 1989).

The amino acid sequence of gB is highly conserved amongst herpesviridae with the hydrophobic domains being almost entirely conserved amongst α-herpesviridae. Gilbert et al. (1994) using HSV-1 gB hydrophobic region deletion mutants showed that the third hydrophobic segment (aa 775-795) of glycoprotein gB was essential for membrane anchoring and nuclear envelope localization of glycoprotein gB. The first and second hydrophobic segments (aa 727-747, aa 752-772 respectively) may be required for stabilizing the glycoprotein within the membrane and may play a role in efficient nuclear envelope targeting (Rasile et al., 1993). Rasile et al. (1993) showed that although mutants with the first and second hydrophobic segments deleted were localized efficiently to the nuclear envelope, they were unable to complement a gB null virus, K082. It was concluded that all three of the hydrophobic domains (aa 727-795) of gB are required for virus infectivity.

In order to determine the role of specific amino acids within the hydrophobic domains of HSV-1 glycoprotein gB, point mutations were created by the method of site directed mutagenesis. The amino acids, alanine 786 (A_{786}), alanine 790 (A_{790}) and alanine 791 (A_{791}) within the third hydrophobic, transmembrane

domain of HSV-1 gB were selected for mutation. These specific alanines, 786, 790 and 791 are the mostly highly conserved alanine residues amongst all of the α-herpesviridae. Doped mutagenesis was used to mutate amino acids A_{786} and A_{790} to four different amino acids in one mutagenesis reaction. This was accomplished by synthesizing a mixture of primers (Table 1) and using these in the mutagenesis reaction. Sequencing reactions were performed to verify that the desired mutations were created. Doped mutagenesis was found to be an excellent tool for the introduction of several point mutations in a single mutagenesis reaction. The use of PCR to introduce point mutations although rapid has low fidelity resulting in the introduction of undesirable point mutations. The introduction of enzymes with proof-reading ability such as PWO DNA polymerase from Boehringer Mannheim, have helped reduce these mistakes, however, the gene fragment which has been synthesized by PCR must be sequenced in it's entirety resulting in numerous sequencing reactions over a long period of time and at a high price.

A_{786} was mutated to serine (S), threonine (T), asparagine (N) and tyrosine (Y); while A_{790} was mutated to serine (S) and glutamine (Q). Sequencing revealed the creation of another mutation along with $A_{786}S$, leucine (L) 788 was mutated to methionine (M) ($A_{786}S;L_{788}M$), this unexpected mutant was studied along with the others. Site directed mutagenesis was used to mutate A_{791} to serine, while introducing a new restriction enzyme site. The creation of this new restriction enzyme site enabled rapid screening of putative mutants by restriction enzyme digestion prior to sequencing resulting in fewer sequencing reactions. Alanine is a hydrophobic, non-polar amino acid which is known to be a strong helix former; serine, threonine, tyrosine, glutamine and asparagine however, are uncharged polar amino acids normally found on the surface of molecules. Leucine and methionine are both non-polar amino acids normally found in the interior of the protein. (Voet and Voet, 1990)

Glycine 743, within the first hydrophobic segment and phenylalanine 770, within the second hydrophobic segment of HSV-1 glycoprotein gB, are conserved amongst all α-herpesviridae. These amino acids were mutated by site directed mutagenesis creating a unique restriction site, and the role of two highly

conserved amino acids within the first and second hydrophobic domains was studied. Glycine, a non-polar amino acid known to be a strong helix breaker, was mutated to arginine (R), a charged polar amino acid usually found on the surface of proteins. Phenylalanine, a non-polar amino acid with a bulky side-chain, usually found within the interior of the molecule, was mutated to serine (S) an uncharged polar amino acid.

The point mutations introduced into the hydrophobic domains of glycoprotein gB were studied to determine their effect on protein expression, localization, targeting and biological activity.

Point mutations can effect the stability of a protein and one of the most important contributions to protein stability is the hydrophobic effect (Alber, 1989). The hydrophobicity of each of the glycoprotein gB mutants was determined by using the Kyte-Doolittle hydrophobic indices (Figure 7). A transmembrane domain should have an average hydrophobic index of 1.65 or greater and the point mutations within the third hydrophobic segment of glycoprotein gB did not reduce the average hydrophobic index below this range (Kyte and Doolittle, 1982). Therefore, the point mutations should not affect hydrophobic interactions, protein stability and the membrane anchoring ability of the third hydrophobic segment. The first and second hydrophobic segments of glycoprotein gB have been shown to exhibit membrane anchoring ability in the absence of the third hydrophobic segment. The point mutations introduced into the first and second hydrophobic regions did not significantly affect the overall hydrophobicity of this region. Since the third hydrophobic segment alone can anchor the protein within the membrane in the absence of the first and second segment, point mutations should have little, if any, effect on membrane anchoring of the protein.

Transfection of COS-1 cells with the plasmids expressing mutant glycoprotein gB resulted in the synthesis of a single species of immunoprecipitatable gB protein for each mutant. Each mutant protein co-migrated with the wild-type glycoprotein on sodium dodecyl sulfate polyacrylamide gels. These proteins were recognized by the anti-gB antibody implying that the epitopes required for recognition by the antibody were still accessible. There are no antigenic domains found within the hydrophobic transmembrane domain of glycoprotein gB, therefore, it was expected that these point mutations would not effect immunoprecipitation

of the protein (Qadri et al., 1991).

Glycoprotein gB localizes to the inner and outer nuclear membrane, Golgi complex, endoplasmic reticulum and the cell surface (Compton and Courtney, 1984; Ali et al., 1987; Raviprakash et al., 1990; Gilbert and Ghosh, 1993; Rasile et al., 1993; Veit et al., 1996). Localization of glycoprotein gB to the inner nuclear membrane of the infected cell is essential for incorporation into the herpesvirus envelope and virus infectivity. The localization of proteins within the nuclear envelope was thought to be due to retention within the endoplasmic reticulum. This hypothesis was tested by Gilbert et al. (1994) who produced a mutant G protein, GH6, which has an insertion of three amino acids at position 244 of the G protein. GH6 does not acquire endoglycosidase H (endo H) resistance and fails to reach the cell surface indicating that it is retained in the endoplasmic reticulum. Indirect immunofluorescence revealed labelling associated exclusively with a cytoplasmic membrane structure which resembled the ER. No labelling was observed at the cell surface and very little labelling was associated with the nuclear envelope. Therefore, localization of a protein at the nuclear envelope is not simply due to retention of the protein within the ER.

