LOCALIZATION TO THE NUCLEAR ENVELOPE AND TRANSPORT OF THE GLYCOPROTEIN B OF HERPES SIMPLEX VIRUS TYPE 1

By

RENALD GILBERT, B.Sc., M.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

NUCLEAR ENVELOPE LOCALIZATION OF GLYCOPROTEIN B

DOCTOR OF PHILOSOPHY (1993) McMASTER UNIVERSITY
(Biochemistry) Hamilton, Ontario

TITLE: Localization to the nuclear envelope and transport of the glycoprotein B of herpes simplex virus type 1

AUTHOR: Rénald Gilbert, B.Sc. (Université Laval)

M.Sc. (Université Laval)

SUPERVISOR: Dr. Hara P. Ghosh, Professor

NUMBER OF PAGES: xxi, 265

ABSTRACT

Herpes simplex virus type 1 (HSV-1), acquires its envelope by budding through a modified inner membrane of the nuclear envelope (NE) which forms thick and dense patches at the site of budding. This suggests that some of the viral envelope constituents, such as the transmembrane glycoprotein B (gB), are specifically transported to the NE in order to be incorporated into the virus. In this thesis, it is shown by immunoelectron microscopy that gB is specifically localized in the NE of HSV-1 infected cells. Glycoprotein B was distributed on the outer and inner membrane of the NE. The distribution was homogeneous and no preferential accumulation was detected around or within the patches where the viruses bud.

To determine which segments specify NE localization of gB, the distribution by immunofluorescence and by immunoelectron microscopy of deletion mutants and chimeras were investigated by transient expression in COS cells. The rate of transport and the oligomeric nature of the mutants were also studied. The chimeras were constructed by fusing domains of gB with domains of glycoprotein G of vesicular stomatitis virus, a transmembrane protein which does not accumulate in the NE. When the ectodomain (EC) of G was replaced with the EC of gB, the resulting chimera did not accumulate in the NE. On the other hand, all the mutants containing a peptide of 75 residues that encompasses the TM of gB were localized to the NE, suggesting that the TM contains the NE localization determinants. However, this domain does not appear

to be essential, because swapping the TM of gB for the TM of G did not abolish NE localization. Furthermore, gB and all the mutants that were localized to the NE were also present in the ER suggesting the presence of an ER localization signal as well.

The results presented in this thesis have appeared in part in the following publications:

Publication:

Gilbert, R., and Ghosh, H.P. (1993). Immunoelectron microscopic localization of herpes simplex virus glycoprotein gB in the nuclear envelope of infected cells. Virus Res. 28, 217-231.

Gilbert, R., Ghosh, K., Rasile, L., and Ghosh, H.P. (1994). Membrane anchoring domain of herpes simplex virus glycoprotein gB is required for nuclear envelope localization. J. Virol. (In press).

Abstracts and presentations at Scientific Meetings:

Ghosh, H.P., Gilbert, R., Ghosh-Choudhury, N., and Snoddy, D. Membrane glycoprotein compartmentalization: targeting of hybrid glycoproteins to nuclear membrane and cell surface. Proc. 19th Ann. Meet. UCLA Symp. Mol. Cell. Biol. p. 51, Taos, USA, Feb. 1990

Gilbert, R., Rasile, L., and Ghosh, H.P. Assembly of HSV glycoprotein in the nuclear envelope is specified by the transmembrane domain. Proc. 11th Ann. Meet. Ame. Soc. Virology., Ithaca, USA, July 1992

ACKNOWLEDGEMENTS

I am grateful to my supervisor Dr. Hara P. Ghosh for allowing me to work in his laboratory. I would like to point out his great availability and his enthusiasm about many aspects of my work. I would like to thank all the members of the laboratory of Dr. Ghosh for their help and friendship, which made my stay at McMaster more enjoyable.

I extend my appreciation to the staff of the electron microscopy facility for their excellent expertise. I enjoyed working in this well organized and efficient EM facility. I am indebted to my supervisory committee members, Dr. Richard Rachubinski, Dr. David Andrews and Dr. Ludwic Prevec for their constructive criticisms and helpful suggestions.

Special thanks to my friend Dr. James Cheetham who critically read portions of the manuscript. Finally, a special thought goes to Linda Holmes for her continual affection and support throughout the redaction of this thesis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ATP:	adenosine 5'-triphosphate
BFA:	brefeldin A
bp:	base pair
BSA:	bovine serum albumin
cDNA:	DNA complementary to RNA
CFU:	colony forming unit
CIP:	calf intestinal alkaline phosphatase
CT:	cytoplasmic domain
D:	dalton
dATP:	deoxyadenosine 5'-triphosphate
dCTP:	deoxycytidine 5'-triphosphate
ddATP:	dideoxyadenosine 5'-triphosphate
ddCTP:	dideoxycytidine 5'-triphosphate
ddGTP:	dideoxyguanosine 5'-triphosphate
ddNTP:	dideoxynucleotides
ddTTP:	dideoxythymidine 5'-triphosphate
dGTP:	deoxyguanosine 5'-triphosphate
dITP:	deoxyinosine 5'-triphosphate
DMSO:	dimethyl sulfoxide
D-MEM:	Dulbecco's modified Eagle's medium
DNA:	deoxyribonucleic acid
dNTP:	deoxynucleotides
DTT:	dithiotreitol
dTTP:	deoxythymidine 5'-triphosphate
EDTA:	ethylene-diaminetetra-acetic acid
EC:	ectodomain
Endo H:	β-endoglycosidase H
ER:	endoplasmic reticulum
FBS:	fetal bovine serum
g:	gram
g:	unit of gravity
G:	glycoprotein G of VSV
gB:	glycoprotein B of HSV-1
HEPES:	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
h:	hour
HSV-1:	herpes simplex virus type 1

IPTG:	isopropyl β-D thiogalactopyranoside
kD:	kilodalton
LBR:	lamin B receptor
M:	molar
mA:	milliampere
mCi:	millicurie
MES:	2-[N-morpholino] ethanesulfonic acid
min:	minute
mg:	milligram
ml:	milliliter
mm:	millimeter
mM:	millimolar
mmol:	millimole
MOI:	multiplicity of infection
mol wt:	molecular weight
MOPS:	3-[N-Morpholino] propanesulfonic acid
N:	newton
NE:	nuclear envelope
ng:	nanogram
OD:	optical density
PFU:	palque forming unit
pg:	picogram
PIPES:	piperazine-N,N-bis [2-ethanesulfonic acid]
pmol:	picomole
PM:	plasma membrane
PMFS:	phenylmethylsulfonide fluoride
PPO:	2,5-diphenyloxazole
RFI:	replicative form I of bacteriophage M13
RNA:	ribonucleic acid
RPM:	rotation per minute
$S_{20,w}$:	sedimentation coefficient at 20°C in water
SDS:	sodium dodecyl sulfate
sec:	second
TEMED:	N,N,N,N,-tetramethylethylenediamine
TM:	transmembrane domain
Tris:	tris(hydroxymethyl)aminomethane
U:	unit
μCi:	microcurie
μg:	microgram
μl:	microliter
μM:	micromolar

vol:	volume
VSV:	vesicular stomatitis virus
wt:	weight
X-gal:	5-bromo 4-chloro 3-indolyl β-D-galactoside

1.0 INTRODUCTION

1.1 Purpose of the present study

Eukaryotic cells are complex living entities divided into various compartments and containing a distinct set of organelles. Protein synthesis occurs in the cytoplasm, and a fundamental biochemical problem is to understand the mechanisms utilized by the cell to sort and to transport the different proteins to their specific sites of action. For example, plasma membrane proteins are integrated into cellular membranes and then transported to the plasma membrane, whereas mitochondrial and nuclear proteins, following their cytoplasmic synthesis, are transported to the mitochondria and the nucleus respectively. The information indicating where a protein is to be localized is contained within its primary structure. This information is decoded by the cell, which uses various strategies to transport the proteins where they belong.

An essential, plurifunctional and dynamic system of membranes, present in all eukaryotic cells, is the secretory pathway. This complex system of membranes is made up of the endoplasmic reticulum (ER), the Golgi complex, the plasma membrane (PM), the lysosomes, the endosomes and the nuclear envelope (NE). Except for the latter compartment, tremendous progress has been made during the past fifteen years concerning the signals and the mechanism used to sort and to transport proteins within the secretory pathway.

The NE is a double system of membranes which surrounds and isolates the cell nucleus from the cytoplasm. When the present work was undertaken in September 1988, little was known about protein targeting to this cellular compartment. What we knew was that synthesis of membrane protein can occur on the outer membrane of the NE (Puddington et al., 1985) and that some membrane proteins can move freely between the outer and inner membrane of the NE by diffusion at the level of a nuclear pore complex (Torrisi et al., 1987). The proteins of the lamina were the sole NE constituents relatively well characterized. No nuclear pore complex protein or specific NE membrane protein had been cloned, and no signal specifying NE localization had been demonstrated. It was not even clear if such a signal existed or was required to specify NE localization.

The main objective of this thesis was therefore to improve our limited knowledge about NE targeting. One way to shed light on this cellular process is to define and characterize the signal specifying the localization to the NE of residents of this compartment. Herpes simplex virus type I (HSV-1) is an envelope virus that acquires its envelope by budding through a modified inner membrane of the NE. Glycoprotein B (gB), which is one of the major components of the envelope of HSV-1, is specifically localized to the NE (Sections 1.4 and 1.5). This relatively well characterized membrane protein was therefore an excellent candidate to study NE targeting.

The approach used consisted of studying the transport of deletion mutants of gB and of chimeras to the NE. The chimeras were constructed by fusing different domains of gB with domains of glycoprotein G (G) of vesicular stomatitis virus (VSV). G was

chosen as a reporter protein, because it is a well characterized cell surface protein and because it does not accumulate in the NE. With this system, I was hoping to show that NE targeting is not different than targeting to other cellular compartments, in a sense that specific signals are required, and I wanted to characterize this NE localization signal.