The effect of point mutations introduced into the hydrophobic segments of glycoprotein gB on nuclear envelope localization was studied by indirect immunofluorescence. COS-1 cells were transfected with each of the mutant plasmids and the localization of the mutant proteins determined by indirect immunofluorescence. All of the third hydrophobic segment mutants were localized to the same cellular location as wild-type gB, the nuclear envelope, ER and Golgi complex. The mutants also showed surface labelling similar to wild-type gB. Therefore, none of the point mutations introduced into the third hydrophobic regions of full length glycoprotein gB, $A_{786}S$, $A_{786}N$, $A_{786}T$, $A_{786}Y$, $A_{790}Q$, $A_{790}S$, and $A_{791}S$ affected nuclear envelope or cell surface localization. Proteins may tolerate certain amino acid substitutions because, some substitutions preserve critical interactions, some interactions apparently do not make large contributions to protein stability and protein structures adjust to compensate for changes in sequence. Classical genetic studies of protein function have suggested that proteins are very tolerant of amino acid substitutions (Alber, 1989). The efficient targeting

and localization of the HSV-1 gB mutants to the nuclear envelope is not surprising since it has been shown that the first and second hydrophobic segments can target the protein to the nuclear envelope in the absence of the third hydrophobic domain although less efficiently (Rasile et al., 1993). The first and second hydrophobic segments may contain signal sequences which facilitate efficient targeting and compensate for the point mutations introduced into the third hydrophobic segment.

Indirect immunofluorescence revealed that point mutations within either the first hydrophobic segment, $G_{743}R$, or the second hydrophobic segment, $F_{770}S$, reduced protein expression at the nuclear envelope. This may be due to the drastic amino acid changes introduced within these regions. Exchanging glycine, a helix breaker which is required for proper conformation of the protein, with arginine, a charged amino acid can be destabilizing. The introduction of charged residues into the hydrophobic or transmembrane domains of a protein often results in misfolding of the protein (Doms et al., 1993). Adams and Rose (1985) found that the replacement of an isoleucine residue within the membrane-spanning domain of VSV G with a polar uncharged amino acid, glutamine, had no effect on membrane anchoring or transport of the protein to the cell surface. However, when the same isoleucine was replaced with a charged amino acid, arginine, the protein was membrane anchored but not transported efficiently to the cell surface. Replacing a non-polar amino acid, phenylalanine with an uncharged polar amino acid, serine, can alter stability and conformation of the protein. Immunoprecipitation assays revealed that the mutant proteins, gB $G_{743}R$ and gB $F_{770}S$ were expressed in COS-1 cells and recognized specifically by anti-gB antibodies. Mutations throughout the gB gene have been known to impair the processing of the glycopeptides (Cai et al., 1988). This impaired processing is mainly due to a block in the movement of the proteins from the nuclear envelope to the Golgi apparatus. An endoglycosidase H assay should be employed in order to study the transport of these HSV-1 gB mutants through the exocytic pathway by analysing a shift in electrophoretic mobility after endoglycosidase treatment. This would enable one to determine whether the mutant proteins were being transported and processed efficiently. The conservation of the amino acids, phenylalanine 770 and glycine 743, amongst all α-herpesviridae is a good

indication of their important role in the overall structure and function of glycoprotein gB.

In order to effectively study the effect of these point mutations introduced into the third hydrophobic segment of HSV-1 glycoprotein gB, the first and second segments must be eliminated. This was accomplished by creating chimeric proteins with Vesicular Stomatitis Virus glycoprotein G (VSV G), a plasma membrane protein, and glycoprotein gB. The chimeric protein Gtm3gBG, otherwise known as GΔ12gBG, has been shown to be expressed and localized efficiently at the nuclear envelope (Rasile et al.,1993). The transmembrane segment of VSV G was therefore replaced with the mutated forms of the third hydrophobic segment of glycoprotein gB. This was accomplished by using the vector, pGemG(AXB) which has unique restriction enzyme sites on either side of the transmembrane region of G. The mutated transmembrane segments of gB were synthesized by PCR using primers which contained the appropriate restriction enzyme sites at the 5' and 3' ends of the transmembrane fragment. These fragments were then subcloned into pGemG(AXB) replacing it's transmembrane segment with the mutated third hydrophobic segments of glycoprotein gB. The use of PCR resulted in fast and efficient subcloning. The resulting genes were then cloned into the vector, pXM, creating the following chimeric mutant plasmids, pXM Gtm3gBG $A_{786}S$, Gtm3gBG $A_{786}N$, Gtm3gBG $A_{786}T$, Gtm3gBG $A_{786}Y$, Gtm3gBG $A_{790}S$, Gtm3gBG $A_{790}Q$, Gtm3gBG $A_{791}S$ and Gtm3gBG $A_{786}S;L_{788}M$. The plasmids were analysed by sequencing and the presence of the desired mutation confirmed.

Expression of the mutant chimeric proteins was analysed by immunoprecipitation. All of the chimeric mutants were expressed in COS-1 cells, for each mutant a single species of immunoprecipitatable G protein was synthesized and comigrated with wild-type Gtm3gBG. The two glycoproteins, $A_{786}S;L_{788}M$ and $A_{786}Y$ appeared to migrate further than wild-type Gtm3gBG and the rest of the mutants. A possible reason for this migration is that the denatured polypeptides may have bound to fewer SDS molecules than wild-type Gtm3gBG and the rest of the mutants. Two of the proteins, Gtm3gBG $A_{790}S$ and $A_{790}Q$, were expressed to a lesser degree than wild-type gB therefore, a pulse-chase experiment should be employed to analyze post-

translational modifications, transport, secretion and the possible degradation of newly synthesized proteins.

The localization of the chimeric mutants was studied by indirect immunofluorescence in order to determine the effect these point mutations have in the absence of the ectodomain, cytoplasmic tail, and first and second hydrophobic segments of glycoprotein gB. Glycoprotein G of vesicular stomatitis virus (VSV) is specifically localized within the plasma membrane, the endoplasmic reticulum and the Golgi complex, and it is not found within the nuclear envelope (Rose and Bergmann, 1983; Adams and Rose, 1985; Schlesinger and Schlesinger, 1987; Rose and Doms, 1988; Doms et al., 1993). It has already been established that when the transmembrane segment of VSV G is replaced with the third hydrophobic segment of HSV-1 glycoprotein gB, the chimeric mutant GΔ12gB is efficiently localized to the nuclear envelope as well as the plasma membrane, Golgi complex and the endoplasmic reticulum. (Rasile et al., 1993). It was concluded, that the third hydrophobic segment of HSV-1 gB is sufficient for nuclear envelope localization and targeting and therefore may contain a signal sequence required for this specific targeting. Signal sequences within the primary structure of proteins, including transmembrane domains, have already been shown to target proteins to specific locations within the cell (Nilsson et al., 1991; Munro, 1991; Wozniak and Blobel, 1992; Masibay et al., 1993; Smith and Blobel, 1993).

Indirect immunofluorescence revealed that all of the Gtm3gBG mutants were localized to the plasma membrane. The mutants Gtm3gBG $A_{786}T$ and $A_{786}N$ were localized to the nuclear envelope as efficiently as Gtm3gBG containing wild-type gB. Therefore, the introduction of threonine or asparagine at amino acid position 786 had no influence on the effective targeting of the hybrid protein to the nuclear envelope as well as the plasma membrane. Threonine residues are quite prevalent in the putative nuclear envelope localization signals of gp210 and the lamin B receptor (Wozniak and Blobel, 1992; Smith and Blobel, 1993) and this may explain the acceptance of this residue within the putative targeting signal of glycoprotein gB.

Two mutant proteins, Gtm3gBG $A_{790}Q$ and $A_{791}S$, were not efficiently localized to the nuclear envelope. Nuclear envelope staining was less than wild-type gB or Gtm3gBG. However, these mutant

glycoproteins were found at the plasma membrane to the same degree as wild-type gB, therefore, they are not being retained within the ER. Pulse-chase labelling experiments need to be performed.

Mutants Gtm3gBG $A_{786}S$, $A_{786}Y$ and $A_{790}S$ were not found to be localized at the nuclear envelope, with staining patterns similar to that of VSV G. These mutant glycoproteins were not retained in the endoplasmic reticulum as they were localized efficiently at the plasma membrane. While the mutant $A_{786}S$ was not localized to the nuclear envelope, the double mutant $A_{786}S;L_{788}M$, was localized efficiently to the nuclear envelope. Conceivably the mutation $L_{788}M$ compensated for the structural changes introduced by the $A_{786}S$ mutation which impaired nuclear envelope localization. The amino acid alanine 786 may be part of a signal sequence, however only when the amino acid was mutated to tyrosine or serine was the localization of the protein altered. The other A_{786} mutations may have been tolerated since these amino acids are found within the putative nuclear envelope localization signals of other proteins and these substitutions may not have affected the native conformation of the protein required for efficient targeting.

When alanine 790 was mutated to serine or glutamine the localization of the protein to the nuclear envelope was impaired indicating that alanine 790 may also be part of a signal sequence. Serine residues are not found within the third hydrophobic segment of HSV-1 glycoprotein gB and it is evident that the presence of serine residues at these amino acid positions within the third hydrophobic domain of gB affects nuclear envelope targeting of the protein. Cai et al. (1988) hypothesized that proper folding of the entire polypeptide is required for efficient processing and transport.

Bretscher and Munro (1993) hypothesized that the cholesterol gradient within the Golgi complex, the length of the transmembrane domain and the spacing arrangement of the side chains specifies protein retention within cellular membranes. In the case of glycoprotein gB the length of the transmembrane domain of glycoprotein gB was not affected by the point mutations created and the amino acids introduced should not affect the bulkiness of the side chains. The secondary structure of glycoprotein gB may play a major role in

targeting.

The introduction of point mutations within the third hydrophobic segment of glycoprotein gB had no effect on protein synthesis or nuclear envelope targeting of the protein. However, when the same point mutations were introduced into the chimeric protein, Gtm3gBG, the amino acid substitutions $A_{790}Q$, $A_{791}S$ affected the efficiency of nuclear envelope localization, while the substitutions of $A_{786}S$, $A_{786}Y$ and $A_{790}S$ abolished nuclear envelope localization all together. It may then be concluded that the first and second hydrophobic segments as well as the cytoplasmic tail and the ectodomain influence efficient nuclear envelope targeting of the protein.

The stable, constitutive expression of glycoprotein gB in the cell line, VB38, allows for the transfer of mutations within cloned genes *in vitro* to HSV-1 viral DNA. A complementation assay was utilized to determine the role specific amino acids within the transmembrane domain of HSV-1 glycoprotein gB play in viral infectivity. Vero cells were transfected with the glycoprotein gB mutants subcloned into the vector pKBXX. The pKBXX vector contains full-length wild-type glycoprotein gB of HSV-1 as well as the 790 bp 5'- and a 498 bp 3'- flanking sequence of HSV-1. The expression of glycoprotein gB within the pKBXX vector is under the control of the HSV-1 promoter. After 17 hours, the Vero cells were infected with K082, a virus which produces no detectable gB-specific polypeptide (Cai et al., 1988), at a concentration of 10^6 pfu. K082 is only able to grow when a transformed cell line expressing gB is employed, in this case the cell line used was VB38. After 24 hours at 37°C, the virus was harvested and titered on both VB38 and Vero cell lines. The ability of K082 virus to infect Vero cells is due to wild-type virus contamination or from the recombination between transfected plasmid and K082 (Cai et al., 1988). The amount of wild-type virus within the K082 stock used in this study was reduced by plaque purification of the virus. Cai et al., (1988) found that when pKBXX was transfected into Vero cells before K082 infection, the number of plaque forming units (pfu) on D6, a gB transformed cell line, was increased by at least two orders of magnitude due to complementation by the plasmid-encoded gB. Previous complementation assays with glycoprotein gB mutants revealed that

glycoprotein gB hydrophobic domain deletion mutants, Δ1, Δ12, Δ13, Δ2, Δ23, Δ3, and Δ123, were unable to complement a gB null virus (Rasile et al., 1993). It had also been shown previously that addition mutations at residue 734 within the first hydrophobic domain of glycoprotein gB and termination mutants within the entire hydrophobic domain of gB abolished complementation (Cai et al., 1988).