The present chapter begins with a description of the secretory pathway and of the mechanisms used by the cellular machinery to transport and sort proteins within this complex system of membranes which includes the NE. The life cycle of HSV-1, with special emphasis on the viral envelopment and the biogenesis and transport of gB, is also discussed. The introduction ends with a presentation of our current knowledge concerning the structure, post-translational modifications and transport of G.

1.2 The secretory pathway

Based on their fate after synthesis in the cytoplasm, proteins can be categorized into two groups: proteins targeted to the secretory pathway and those which are not targeted to this complex system of membranes. The latter category includes cellular cytosolic proteins, mitochondrial and chloroplastic proteins, proteins targeted to the peroxisomes and to the cell nucleus. The sorting mechanism of members of the latter group will not be discussed and readers interested in protein targeting to these cellular organelles should consult recent reviews (Andrews and Rachubinski, 1990; Silver, 1991; de Boer and Weisbeek, 1991; Goldfarb, 1992; Pfanner et al., 1992; Glick et al., 1992).

The secretory pathway is a complex and dynamic system of membranes and recent

biochemical evidence indicates that the different compartments making up the secretory pathway are closely linked with each other by a constant and dynamic exchange of vesicles (see Fig. 1.2.7). Proteins enter the secretory pathway by specific synthesis on the ER membranes. It is now well recognized that a degenerate segment of hydrophobic amino acids which makes up the core of the signal sequence and the signal/anchor sequence (see below), is the main determinant specifying targeting to the secretory pathway (Kaiser et al., 1987; von Heijne and Gavel, 1988; Haeuptle et al., 1989; Kaiser and Botstein, 1990).

1.2.1 The signal sequence

Secretory proteins and most membrane proteins possess a specific sorting determinant at their amino terminus (the signal sequence), which directs their synthesis and translocation in the ER (reviewed in von Heijne, 1988; Walter and Lingappa, 1986). The signal sequence is a segment of 13 to 30 residues located at the amino terminus of the newly synthesized protein, which is then cleaved in the ER by signal peptidase. Comparison of the primary structure of different signal sequences has led to their dissection into 3 regions: the amino terminus (N region) composed of one or more positively charged amino acids, followed by a stretch of hydrophobic residues (H region) that ends with a segment made of more polar residues (C region). The length of the C region, which is about 5 amino acids, is less variable than the N and H regions which can range from 1 to 17 and from 7 to 16 residues respectively (von Heijne, 1985). No

conserved amino acid segment determines the site of cleavage. However, a comparison among eukaryotic signal sequences indicates that the last residue of the signal sequence (-1) and the third residue upstream from the cleavage site (-3), are strongly selected for small uncharged amino acids such as alanine and serine (von Heijne, 1983).

1.2.2 Targeting and translocation in the ER

Excellent reviews exist concerning our current knowledge of the first steps of ER targeting and of the components making up the translocation sites (Walter and Lingappa, 1986; Andrews and Rachubinski, 1990; Lingappa, 1991; Rapoport, 1992; Sanders and Schekman, 1992). For this reason, only the most important steps of this fundamental biological process are briefly presented here.

Protein synthesis is a vectorial process beginning at the amino terminus and ending at the carboxy terminus. During synthesis, the signal recognition particle (SRP), by binding to the newly made and emerging signal sequence, halts or slows down polypeptide synthesis. The SRP is a rod shaped particle of 25 x 5 nm (Andrews et al., 1985) composed of six different polypeptides and one molecule of RNA (Walter and Lingappa, 1986). The interaction with the signal sequence or any specific segments fulfilling the criteria of a signal sequence or signal/anchor sequence (see below) is accomplished by a methionine rich hydrophobic groove on the surface of the SRP (Bernstein et al., 1989; High and Dobberstein, 1991).

Following binding with SRP, the SRP-ribosome complex is targeted to the ER and

becomes anchored on the ER membrane by association with the SRP receptor, an integral membrane protein composed of two subunits. The ribosome is then docked on the ribosome receptor, with the concurrent release of the SRP. Protein synthesis is resumed and the nascent polypeptide chain is translocated into the ER. Evidence exists indicating that translocation occurs through an aqueous proteinaceous channel (Simon and Blobel, 1991). At least 6 different proteins are believed to make up the mammalian translocation sites (reviewed in Lingappa, 1991; Rapoport, 1992; Sanders and Schekman, 1992). Among these, the signal peptidase, which cleaves the signal sequence, and oligosaccharide transferase, an enzyme involved in N-linked glycosylation, are probably the best characterized. The mechanism of translocation is unknown and the precise functions of the proteins associated with the translocation sites are not well defined. The overall organization of the translocation sites is also unknown and it is not clear if the translocation sites exist on their own or if they are assembled solely during translocation.

1.2.3 Membrane proteins and their topology

Integral membrane proteins are anchored in the membrane because of the existence in their primary structure of stretches of hydrophobic amino acids that stop translocation and remain integrated within the membrane, thus forming transmembrane domains (TM). Depending on their topology and their insertion mechanism, integral membrane proteins can be classified into four groups (von Heijne, 1988; von Heijne and Gavel, 1988). Type I span the membrane only once, have their amino and carboxy

termini facing the ER and the cytoplasm respectively, and possess a typical signal sequence (Fig. 1.2.3). Type II span the membrane once, but possess an orientation opposite to Type I. Type III possess the same orientation as Type I, but lack a typical signal sequence. Finally, Type IV span the membrane more than once and can have their amino and carboxy termini facing the ER or the cytoplasm.

Secretory proteins and Type I membrane proteins are targeted to the ER and translocated into this organelle, because they possess a typical signal sequence at their amino terminus. In the case of Type I membrane proteins, a short segment of 15 to 25 hydrophobic residues (stop/transfer signal) down stream from the signal sequence, halts translocation and anchors the protein in the membrane. Charged amino acids, which increase the stability, are usually found on both sides of the stop/transfer signal (reviewed in Jennings, 1989; Singer, 1990).

Type II membrane proteins can be viewed as proteins in which the signal sequence is not cleaved. The protein is thus anchored in the membrane by the signal sequence which performs the dual function of a signal/anchor sequence (von Heijne, 1988; Singer, 1990). Evidence exists indicating that a typical signal sequence can be converted into a signal/anchor sequence by disruption of the cleavage site, and that a type II membrane protein can become a secretory protein by inserting or unmasking a cleavage site (Lipp and Dobberstein, 1986; Haeuple et al., 1989; Sakaguchi et al., 1992).

Type III membrane proteins are targeted and anchored in the ER membrane by a signal/anchor sequence, that is however inserted in an orientation opposite to the

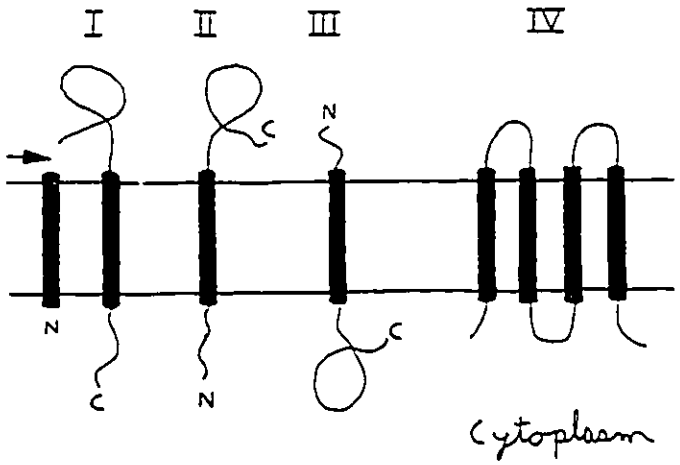

Figure 1.2.3. Classification of integral membrane proteins. The signal sequence and the transmembrane domain are black. The arrow indicates cleavage of the signal sequence. Adapted from von Heijne and Gavel (1988).

signal/anchor sequence of Type II. A current challenging problem is to find the determinants specifying the orientation of Type II and Type III membrane proteins, since the only obvious characteristic of their signal/anchor sequences is the presence of a hydrophobic stretch of amino acids. Evidence exists indicating that one of the main topologic determinants is the presence of positive residues on the cytoplasmic side of the signal/anchor sequence (von Heijne, 1988; reviewed in Boyd and Beckwith, 1990). It has been hypothesized that positively charged residues are more difficultly translocated across the bilayer than negative residues. Consequently, the topology of Type II and Type III membrane proteins could be governed by the charge distribution around their hydrophobic core: more positively charged segments being localized on the cytoplasmic side. This model has been tested successfully by some researchers, who have inverted the orientation of a membrane protein by modification of the charges around the hydrophobic core of the signal/anchor sequences (Haeuptle et al., 1989; Sato et al., 1990; Parks and Lamb, 1991; Sakaguchi et al., 1992). However other factors, such as the amino acid composition of the adjacent segments, also influence the topology (Andrews et al., 1992). It has also been observed that Type III membrane proteins are favoured by longer hydrophobic segments (Hong and Doyle, 1990; Sato et al., 1990; Sakaguchi et al., 1992).

The topology of type IV membrane proteins is generally believed to result from the consecutive insertion of TM, acting alternatively as signal/anchor and stop/transfer sequences (Singer, 1990). Positively charged amino acids are usually more abundant on

the cytoplasmic side of the TM of Type IV membrane proteins (von Heijne and Gavel, 1988). Several experiments suggest a direct involvement of these positive residues in determining the protein topology of Type IV membrane proteins (von Heijne, 1989; Yamane et al., 1990; Kuroiwa T. et al., 1991; McGovern et al., 1991; Andersson et al., 1992). However, there are some exceptions (Jennings, 1989; Locker et al., 1992). The insertion mechanism of the successive signal/anchor and stop transfer sequences remains unknown. Clearly, more research is needed to understand this fundamental biochemical process.