The ability of each of the full-length glycoprotein gB mutants to complement a gB null virus was determined. The following mutants, gB $A_{786}T$, $A_{786}S;L_{788}M$ and $A_{790}S$, were able to efficiently complement the gB null virus. Mutant proteins gB $A_{786}N$ and gB $A_{791}S$ were unable to efficiently complement the null virus with complementation efficiencies less than 50%. While, the gB mutants $A_{786}S$, $A_{786}Y$, $A_{790}Q$, $F_{770}S$ and $G_{743}R$ were unable to complement the gB null virus having complementation efficiencies less than 25%.

No effect on nuclear envelope localization was observed with any of the point mutations introduced into full-length glycoprotein gB. Therefore these mutant glycoproteins should be available at the nuclear envelope for incorporation into the herpes simplex virus envelope. However, when the same mutations were introduced into Gtm3gBG, several of the mutants were either not localized to the nuclear envelope or not targeted efficiently. This may have been caused by a change in structure which inhibits or impairs protein transport, or due to the substitution of an essential amino acid within a targeting sequence. Nuclear envelope localization of glycoprotein gB is vital for its incorporation into the viral envelope and virus infectivity. The mutants $A_{786}T$ and $A_{786}S;L_{788}M$ were shown to be synthesized, transported and localized efficiently at the nuclear envelope and were found to effectively complement a gB null virus. The mutants Gtm3gBG $A_{786}S$, $A_{786}Y$, $A_{790}Q$, and $A_{791}S$ were not targeted or not targeted efficiently to the nuclear envelope and when these amino acid substitutions were made in wild-type glycoprotein gB they were unable to complement a null virus. These mutant proteins although present at the nuclear envelope in the context of glycoprotein gB, may not have been incorporated into the viral capsid due to misfolding or due to the fact that sequences required for interaction with other viral proteins was lost. Another possibility is that the mutant proteins were incorporated into the HSV virion but were not biologically active due to the point mutations.

The glycoprotein gB mutants $F_{770}S$ and $G_{743}R$ were unable to complement a gB-null virus. These glycoprotein gB mutants were not targeted efficiently to the nuclear envelope therefore, perhaps they were not present in sufficient number at the nuclear envelope resulting in fewer proteins being incorporated into the viral envelope and a reduction in infectious virus.

The mutant, Gtm3gBG $A_{790}S$, was not found to be localized at the nuclear envelope, however, the mutant gB $A_{790}S$ was able to complement the gB null virus. Perhaps when this mutation was present in the context of full length glycoprotein gB, the adverse structural effects it had within Gtm3gBG were abolished and it was incorporated into the viral envelope and was biologically active.

The mutant, gB $A_{786}N$, was unable to complement the null virus even though gB $A_{786}N$ and Gtm3gBG $A_{786}N$ were targeted efficiently to the nuclear envelope. Previous complementation assays with glycoprotein gB mutants revealed that glycoprotein gB hydrophobic domain deletion mutants, Δ1, Δ12, Δ2, were unable to complement a gB null virus even though these deletion mutants were targeted efficiently to the nuclear envelope (Rasile et al., 1993). The mutant glycoproteins inability to complement a gB-null virus may be because they were not incorporated into the viral capsid due to misfolding or due to the fact that sequences required for interaction with other viral proteins was lost. Another possibility is that the mutant proteins were incorporated into the HSV virion but were not biologically active.

It must be determined whether these glycoproteins containing point mutations within their hydrophobic regions, were incorporated into the viral envelope. If they were incorporated successfully, then perhaps these point mutations are affecting the ability of glycoprotein gB to mediate viral entry and /or cell fusion.

FUTURE DIRECTIONS

The creation of these point mutations within the hydrophobic regions of glycoprotein gB enables one to study the role of these specific amino acids in nuclear envelope targeting as well as their role in the life cycle of HSV-1.

Pulse-chase experiments must be performed in order to determine post-translational modifications, transport, secretion and degradation of newly synthesized proteins for each of the mutant glycoproteins. Determining the secondary structure of each of these mutant glycoproteins and chimeric proteins would evaluate whether a change in structure is the reason for their inability to target the nuclear envelope or complement a gB-null virus.

The inability of some of these mutants to complement a gB-null virus should be further analyzed. It must first be determined whether these mutant glycoproteins were incorporated into the virion particle. If they are incorporated, the fusiogenic ability of each of the mutants as well as their ability to mediate cell entry must be analyzed. If the mutant glycoproteins are not incorporated into the virion envelope, the reason for their inability to be incorporated must be resolved.

In order to further define the nuclear envelope targeting signal of glycoprotein gB distinct mutations need to introduced into this highly conserved domain. Mutations within the chimeric protein, Gtm3gBG, must also be studied in order to eliminate the neutralizing effects of the cytoplasmic domain, ectodomain, and first and second hydrophobic domains.

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APPENDICES

APPENDIX A: MOLECULAR CLONING

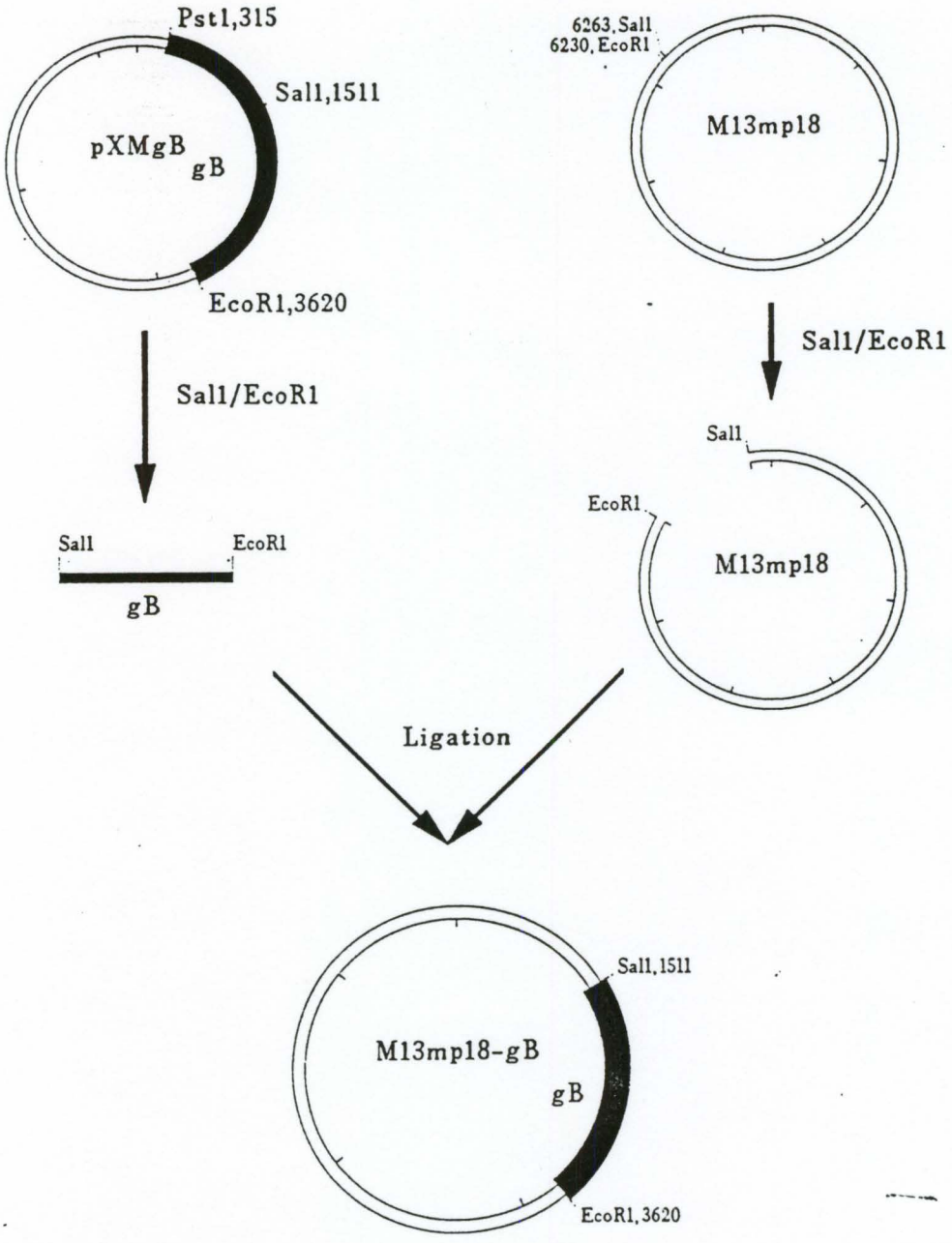


Figure A1: A schematic diagram representing the subcloning of HSV-1 glycoprotein gB into M13mp18

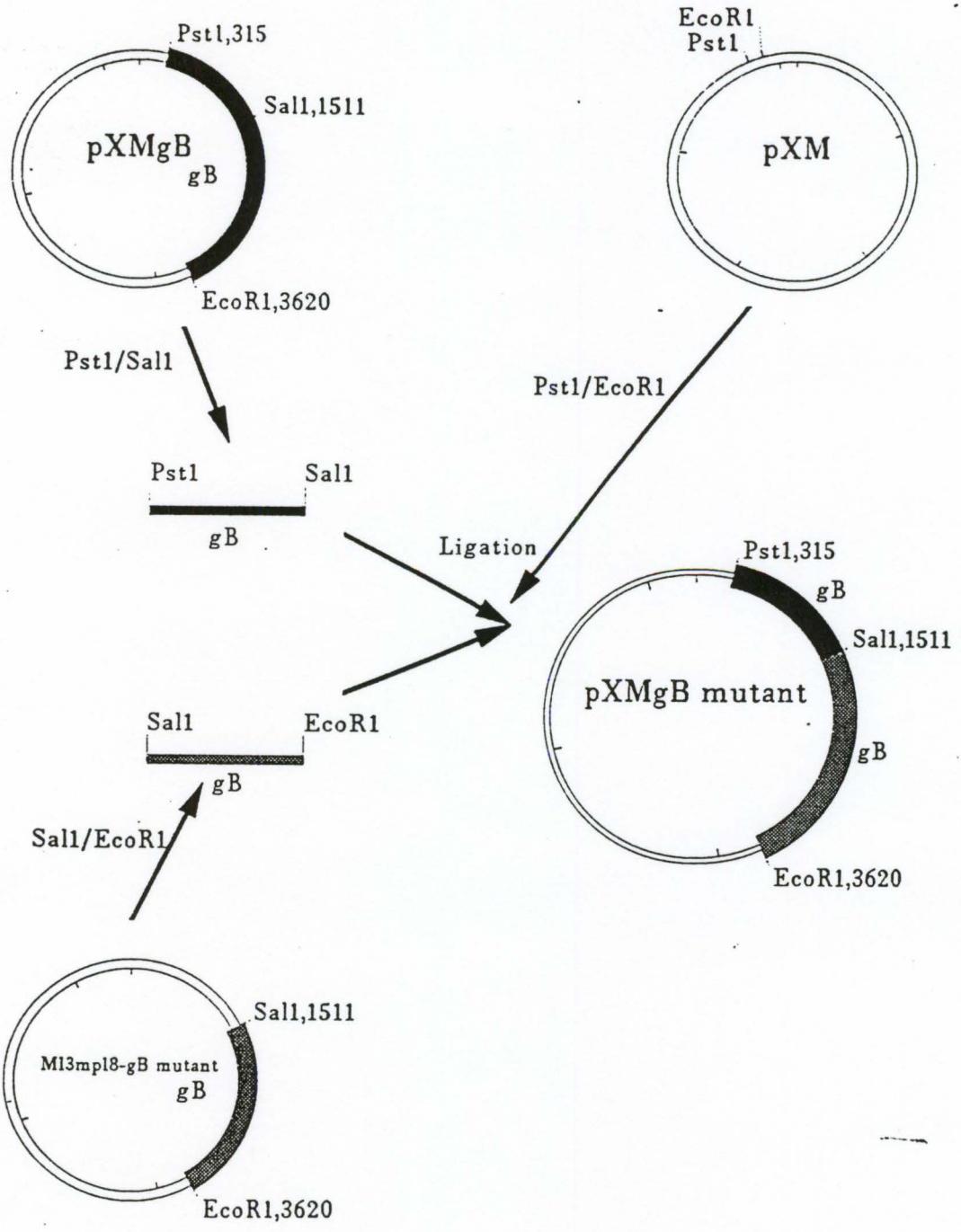


Figure A2: A schematic diagram representing the subcloning of M13mp18gB mutants into the expression vector, pXM.