1.2.4 Structures and function of the ER

The ER appears under the light and electron microscope as an interconnected network of tubules and flatten cisternae. Depending on the method used to visualize the ER and on the cell type under investigation, the relative proportion of cisternae and tubules is quite variable (Fawcett, 1981; Song et al., 1991). The ER is a dynamic membranous system and impressive studies with living cells, have demonstrated that ER tubules slide along microtubules, fused with each other and can be absorbed by preexisting tubules (Lee and Chen, 1988). The ER can be further divided into two functionally and morphologically distinct but continuous substructures: the rough ER which is studded with ribosomes and the smooth ER devoid of ribosomes. The rough ER is mainly involved in protein synthesis and post-translational modifications, whereas the smooth ER, which is generally made of shorter but highly interconnected tubules, is

thought to correspond to regions of exit of newly synthesized proteins and of lipid metabolism (Fawcett, 1981).

The functions of the ER are numerous. They include synthesis and transport of proteins, N-linked glycosylation and possibly O-linked glycosylation (Kornfeld and Kornfeld, 1985; Roth, 1987; Perez-Vilas, 1991), protein degradation (Klausner and Sitia, 1990), and synthesis of phospholipid and cholesterol (Dawidowicz, 1987).

1.2.5 Structure and function of the Golgi complex

Several reviews have been published concerning the structure and functions of the Golgi complex (Farquhar and Palade, 1981; Simons and Griffiths, 1986; Roth, 1987; Rambourg and Clermont, 1990; Mellman and Simons, 1992). Consequently, I will limit myself to discuss the major characteristics and the most recent findings about this organelle. A model of the Golgi complex has been recently proposed in which this elaborate structure of smooth cisternae and tubules is divided into three functionally and morphologically distinct compartments namely the cis-Golgi network, the Golgi stacks and the trans-Golgi network (Mellman and Simons, 1992; see also Rothman and Orci, 1992; Pryer et al., 1992).

In contrast with the ER, which spreads its tubular network over the entire cytoplasm, the Golgi complex is a relatively compact structure close to the nucleus, which can sometimes appear as a complex network of cisternae and tubules surrounding the nucleus (Rambourg and Clermont, 1990). The functions of the Golgi complex are

numerous and they include: the transport and sorting of proteins targeted to the plasma membrane, lysosomes and endosomes (Farquhar and Palade, 1981; Mellman and Simons, 1992); the processing of N-linked and O-linked glycosylation (Johnson and Spear, 1983; Kornfeld and Kornfeld, 1985; Roth, 1987; Tooze et al., 1988); fatty acylation of proteins (Bonatti et al., 1989); and synthesis of glycolipids (Dawidowicz, 1987).

One of the main characteristics of the Golgi complex is its polarity. The different compartments and subcompartments making up the Golgi complex are lined up in a cis to trans direction. Proteins and lipids synthesized in the ER enter and exit the Golgi complex from its cis and trans faces respectively. During their journey, they visit each Golgi subcompartment in a cis to trans direction. The proteins are thus processed sequentially by the different set of enzymes enclosed by each of these subcompartments.

1.2.5.1 The cis-Golgi network

This ill defined compartment, also known as the intermediate or the salvage compartment, receives the proteins and lipids synthesized in the ER before their subsequent movement to the Golgi stacks (Pelham, 1989; Mellman and Simons, 1992; Pryer et al., 1992). The cis-Golgi network was described by Saraste and Kuismanen (1984) as the structure where a viral glycoprotein accumulates following a block of transport by incubation at 14°C. At the level of the light microscope, the cis-Golgi network appears as a set of perinuclear vesicles in close association with the Golgi stacks (Lippincott-Schwartz et al., 1990; Saraste and Svensson, 1991; Huovila et al., 1992;

Tang et al., 1993). At the ultrastructural level, it is composed of a tight network of vesicles and tubules adjacent and facing the cis-face of the Golgi stacks (Saraste and Svensson, 1991; Mellman and Simons, 1992; Lotti et al., 1992; Tang et al., 1993).

One of the main functions of the cis-Golgi network is to return back to the ER the proteins that have escaped the ER retention mechanism, because they were nonselectively incorporated into the transport vesicles continuously budding from the ER (Pelham 1989; Mellman and Simons, 1992; see also Section 1.2.10.2). Other biochemical functions, such as the first step of O-linked glycosylation and fatty acylation have also been assigned to the cis-Golgi network (Tooze et al., 1988; Bonatti et al., 1989). A clear demonstration that the cis-Golgi network is distinct from the ER and other Golgi compartments was provided by Schweizer et al. (1991) whom, after isolation and purification of the cis-Golgi network, showed that it possesses a unique morphology and composition.

1.2.5.2 The Golgi stacks

This compartment, which is mainly involved in the processing of glycoproteins and glycolipids, consists of several stacks of flattened cisternae (Mellman and Simons, 1992). The cisternae are connected by short tubules with the homologous cisterna of an adjacent stack (Roth, 1987; Rambourg and Clermont, 1990; Mellman and Simons, 1992). Although the exact number of cisternae in a stack is quite variable, it is common to classify them as belonging to the cis-, medial- and trans- Golgi according to their relative

position within the stack. Cisternae of the cis-Golgi are involved in early glycosylation events such as processing by α-mannosidase I, whereas those of the trans-Golgi are involved in late events such as the terminal transfer of galactose residues (Kornfeld and Kornfeld, 1985; Roth, 1987).

1.2.5.3 The trans-Golgi network

This organelle appears under the electron microscope as a network of tubules derived from the trans-face of the Golgi stacks (Rambourg and Clermont, 1990). The trans-Golgi network is distinct from the other Golgi compartments, because it is composed of a particular set of proteins and because it behaves differently following treatment with brefeldin A (Chege and Pfeffer, 1990; Reaves and Banting, 1992; Section 1.2.7). Two major functions have been associated with the trans-Golgi network. First, it is the site where proteins targeted to the plasma membrane, secretory granules, endosomes and lysosomes are sorted from each other and packed into specific transport vesicles (Simons and Griffiths, 1986; Mellman and Simons, 1992). Second, it participates in the recycling of plasma membrane receptors that were internalized by endocytosis (Duncan and Kornfeld, 1988).

1.2.6 Glycosylation in the secretory pathway

During their transport to the plasma membrane, some proteins are glycosylated on specific asparagine residues (N-linked glycosylation) or on specific serine and

threonine residues (O-linked glycosylation). The initial attachment of oligosaccharides to asparagine residues occurs during translation as the growing polypeptide chains emerge in the lumen of the ER (reviewed in Kornfeld and Kornfeld, 1985; Roth, 1987). Glycosylation begins with the transfer of a preassembled oligosaccharide chain termed the high mannose core oligosaccharide, because 9 of its 14 units consist of mannose residues. Sequence analysis of numerous glycoproteins have indicated that the glycosylated asparagine is almost exclusively found in the context of the tripeptide ASN-X-SER/THR where X corresponds to any amino acids except for proline and aspartic acid. Only 1/3 of the potential asparagine residues are glycosylated, most likely because many of them, although in the right context, are not accessible to the oligosaccharide transferase during protein folding.

Following attachment, the high mannose core oligosaccharides are modified by digestion of the terminal glucose and mannose residues in the ER and also during transport in the Golgi complex. In the latter organelle, the processing is accomplished by various glycosidases and glycosyl transferases located within specific Golgi subcompartments. The Golgi enzymes act subsequently on the glycoproteins as they traverse the Golgi complex in a cis to trans direction. Examples of such modifications are digestion of mannose residues by a cis-Golgi mannosidase, addition of acetylglucosamine in the med-Golgi, and addition of fucose and sialic residues in the trans-Golgi. The oligosaccharide-containing terminal sialic and fucose residues are commonly referred to as the complex type, as opposed to the high mannose core

oligosaccharide, which belongs to the simple type.

O-linked glycosylation however, begins after completion of protein synthesis (reviewed in Roth, 1987). There are considerable disagreements concerning the site where the initial glycosylation step occurs, since this event has been localized to an early Golgi compartment (Roth, 1987; Tooze et al.,1988) or in the ER (Perez-Vilas, 1991). The initial event involves the addition of N-acetylgalactosamine to a serine or a threonine residue. Glycosylation then proceeds in the Golgi complex by addition of galactoses, sialic acids and sometimes other sugars such as fucose, N-acetylglucosamine and N-acetylgalactosamine. No signal for O-linked glycosylation has been found and the determinants specifying glycosylation of selected serines and threonines remain to be discovered.

1.2.7 The transport machinery

Growing evidence suggests that lipids and proteins are transported within the secretory pathway by means of coated vesicles of 80-100 nm diameter (Lodish, 1988; Rothman and Orci, 1992; Pryer et al., 1992). The formation and transport of these coated vesicles is a complex process involving the following steps: assembly of a proteinaceous coat around a budding vesicle, transport and targeting of the newly formed vesicles to the correct membrane, uncoating, and fusion followed by transfer of the cargo. The formation, transport and fusion of these vesicles is tightly regulated by a number of GTP binding proteins that function either to amplify a signal required for a

subsequent steps in the vesicular process, or to confer specificity to the targeting and fusion machinery.

The coat is assembled on the membrane from a cytoplasmic pool of proteins. According to the composition of their coats, transport vesicles can be classified into two major groups. First, the well characterized clathrin vesicles, which mediate the selective transport of lysosomal and endosomal proteins, and endocytosis (reviewed in Pearse and Robinson, 1990). Second, the nonclathrin vesicles, composed of at least 7 distinct coat proteins, which mediate the nonselective transport from the ER to the plasma membrane (Rothman and Orci, 1992; Pryer et al., 1992).