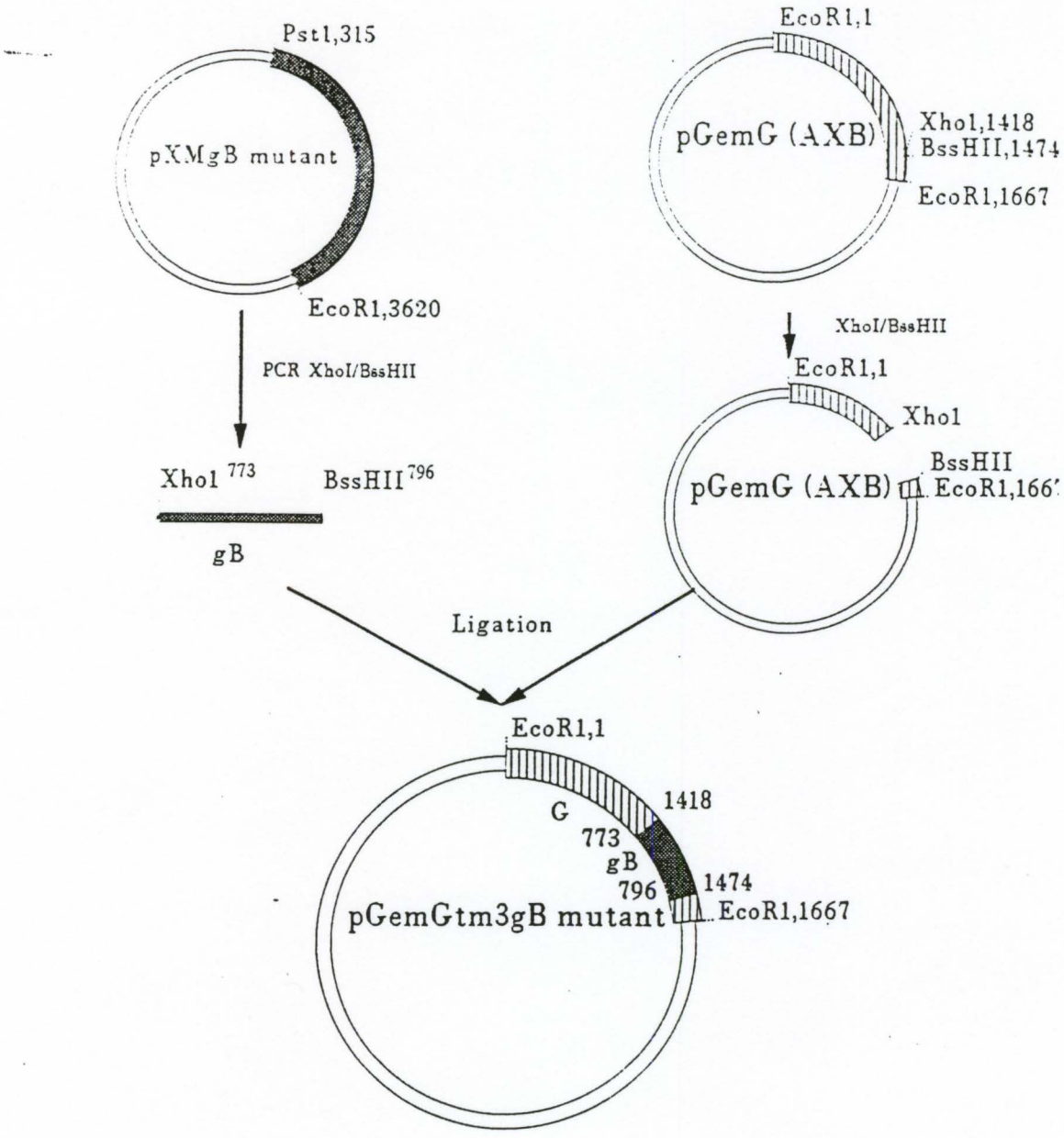


Figure A3: A schematic diagram representing the subcloning of pXM-gB mutants into pGemGtm3gBG by PCR.

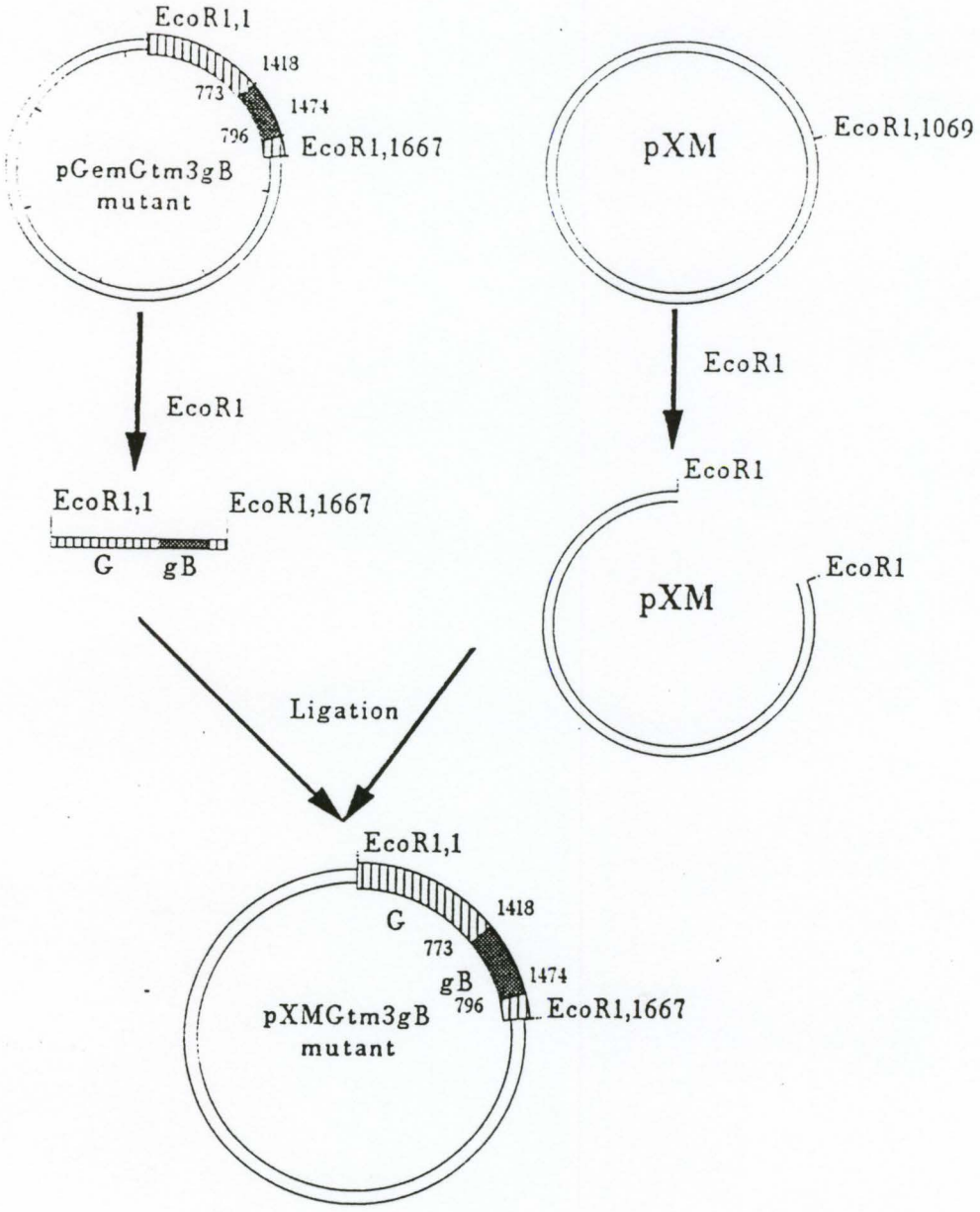


Figure A4: A schematic diagram representing the subcloning of pGemGtm3gBG mutants into pXMGtmgBG.

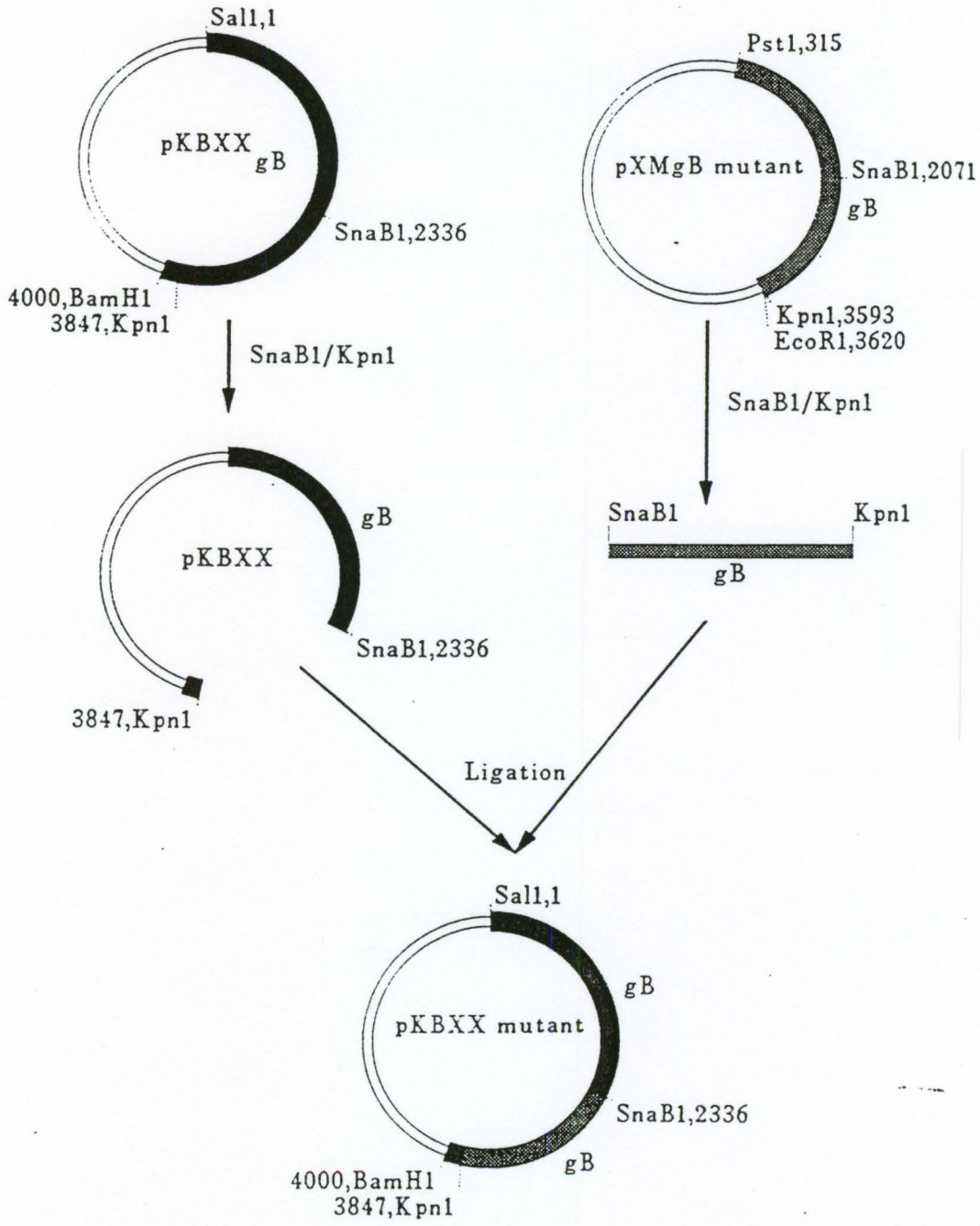
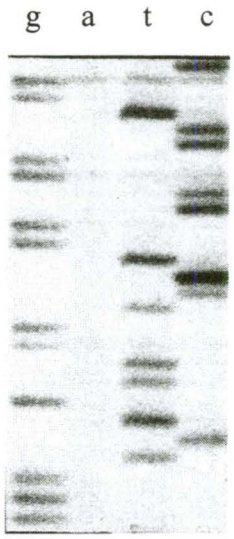
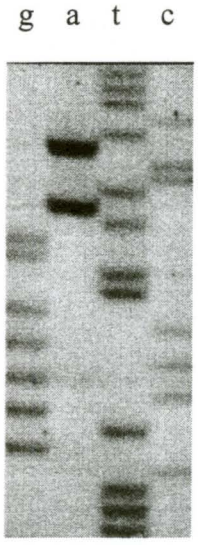


Figure A5: A schematic diagram representing the subcloning of the pXM-gB mutants into pKBXX.