Experiments with brefeldin A (BFA), an antiviral antibiotic, have demonstrated the existence of a recycling pathway between the Golgi and the ER, and between the trans-Golgi network and the endosomal/prelysosomal compartment (reviewed in Pelham, 1991; Klausner et al., 1992). When cells are treated with BFA, a relocation of the constituents of the Golgi stacks into the ER and a relocation of constituents of the trans-Golgi network into the endosomes are observed (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989; Lippincott-Schwartz et al., 1991; Wood et al., 1991). This atypical movement of proteins and lipids is mediated by tubulovesicular processes and uncoated vesicles (Lippincott-Schwartz et al., 1990; Wood et al., 1991).

One of the first actions of BFA is to block the formation of the coated vesicles by preventing the assembly of the coat on the membrane (Donaldson et al., 1990; Orci et al., 1991). The association of the coat proteins with the membrane requires the activity

of a low molecular GTP binding protein (ARF). BFA mediates its action by inhibiting an enzyme involved in the activation of ARF (Donaldson et al., 1992; Helms and Rothman, 1992). The overall effect of BFA can be easily explained if we assume a bidirectional movement of lipids and proteins between the ER and the Golgi complex, and between the trans-Golgi network and the endosomes (Fig. 1.2.7). BFA would abolish the transport in only one direction, the one mediated by the coated vesicles, resulting in the mixing of components between the ER and Golgi, and between the trans- Golgi network and endosomes.

Interestingly the distribution of the components of the cis-Golgi network is not overly altered by treatment with BFA (Lippincott-Schwartz, 1990; Saraste and Svensson, 1991; Tang et al., 1993). Cycling between the ER and the cis-Golgi network has been described after treatment with BFA, suggesting a different mechanism of transport between the ER and the cis-Golgi network (Lippincott-Schwartz, 1990). Similarly, transport between the endosomes and the PM is not affected by BFA (Lippincott-Schwartz et al., 1991; Wood et al., 1991; Miller et al., 1992).

The conspicuous tubulovesicular processes that mediate the relocation of Golgi constituents during BFA treatment are not observed under normal conditions. However, many researchers agree that these tubulovesicular processes correspond to a normal and alternative transport pathway, which is greatly amplified following treatment with BFA (Klausner et al., 1992). In agreement with this hypothesis is the fact that analogue tubular processes were described connecting and mixing adjacent Golgi elements under normal

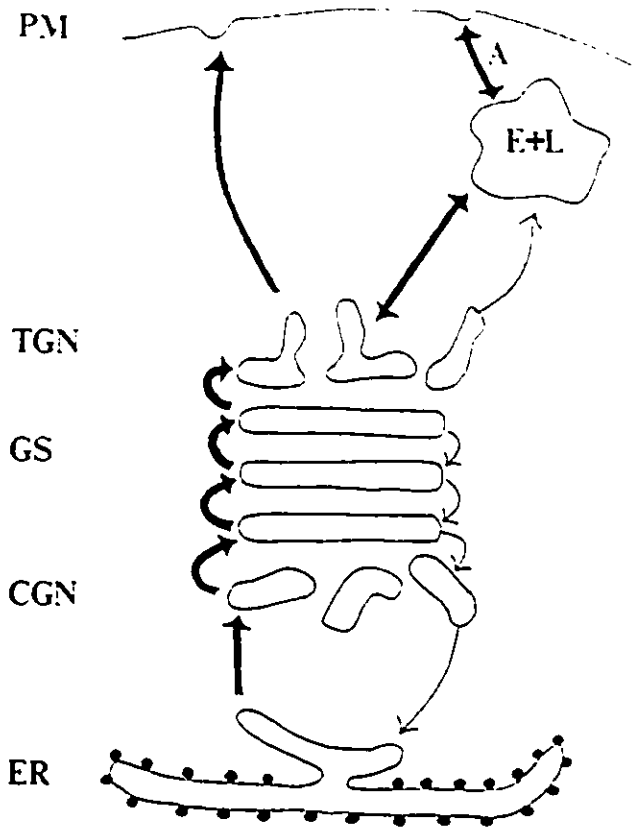

Figure 1.2.7. Transport within the secretory pathway. Transport between the different compartments of the secretory pathway is mediated by coated vesicles (thick arrows) and by noncoated vesicles or tubules (thin arrows). Treatment with BFA abolishes the transport mediated by the coated vesicles except between the endosomes and the plasma membrane (A). CGN: cis-Golgi network; E+L endosomes and lysosomes; ER: endoplasmic reticulum; GS: Golgi stacks; PM: plasma membrane; TGN: trans-Golgi network. Adapted from Saraste and Kuismanen (1991); Wood et al. (1991); Lippincott-Schwartz et al. (1990).

conditions (Cooper et al., 1990) or following treatment that blocks the association of the coat proteins with the membrane (Cluett et al., 1993).

1.2.8 The default pathway

Transport from the ER to the Golgi complex and plasma membrane, is generally assumed to occur by default. That is, proteins exit the ER and end up at the plasma membrane or outside the cell unless they contain a specific targeting signal, and there is much evidence to support this model. After more than 10 years of intensive research no specific signal for transport to the cell surface has been found. ER and Golgi resident proteins are targeted to these organelles by specific retention signals (Sections 1.2.10 and 1.2.11). Destruction of these retention signals results in transport to the cell surface. Lysosomal, endosomal and constituents of secretory granules escape the bulk flow at the level of the trans-Golgi, because they posses specific targeting signals (Section 1.2.12).

1.2.9 The rate of bulk flow

Experiments have been undertaken to measure the speed by which a certain component is nonselectively transported to the cell surface by the constant flow of membranes originating from the ER. Two markers were used for these studies. In one case, the membrane flow was monitored using a small tripeptide encoding a glycosylation site. Glycosylation of the peptide and processing of the oligosaccharides, as well as secretion into the medium, were used to follow its course within the cell (Wieland et al.,

1987). In the second case, integral membrane components, such as modified sphingomyelins or radiolabeled glycosphingolipids, were used as markers for the rate of bulk flow (Karrenbauer et al., 1990; Young et al., 1992). As predicted by the model, these markers were transported efficiently and relatively rapidly from their site of synthesis in the ER or Golgi to the cell surface, with an average time between 10 and 20 min. They were hence transported at a speed that was either slightly higher or similar to the faster moving proteins analyzed so far.

One of the major criticisms of the bulk flow is that different proteins are transported at different rates to the plasma membrane. According to the bulk flow model, the transport vesicles move at a similar rate and therefore, the same properties should be applied to the components they transport. The empirical rate of protein transport is however quite variable. For example, the plasma membrane is reached by some proteins within 15 min following synthesis, whereas other proteins are transported to the cell surface with a half time of more than 3 h (Rose and Doms, 1988). As pointed out by Pfeffer and Rothman (1987), this is not a real problem, because the rate of transport is also dependent upon several factors such as folding, oligomerization and post-translational modifications, which vary greatly among different proteins. The transport rate is also dependent upon the various interactions encountered by a protein during its journey to the cell surface.

1.2.10 Regulation of export from the ER

1.2.10.1 The system of quality control

Although no positive signal seems required for transport out of the ER, this process is highly regulated, because unassembled oligomers and misfolded proteins do not usually leave the ER (reviewed in Rose and Doms, 1988; Pelham, 1989; Hurtley and Helenius, 1989). In the ER, protein folding occurs during translation as the growing polypeptide chain emerges in the ER lumen. Folding is a complex and not well understood process requiring ATP (Braakman et al., 1992) and the action of specific enzymes such as protein disulphide isomerase and peptidyl prolyl isomerase, and the presence of chaperons (reviewed in Gething and Sambrook, 1992). At least three molecular chaperons, BiP, GRP94, and IP90, have been found in association with folding intermediates in the ER, and the role of BiP is by far the best documented (Gething and Sambrook, 1992; Hochstenbach et al., 1992; Melnick et al., 1992). The main function of BiP is to prevent the inappropriate interaction of unfolded polypeptide stretches with other protein segments, which could promote misfolding or aggregation. The action of BiP is mediated by cycles of binding and release which require ATP (Gething and Sambrook, 1992).

Misfolded proteins are produced during folding in the ER under normal conditions (Gething et al., 1986; Hurtley et al., 1989; Kim et al., 1992; De Silva et al., 1993). Misfolded proteins are also generated in large amounts under stress conditions such as heat shock or glucose deprivation, or after inhibition of glycosylation. Many

chimeric proteins, deletion or point mutation mutants also do not fold properly (Hurtley and Helenius, 1989; Pelham, 1989; Rose and Doms, 1988). Misfolded proteins are usually retained in the ER and are often degraded within this organelle, since their normal expression could have deleterious effects (Klausner and Sitia, 1990).

Misfolded proteins and incomplete oligomers can be retained in the ER by three different mechanisms. Often these proteins become associated into large protein aggregates that can attain a size of several million daltons (Doms et al., 1988; Hurtley and Helenius, 1989; Singh et al., 1990; Marquardt and Helenius, 1992). The proteins in these aggregates often contain interchain disulphide bonds and are usually associated with BiP. The aggregates do not exit the ER, most likely because they are to large to be incorporated into the transport vesicles (Pfeffer and Rothman, 1987; Rose and Doms, 1988).

Many misfolded proteins or incomplete oligomers do not form aggregates and are retained in the ER, because they remain continuously associated with BiP (Gething et al., 1986; Pelham, 1989; Knittler and Haas, 1992). These proteins are thus indirectly retained in the ER, because they are bound to ER residents (BiP) which possess a positive ER retention signal (Section 1.2.9).

The third mechanism is exemplified by few mutants that do not form aggregates and that are not physically associated with BiP (Machamer et al., 1990a; Gething and Sambrook, 1992). It is not understood why these proteins do not leave the ER, but the inhibition of transport is most likely the result of binding with some ER residents that are

not characterized or with some components of the cytoskeleton. These mutants could also possess an abnormal conformation that blocks incorporation into the transport vesicles.