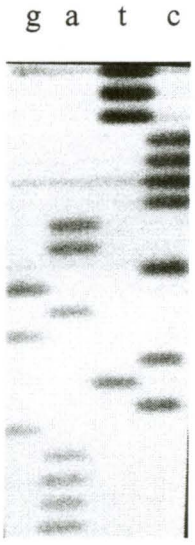
APPENDIX B: SEQUENCING RESULTS



Wt gB



Gtm3gBG 3'



Gtm3gBG 5'

Figure B1: Autoradiographs representing the sequence of HSV-1 glycoprotein gB (Wt gB), the 3' end of transmembrane domain of the chimeric mutant Gtm3gBG (Gtm3gBG 3') and the 5' end of the transmembrane domain of Gtm3gBG (Gtm3gBG 5').

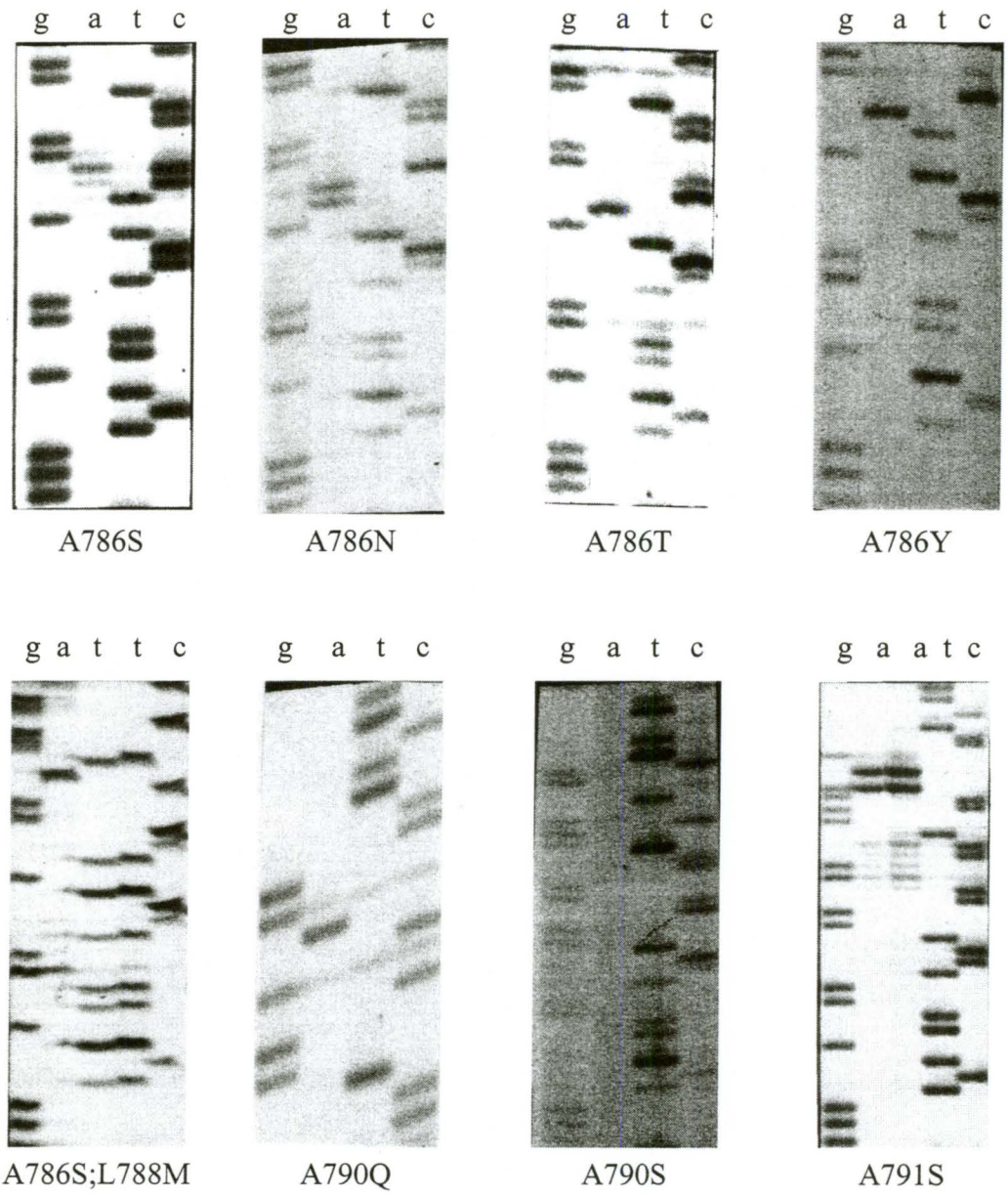


Figure B2: Autoradiographs showing the HSV-1 glycoprotein gB mutant sequences, $A_{786}S$, $A_{786}N$, $A_{786}T$, $A_{786}Y$, $A_{786}S;L_{788}M$, $A_{790}Q$, $A_{790}S$ and $A_{791}S$.

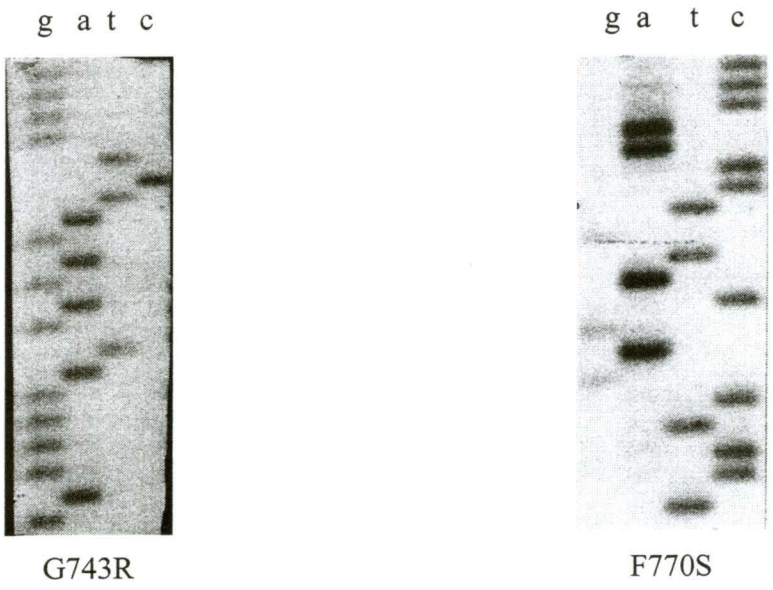


Figure B3: Autoradiographs showing the HSV-1 glycoprotein gB mutant sequences, $G_{743}R$ and $F_{770}S$.