1.2.10.2 Signal mediated retention in the ER

Soluble proteins

Many soluble ER resident proteins possess the tetrapeptide KDEL, or a related sequence, at their carboxy terminus. Truncation or mutation of this motif results in slow secretion. On the other hand, addition of this conserved sequence to the carboxy terminus of secretory proteins abolishes their secretion and generates proteins that remain in the ER, indicating that the KDEL motif is both essential and sufficient for ER localization (reviewed in Pelham, 1989; Pelham, 1990).

Secretory proteins that were converted into ER residents by addition of a KDEL motif, are also modified by early Golgi enzymes, suggesting that they are transiently transported to this compartment (Pelham, 1987). This observation led to the hypothesis that the main function of the KDEL motif is not to impair movement out of the ER, but instead to promote the recycling to the ER of proteins that have escaped this compartment. In this model, the ER proteins are transported to the cis-Golgi network or the Golgi stacks by default, because they are trapped, like most proteins, in the transport vesicles exiting the ER. In the Golgi complex however, the KDEL bearing proteins are specifically recognized by a membrane protein, the KDEL receptor. The newly formed complex is then recycled back to the ER, most likely using the retrograde flow of

membrane made obvious by treatment with BFA (Section 1.2.7; Pelham, 1989; Pelham, 1990; Lewis and Pelham, 1992).

ER resident proteins are secreted very slowly after destruction of their KDEL motif in comparison with secretory or plasma membrane proteins. For example, the truncated BiP is secreted with a half time of 3 h (Munro and Pelham, 1987), whereas many proteins are transported to the cell surface with a half time between 15 to 30 min (Rose and Doms, 1988). This suggests that ER residents possess some unknown characteristic which impairs their efficient transport.

Membrane proteins

Integral ER membrane proteins remain specifically associated with the ER because they possess specific retention signals. Destruction of these retention signals results in transport to the plasma membrane, most likely by passive incorporation into the transport vesicles. One of the first characterizations of the retention signal of an ER membrane protein was accomplished by studying the protein VP7 of rotavirus. VP7 is targeted to the ER by the presence of a signal sequence which is cleaved normally during protein synthesis. Interestingly, the cleaved signal sequence in conjunction with the first 60 residues of VP7 are essential for membrane anchoring and ER retention (Poruchynsky and Atkinson, 1988; Stirzaker and Both, 1989; Stirzaker et al., 1990). Membrane anchoring and retention of VP7 are mediated by the association of the signal sequence with the first 60 residues of VP7. Deletion of one of these two regions results in the

secretion of the protein.

The ER retention signal of some type I membrane proteins is localized in their cytoplasmic domains (CT). It has been shown that the last six residues of the short CT of adenovirus E3/19K glycoprotein are both necessary and sufficient for ER retention (Nilsson et al., 1989). Furthermore, extensive and very elegant mutagenesis studies showed that two lysines, one located three residues (position -3) and the other one four or five residues (position -4 or -5) from the carboxy terminus, were the only requirement to confer retention in the ER (Jackson et al., 1990). Similar conclusions were reached by Shin et al. (1991), who studied the ER retention of a truncation mutant of CD4. They observed that arginine residues could replace, without disturbing retention, the lysines at position -4 and -5 but not at position -3. The consensus sequence was thus defined as KKXX, KXKXX, RKXX and RXKXX, X being any amino acid. A search in the gene data bank has confirmed this hypothesis, since each of the 15 membrane proteins possessing one of these motifs is a resident of the ER.

A different retention motif was recently described in the CT of a type I membrane protein (Mallabiabarrena et al., 1992). When expressed alone, the polypeptide chains constituting the T-cell antigen receptor complex such as the CDC3ϵ unit, are retained and degraded in the ER. It was shown that the peptide $^{-15}$KGQRDLYSGL^{-6}, which is located 5 residues before the carboxy terminus of CDC3ϵ, is essential and sufficient to specify ER retention. To be functional however, this segment must be located 5 residues before the carboxy terminus. Replacement of Y^{-10} by C, or S^{-8} by N abolishes retention,

illustrating the importance played by these two polar residues.

Tang et al. (1992b) observed that the KDEL motif, when appended to the carboxy terminus of a type II membrane protein, namely protein dipeptidyl peptidase IV (DPPIV), could promote efficient ER retention. This observation indicates that the mechanism involved in the ER retention of soluble proteins can also be used to retain Type II membrane proteins. The ER retention motif of the adenovirus E3/19K glycoprotein can bind to microtubules and can promote tubulin polymerization (Dahllöf et al., 1991). This interesting observation led to the attractive hypothesis that type I membrane proteins with a retention motif of the E3/19K type, are retained in the ER because their CT binds to the microtubule network closely associated with the ER.

1.2.11 Retention in the Golgi

The signal and the mechanism of localization of four Golgi membrane proteins have been studied. These proteins are the coronavirus E1 glycoprotein, a component of the cis-Golgi (Swift and Machamer, 1991), N-acetylglucosaminyltranspherase a medial-Golgi constituent (Tang et al., 1992a; Burke et al., 1992), galactosyltransferase a component of the trans-Golgi (Nilsson et al., 1991; Teasdale et al., 1992; Russo et al., 1992; Aoiki et al., 1992), and a sialyltransferase a trans-Golgi and trans-Golgi network constituent (Munro, 1991; Wong et al., 1992; Colley et al., 1992). Except for the coronavirus E1 glycoprotein (E1), a Type IV membrane protein, the other three Golgi residents are Type II membrane proteins.

When these proteins were expressed in mammalian cells, they were transported and localized efficiently to the Golgi complex and either no protein or a negligible amount of protein was expressed on the plasma membrane. In order to find the segment responsible for Golgi localization, extensive studies involving the construction and localization of chimeras using plasma membrane proteins as reporters were undertaken. It was found that the TM of these proteins encodes sufficient information to specify Golgi localization, because all the chimeras containing the TM were localized efficiently to the Golgi complex with little negligible expression on the plasma membrane.

In the case of α1,2 sialyltransferase (ST), protein segments other than the TM are also sufficient to specify Golgi localization (Munro, 1991; Colley et al., 1992). ST is composed of a short CT of 9 residues, followed by a signal/anchor sequence of 17 residues and a stem region of 35 amino acids, which is attached to a large luminal catalytic domain (Weinstein et al., 1987). The information essential for localization of ST to the Golgi complex is contained within the first 63 residues, because truncation after amino acid 63 results in secretion of the catalytic domain (Weinstein et al., 1987). It was also observed that chimeras containing the TM and CT, or the TM and portion of the stem, were localized more efficiently to the Golgi than a chimera containing only the TM of ST (Munro, 1991).

A chimera made with the first 43 residues of ST fused to lysozyme was constructed. When the TM of this protein was replaced with a peptide of polyleucine of similar length, the resulting mutant was strongly localized to the Golgi. Replacing the CT

of this mutant with the CT of a plasma membrane protein did not affect Golgi localization, indicating that the stem region close to the membrane is sufficient to specify Golgi localization. Similar observations were made by Colley et al. (1992), who showed that deletion of the CT and TM of ST generates a soluble protein that was still targeted to the Golgi complex.

The Golgi localization signal acts as a retention signal, because the Golgi proteins were transported passively to the plasma membrane wnen their targeting signal was destroyed. Over expression of the wild types and mutants did not result in cell surface expression, indicating that the retention mechanism was not saturable.

The E1 membrane glycoprotein is the only known Golgi resident having the amino and the carboxy termini facing the lumen of the Golgi complex and the cytoplasm respectively. This membrane protein is characterized by an unusually long TM that can span the membrane three times. In the absence of other viral protein expression, E1 has been localized by immunoelectron microscopy to the cis-Golgi (Machamer et al., 1990b). Deletion of the first TM of E1 (segment 1) abolished Golgi retention and results in transport to the plasma membrane, whereas deletion of the second and third TM, which generated mutant m2,3, did not alter Golgi localization, suggesting that segment 1 contains the information essential for Golgi retention (Machamer and Rose, 1987). It was subsequently demonstrated that segment 1 is sufficient for Golgi retention, because replacement of the TM of glycoprotein G of VSV with segment 1, produced a chimera (Gm1) that was retained completely in the Golgi (Swift and Machamer, 1991). Single

point mutations, which replaced polar with hydrophobic residues, or insertion of two hydrophobic residues within segment 1, were able to abolish Golgi retention of mutants Gm1 and m2,3. Unfortunately, the effects of these mutations could not be tested on the wild type E1, because they were interfering with the folding of E1.

The essential polar residues are conserved among different strains of coronavirus and they are lined up on the same face of the α-helix making up segment 1. Two mechanisms were proposed to explain retention in the Golgi complex (Swift and Machamer, 1991; Machamer, 1991). Retention could be accomplished by association or binding of the TM to a receptor buried in the Golgi membrane. Alternatively, the TM could promote the formation of aggregates in the Golgi complex that could not be incorporated into the transport vesicles. In these two models, binding to a potential receptor as well as aggregation would involve polar residues.

Specific residues within the TM of galactosyltransferase (GT) were also shown to be important for retention in the Golgi complex (Aoki et al., 1992). When CYS^{25} was replaced by SER, or when HIS^{32} was replaced by LEU, the retention of GT in the Golgi complex was partly abolished. When a double mutant containing these two mutations was studied, the protein was no longer retained in the Golgi complex and most of it was transported to the cell surface. It was also shown that only half of the TM of GT were sufficient to confer Golgi retention of a reporter protein.

1.2.12 Trans-Golgi network and sorting to lysosomes

In higher eukaryotes, lysosomal proteins are sorted from plasma membrane and secretory proteins in the trans-Golgi network (Simons and Griffiths, 1986; Mellman and Simons, 1992). The sorting of lysosomal constituents involves the recognition of specific signals and incorporation into clathrin coated vesicles which are then targeted to a prelysosomal compartment (reviewed in Pearse and Robinson, 1990; Fukuda, 1991). Proteins targeted to the regulated secretion, as well as basolateral and apical membrane proteins of polarized epithelia, are also sorted in the trans-Golgi network by specific targeting signals (Gerdes et al., 1989; Chung et al., 1989; Chanat and Huttner, 1991; Castle et al., 1992).

The sorting of lysosomal enzymes is by far the best documented. In the cis-Golgi, these enzymes are modified by the addition of a mannose 6-phosphate residues. Proteins harbouring this modification are recognized and become associated with the mannose 6-phosphate receptor, a trans-Golgi network membrane protein. The complex is then incorporated into clathrin coated vesicles, followed by transport to a prelysosomal compartment, where an acid pH triggers the release of the lysosomal enzymes. These enzymes are then transported to the lysosomes and the receptor is returned back to the trans-Golgi network. Recent evidence indicates that the CT of the mannose 6-phosphate receptor and other lysosomal membrane proteins contains the information essential for their selective transport to the lysosomes. In this domain, two different motifs were

shown to be essential for proper targeting. One motif is centred around a tyrosine with no other apparent amino acid homology, while part of the second motif consists of two adjacent leucines. In some cases the two signals are present on the same protein (Fukuda, 1991; Johnson and Kornfeld, 1992; Letourneur and Klausner, 1992).

1.3 The nuclear envelope (NE)

1.3.1 Structure and composition of the NE

The NE can be viewed as a double system of membranes, the inner and outer membranes, that surrounds and partly isolates the cell nucleus from the cytoplasm (reviewed in Franke et al., 1981; Gerace and Burke, 1988; Dingwall and Laskey, 1992; Dessev, 1992). The outer and inner membranes are in continuity with each other at the level of numerous pore complexes distributed over the entire surface of the envelope. The inner membrane is associated on its nucleoplasmic side with the lamina, a well organized meshwork of proteinaceous filaments underlying the inner membrane and interconnecting the pore complexes (Fig. 1.3.1).

1.3.1.1 The outer membrane

The outer and inner membranes are separated from each other by a space of 14 nm termed the perinuclear cisterna. The outer membrane is studded with numerous ribosomes and morphological evidence indicates the outer membrane and the perinuclear

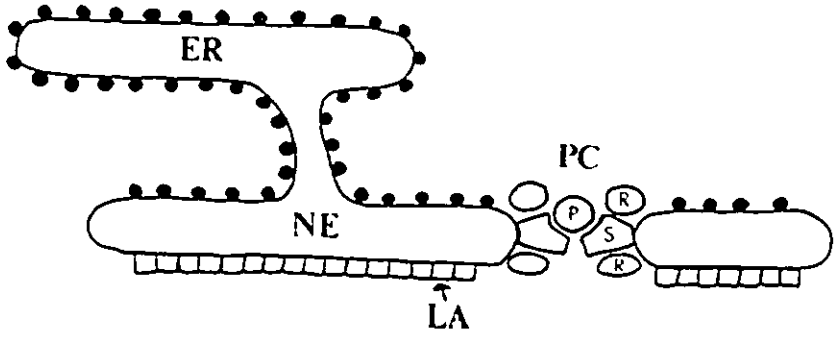

Figure 1.3.1. Structure of the nuclear envelope. The endoplasmic reticulum (ER) and the outer membrane of the nuclear envelope (NE) are studded with ribosomes (black dots). The inner membrane of the NE is associated with the lamina (LA). The nuclear pore complex (PC) is composed of two rings (R), height spokes (S) and a central plug (P). Adapted from Frank et al. (1981); Reichelt et al. (1990).

cisterna are in continuity with the membranes and lumen of the ER (Fawcett, 1981; Frank et al., 1981). Evidence exists indicating that the outer membrane of the NE is biochemically and functionally equivalent to the ER and should consequently be perceived as a subdomain of the ER. For example, it has been shown that the ribosomes on the outer membrane are fully competent for the synthesis of membrane proteins (Puddington et al., 1985); that some ER membrane constituents, such as the signal sequence receptor α-subunit and the rat liver calcium pump, are present in the NE (Smith and Blobel, 1993; Lanini et al., 1992); and that some soluble ER proteins, such as BiP have been localized by immunoelectron microscopy within the perinuclear cisterna (Bole et al., 1989).

1.3.1.2 The nuclear pore complex

The nuclear pore complex is a symmetrical proteinaceous structure made of transmembrane and peripheral membrane proteins. Its complexity can be envisioned, when it is realized that the pore complex, which possesses an estimated molecular weight of 125 000 kD, is believed to be composed of more than 100 distinct proteins (Davis and Blobel, 1986; Reichelt et al., 1990; Sukegawa and Blobel, 1993). The structure of the nuclear pore complex has been recently revised using high resolution electron microscopy (Reichelt et al., 1990; Jarnik and Aebi, 1991; Hinshaw et al., 1992). Briefly, the pore complex consists of two rings of 120 nm diameter, one associated with the outer membrane and the other with the inner membrane, that are connected to each other by

eight columns or spokes (Fig. 1.3.1). In non-detergent treated envelope, a central plug is also observed. Filaments originating from the rings and projecting into the nucleoplasm and cytoplasm have also been described. Two major functions have been assigned to the nuclear pore complex. One is to act as a scaffold that maintains the continuity between the outer and inner membranes, and the other one is to allow diffusion of small molecules and to regulate the transport of larger macromolecules between the cytoplasm and the nucleoplasm (Dingwall and Laskey, 1992).

Of the 100 different proteins believed to make up the pore complex, only 8 peripheral membrane proteins and two transmembrane proteins (gp210 and POM 121) have been cloned and sequenced (Wozniak et al., 1989; Greber et al., 1990; Wente et al., 1992; Sukegawa and Blobel, 1993; Radu et al., 1993; Hallberg et al., 1993). The peripheral membrane proteins are synthesized on free ribosomes. Their function is presently not well understood, but they are believed to have either a structural function or to be directly involved in the regulation of transport.

1.3.1.3 The inner membrane and lamina

The inner membrane of the NE is a distinct cellular compartment composed of a specific set of integral and peripheral membrane proteins. Some of these peripheral membrane proteins belong to the nuclear lamina, which is by far the best characterized constituent of the inner membrane (reviewed in Gerace and Burk, 1988; Nigg, 1992). In mammals, the lamina is made up of three major peripheral membrane proteins, termed

lamin A, B, and C, which belong to the large group of intermediate filaments. Under the electron microscope, the lamina appears as a well organized orthogonal network of filaments, underlying the inner membrane and connected to the nuclear pore complexes (Aebi et al., 1986). The lamina is viewed by most researchers as a supporting framework maintaining the NE integrity and providing anchorage for the chromatin.

Six inner membrane proteins have been characterized to some extent. Of these proteins, four (LAP1a, LAP1b, LAP1c and LAP2) are integral components of the mammalian NE (Senior and Gerace, 1988; Foisner and Gerace, 1993), one (p58/54) was isolated from the avian NE by two independent groups (Worman et al., 1988; Bailer et al., 1991), and one (p53) belongs to the NE of drosophila (Padan et al., 1990). The best characterized membrane protein is p58/54, also referred as the lamin B receptor (LBR), because of its strong affinity to lamin B (Worman et al., 1988; Bailer et al., 1991; Simos and Georgatos, 1992). It has been hypothesized that the main function of LBR is to attach the lamina to the inner membrane of the NE. The complete nucleotide sequence of LBR has been determined (Worman et al., 1990). The gene encodes a protein of 637 residues containing 8 potential TM. LBR does not contain a typical signal sequence and the first of the 8 TM is thought to act as a signal/anchor sequence (Smith and Blobel, 1993). The amino terminal domain, a segment of 205 residues, most likely faces the nucleoplasm, because it is phosphorylated by nucleoplasmic kinases (Simos and Georgatos, 1992). The exact orientation of the short CT is not known.

In analogy with LBR, three of the four inner membrane proteins of the

mammalian NE (LAP1a, LAP1b and LAP2) possess a strong affinity for the lamina, suggesting that they may be involved in the attachment of the lamina to the NE (Foisner and Gerace, 1993). The sequence of the gene encoding the inner membrane protein p53 has also been determined. The product of this gene is a hydrophilic protein of 406 residues with a unique hydrophobic segment of 18 residues at its carboxy terminus, which could theoretically act as a signal/anchor sequence. It has been postulated that p53 is anchored in the inner membrane by its carboxy terminal hydrophobic domain, leaving the bulk of the protein free to interact with some nucleoplasmic constituents (Padan et al., 1990).

1.3.2 Targeting to the NE

The number of studies dealing with NE localization is amazingly scarce in comparison with the literature presently available about targeting to other cellular systems. This observation stems from the fact that few NE constituents have been characterized, and because no easy and quick biochemical assays exist to measure NE localization.

The signal and mechanism for the NE targeting of the lamins are probably the best characterized (Senior and Gerace, 1988; Holtz et al., 1989; Nigg, 1992; Goldman et al., 1992). The lamins are synthesized on free ribosomes and they possess in their primary structure a typical nuclear localization signal, allowing accumulation in the nucleus by selective transport through the nuclear pore complexes (Nigg, 1992). Once

inside the nucleus, the lamins become tightly associated with the NE by direct binding to the inner membrane or via the interaction of specific inner membrane receptors, such as LBR. The lamins are post-translationaly modified by the covalent addition of a farnesyl group to a cysteine residue via a thioether linkage, a process known as isoprenylation. This modification occurs at their carboxy terminal consensus sequence CAAX, where A and X correspond to an aliphatic and any amino acids respectively (Farnsworth et al., 1989; Kitten and Nigg, 1991). This modification is then followed by cleavage of the last three residues and methylation of the cysteine (reviewed in Glomset et al., 1990; Clark, 1992). Isoprenylation is essential for the association of the lamins with the inner membrane. Lamins lacking the terminal farnesyl group were not attached to the inner membrane of the NE, although they were transported efficiently inside the nucleus (Krohne et al., 1989; Holtz et al., 1989; Kitten and Nigg, 1991).

The nuclear pore complex proteins, in contrast with the lamins, do not have to enter the nucleus to reach their correct subcellular location, and therefore lack a typical nuclear localization signal. In an elegant study using NSP1 (a yeast nuclear pore complex protein) it was demonstrated that the carboxy terminal moiety of NSP1 encodes sufficient information for proper localization to the pore complex (Hurt, 1990). NSP1 is a peripheral membrane protein of 823 residues. When the last 220 residues of NSP1 are appended to a cytosolic protein, the resulting chimera is targeted to the pore complex and is able to complement yeasts deficient in NSP1. Although the exact targeting mechanism remains to be unveiled, NSP1 is most likely transported by diffusion to the NE, where

it binds to the nuclear pore complex via its carboxy terminal tail. The precise binding motif of NSP1 and the potential receptor are unknown.

The NE localization determinants of two integral membrane proteins of the NE, the lamin B receptor (LBR) and the pore complex protein gp210, were recently investigated (Wozniak and Blobel, 1992; Smith and Blobel, 1993; Soullam and Worman, 1993). When LBR is expressed in COS cells, it is specifically localized to the NE as determined by immunofluorescence (Smith and Blobel, 1993; Soullam and Worman, 1993). Conflicting results exist concerning the nature of the determinant specifying inner membrane localization of LBR. According to Smith and Blobel (1993), the first TM of LBR, which acts as a signal/anchor sequence, encodes the sorting information, because this domain is able to target a reporter protein to the NE, and because deletion of the first TM abolishes NE localization. This is in clear contradiction with the study published by Soullam and Worman (1993). For these two researchers, who were using the same protein and a similar system, the nucleoplasmic tail of LBR encodes the sorting determinant for the following reasons: First, because deletion of the nucleoplasmic tail of LBR abolishes NE localization. Second, because a chimera made by fusing the nucleoplasmic tail of LBR to the TM of a type II membrane protein is efficiently targeted to the inner membrane of the NE.

Glycoprotein gp210 is a large type I membrane protein of 1 886 residues. The protein, which possesses a typical signal sequence, is anchored in the membrane by a single carboxy terminal TM of 20 residues (Wozniak et al., 1989; Greber et al., 1990).

The amino terminus of gp210 represents more then 96% of the total protein mass. When gp210 is associated with the pore complex, only a short CT of 58 residues can directly interact with the pore constituents (Greber et al., 1990). Although the function of gp210 remains unknown, gp210 is thought to mediate anchoring of the pore complex into the membrane (Wozniak et al., 1989; Greber et al., 1990; Jarnik and Aebi, 1991).

It was shown by Wozniak and Blobel (1992) that the major sorting determinant of gp210 resides within the TM. Following expression of gp210 in mammalian cells by microinjection of the cDNA, gp210 is specifically localized to the pore complex as determined by immunofluorescence. Deletion of the TM and CT of gp210 abolishes completely nuclear pore complex localization, whereas the TM and the CT of gp210 are both independently sufficient to target a plasma membrane protein to the nuclear pore complexes.

1.3.3 Mechanism of NE localization

It is generally thought that LBR and gp220 reach the inner membrane and nuclear pore complex respectively, by lateral diffusion in the membrane from their site of synthesis, which occurs in the ER and outer membrane of the NE (Wozniak and Blobel, 1992; Smith and Blobel, 1993; Soullam and Worman, 1993). It is presently not known if this process is passive, or if it involves specific receptors that shuffle back and forth between the ER and the NE. It is also unclear how these proteins are retained in the pore complex and the inner membrane. Although the model of lateral diffusion is generally

favoured, we cannot rule out that NE membrane proteins are transported by specific vesicles budding from the ER. Clearly, more studies are required to answer these intriguing and fundamental questions.

The hypothesis that targeting to the NE involves transfer from the ER, is in agreement with the elegant study of Powell and Burk (1990). It was observed that the inner membrane protein LAP1c (formerly p55), can be relocated from the NE of a donor cell to the NE of an acceptor cell following fusion of the two cells in the absence of protein synthesis. The transfer is slow (average equilibration time between 3 to 6 h) and it does not result from fusion of the two NE, because the nuclei remain intact and because lamin A is not exchanged during the event. A model was proposed in which the transfer of LAP1c is mediated by lateral diffusion in the ER membranes of the donor and acceptor cells which have fused with each other.

1.4 Herpes simplex virus

1.4.1 Structure of herpes simplex virus

Members of the herpesvirus family, such as HSV-1, are relatively large and complex DNA virus (Roizman, 1991; Roizman and Sears, 1991). For example, the genome of HSV-1, which encodes about 75 proteins, is composed of a linear DNA molecule with a length of 152 kb. The mature virus, whose average diameter is about 120 nm (Roizman and Furlong, 1974), is made of the following morphological elements (Fig. 1.4.1). An internal and central opaque core, containing the genomic DNA tightly

associated with proteins. The core is surround by a proteinaceous symmetric capsid, made of 162 similar subunits (the capsomers). The capsid itself is surrounded by a membrane, the envelope, constituted of lipids and numerous glycoproteins forming the projections or spikes at the surface of the virus. The space between the envelope and the capsid is filled with an amorphous substance known as the tegument.

1.4.2 The life cycle of herpes simplex virus

Excellent reviews have been written about the diverse aspects of the life cycle of HSV-1 and other herpesviruses (Roizman and Sears, 1991; Spear and Roizman, 1981; Roizman and Furlong, 1974). It is not the purpose of this brief discussion to present a thorough analysis of this fascinating biological process, and the readers requiring more information should consult these reviews. Viral envelopment however is treated with more detail, because this process is more directly related to the experimental section of the present study.

The life cycle of HSV-1 can be dissected into the following events (Fig. 1.4.2). A) For productive infection, the virions must attach to the cell surface and penetrate inside the cell. Penetration is generally thought to occur by fusion of the viral envelope with the plasma membrane (PM), with the release of the capsid inside the cytoplasm. B) The newly de-enveloped capsids are translocated to the pore complexes, where the DNA and regulatory proteins are released inside the nucleus. C) Specific genes are transcribed and the viral DNA is replicated. D) The proteins encoded by the virus are synthesized

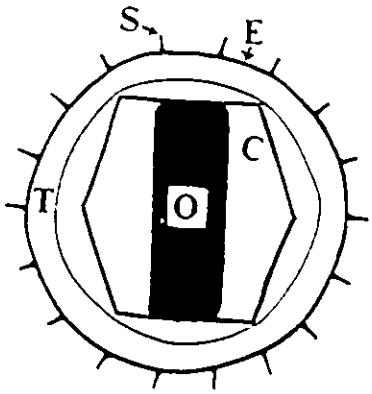

Figure 1.4.1. Structure of HSV-1. C: capsid; E: viral envelope; O: core; S: spikes; T: tegument. Adapted from Roizman (1991).

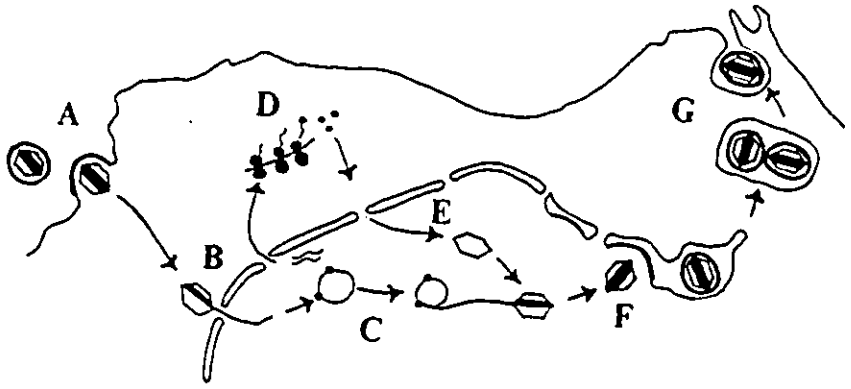

Figure 1.4.2. The life cycle of HSV-1. A penetration; B: Release of the DNA and regulatory proteins; C: Transcription and replication; D: Viral protein synthesis; E: Capsid assembly; F: Envelopment; G: Transport to the cell surface. Adapted from Roizman and Sears (1991).

in the cytoplasm. E) DNA associated and structural proteins are transported inside the nucleus, where they are used as building blocks for the assembly of the capsids. F) The DNA and its associated proteins are packaged inside the newly made capsids, which are enveloped by budding through a modified inner membrane of NE. G) Following envelopment, the virions are transported to the cell surface inside vesicles formed by membranes derived from the outer membrane of NE or the ER. The mature virions are finally released outside the cell by fusion of the vesicles with the plasma membrane.

1.4.3 Envelopment of herpes simplex virus

One of the first cell alterations generated by infection with HSV-1 and other herpesviruses, is a shut off of the host cell DNA and protein synthesis, concurrently with specific synthesis of viral constituents (Roizman and Furlong, 1974; Roizman and Sears, 1991). The morphology of the cells is also markedly altered during infection. The cell becomes round, the chromatin condenses and forms a thick ring at the nuclear periphery. The nucleolus looses is granular component and becomes fragmented.

The morphology of the NE is also modified by the infection. Portions of the NE become duplicated and are piled into stacks composed of several layers of membrane. Certain parts of the inner membrane invaginate deeply inside the nucleus, occasionally generating intranuclear vacuole-like structures (Nii et al., 1968; Darlington and Moss, 1968; Morgan et al., 1959). Also observed is the presence of dense patches in the NE corresponding to thicker, denser and curved portions of the inner membrane.

Morphological evidence indicates that herpes simplex viruses acquire their envelope by budding through the dense patches of the inner membrane (Nii et al., 1968; Darlington and Moss, 1968). Enveloped herpes simplex viruses were also described inside intranuclear vacuoles (Darlington and Moss, 1968; Torrisi et al., 1992) and it was recently hypothesised that envelopment for cytomegalovirus can occur by budding inside these intranuclear vacuoles (Tumilowicz and Powell, 1990).

The inner membrane of the NE is composed of a number of specific cellular proteins (Section 1.3.1.3). A mechanism must exist to exclude the host membrane proteins from the budding sites, since the envelope of mature virions does not contain a detectable amount of cellular proteins (Spear, 1976; Spear and Roizman, 1972). However, no study exists concerning this fundamental and interesting phenomenon.

1.4.4 The envelope of HSV-1

The envelope of HSV-1 is composed of lipids and of a certain number of glycoproteins. Few things are known about the lipid composition of the viral envelope. Earlier studies have presented evidence indicating that the phospholipid composition of the viral envelope is similar to the inner membrane of the NE, and is most likely derived from it (Ben-Porat and Kaplan, 1971; Roizman and Furlong, 1974). At least 11 glycoproteins known as gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL and gM are encoded by the genome of HSV-1 and are synthesized during HSV-1 infection (Spear, 1984; Gompels and Minson, 1986; Richman et al., 1986; Longnecker et al., 1987; Roizman

and Sears, 1991; Hutchinson et al., 1992a,b; Baines and Roizman, 1993). Among these glycoproteins, direct evidence exists that glycoproteins gB, gC, gD (Spear, 1976), gE (Para et al., 1982), gG (Richman et al., 1986), gH (Desai et al., 1988), gL (Roop et al., 1993) and gM (Baines and Roizman, 1993) are present in the envelope of the mature virion. Although the presence of gI in the virion has not been directly demonstrated, it is reasonable to assume that this glycoprotein is also associated with the viral envelope, because gI forms a complex with gE (Johnson et al., 1988a). A recent analysis by immunoelectron microscopy of the composition of the spikes of the envelope of HSV-1, suggests that different glycoproteins are associated with structurally distinct spikes having a specific distribution pattern on the viral surface. For example, the gB spikes are clustered, whereas the gC spikes are randomly distributed and widely spaced (Stannard et al., 1987).

1.4.5 NE distribution of viral glycoproteins

The presence of specific viral glycoproteins in the NE of cells infected with HSV-1 and other herpesviruses has been demonstrated by cell fractionation (Compton and Courtney, 1984a; Radsak et al., 1990), immunofluorescence (Koga et al., 1986) and electron microscopy (Poliquin et al., 1988; Torrisi et al., 1989; Gong et Kieff, 1990; Radsak et al., 1990; Torrisi et al., 1992).

The high resolution provided by the use of colloidal gold as a probe for electron microscopy, has allowed researchers to demonstrate the association with the inner

membrane of glycoproteins belonging to three different herpesviruses. These proteins are glycoproteins gB and gD of HSV-1 (Torrisi et al., 1992), gp350/220 (Torrisi et al., 1989) and gp110 (Gong and Kieff, 1990; Gong et al., 1987) of Epstein-Barr virus, and glycoprotein B of cytomegalovirus (Radsak et al., 1990). An interesting observation was made by Torrisi et al. (1989) who claimed that glycoprotein gp350/320 was specifically concentrated at the budding sites on the inner membrane. This is in disagreement with the study published by Gong and Kieff (1990) who did not observed a specific distribution of gp350/320 in the NE, although they showed that gp110 is strongly localized to this compartment.

The viral glycoproteins present in the NE, are associated exclusively with the high mannose core oligosaccharide. This observation was made by various groups using different technics, such as treatment with specific endoglycosidases (Compton and Courtney, 1984a; Gong and Kieff, 1990; Radsak et al., 1990), labeling with antibodies or lectin that recognize specific glycosylation intermediates (Torrisi et al., 1989; Torrisi et al., 1992), or labeling with preferential stains, and radioactive precursors (Lopez-Iglesias and Puvion-Dutilleul, 1988; Poliquin et al., 1985).

On the other hand, the glycoproteins of the mature virions are associated with oligosaccharides of the complex type indicating that they were processed by the Golgi enzymes (Johnson and Spear, 1982; Campadelli-Fiume and Serafini-Cessi, 1985). For this reason, most researchers believe that maturation of the glycoproteins occurs after envelopment, during transport to the cell surface, most likely by fusion of the vesicles

carrying the virions with Golgi cisternae (Spear, 1984; Torrisi et al., 1992).

Treatment of cells infected with herpes simplex virus with monensin, a drug that disrupts the function and the morphology of the Golgi complex, abolishes transport to the cell surface and results in the production of non-infectious virions containing incompletely processed glycoproteins (Johnson and Spear, 1982; Ghosh-Choudhury et al., 1987). This illustrates the significant role played by the Golgi complex for transport and maturation of herpesviruses.

1.5. Glycoprotein B

1.5.1 Structure and topology of gB

The nucleotide sequences of the gene encoding gB are presently known for at least four strains of HSV-1. (KOS: Bzik et al., 1984, 1986; F: Pellet et al., 1985; Patton: Stuve et al., 1987; and 17: McGeoch et al., 1988). These genes are predicted to encode a protein of 904 amino acids, except for strain F which is predicted to be one residue smaller.

Very minor differences exist between the primary structure of gB of the different strains. The 4 proteins differ by an average of 6 to 11 amino acids, and more than half of these differences are located at the amino terminus before codon 79, mainly in the signal sequence (Bzik et al., 1986; Stuve et al., 1987). The gB gene is conserved among all herpesviruses examined (Griffin, 1991), and the protein shares about 85% amino acid homology with glycoprotein B of herpes simplex virus type 2 (Bzik et al., 1986; Stuve

et al., 1987). This high degree of conservation suggests an essential and common function of gB in the life cycle of herpesviruses.

The amino terminus of gB encodes a region with all the characteristics of a typical signal sequence. The cleavage site was theoretically mapped after amino 30 for strain KOS, Patton and 17, and after amino acid 29 for strain F. The mature gB was therefore expected to be composed of 874 amino acids for the four strains of HSV-1. Sequence analysis of the amino terminus of the mature gB, have later on confirmed these predictions (Claesson-Welsh and Spear, 1987; Pachl et al., 1987).

Glycoprotein B is a major constituent of the viral envelope and is also present in the membranes of the infected cells (Spear, 1976; Heine et al., 1972; Spear and Roizman, 1972). Studies of the hydropathic profile of gB has allowed the delineation of a relatively hydrophobic segment of 69 amino acids near the carboxy terminus, that could serve as a membrane anchoring domain (Pellet et al., 1985; Bzik et al., 1986). A model was proposed by Pellet et al. (1985) in which gB is anchored in the membrane by this long hydrophobic segment, spanning the membrane three times, and which could be dissected into segments 1, 2 and 3 (Figs 1.5.1a and 3.1.1). Because of the presence of a typical signal sequence, the carboxy and amino termini are presumed to face the cytoplasm and ER respectively.

According to the model of Fig. 1.5.1a., the EC of gB, which faces the ER or the external environment, is 696 amino acids long and its highly charged CT is made of 109 residues. Analysis of the primary structure of gB indicates the presence of 6 potential N-

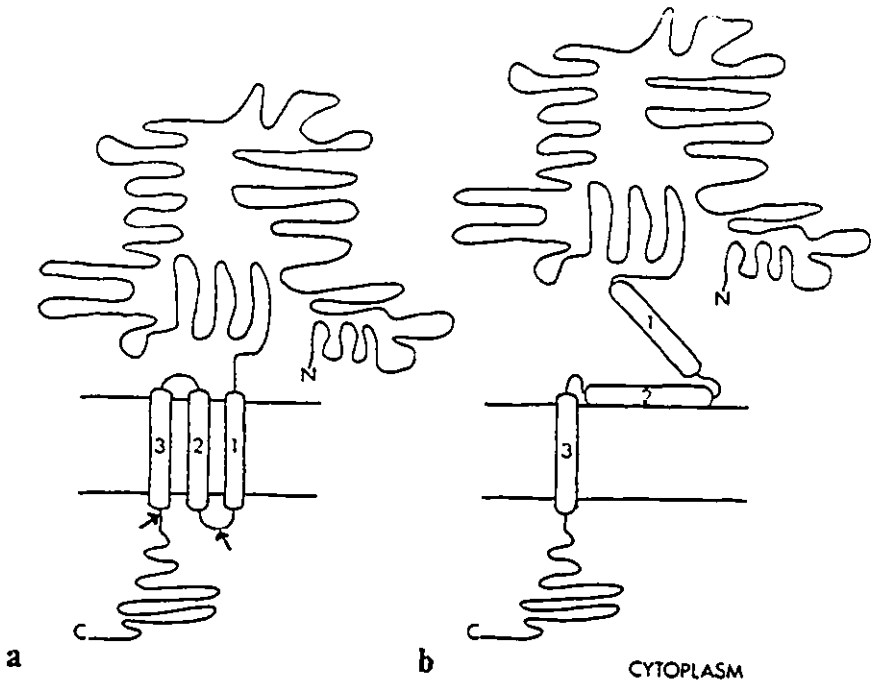

Figure 1.5.1. Topology of glycoprotein gB. a) Model proposed by Pellet et al. (1985). b) Model suggested from the results of Rasile et al. (1993). The position of Segments 1, 2 and 3 is indicated. Arrows in a) indicate potential trypsin/chymotrypsin cleavage sites.

linked glycosylation sites within the EC (Bzik et al., 1986., Pellet et al., 1985, Stuve et al., 1987; Griffin, 1991). Partial digestion with a glycosidase showed that five of these sites are used for glycosylation (Cai et al., 1988b). A deletion mutant lacking the TM and CT of gB and the most carboxy terminal glycosylation site, was glycosylated at the 5 sites that were left, suggesting that these sites are normally used for glycosylation (Cai et al., 1988b).

Digestion of gB with trypsin/chymotrypsin after synthesis in vivo, or in vitro in presence of membranes, resulted in the protection of fragments of 93 and 98 kD (Claesson-Welsh and Spear, 1987). The protected fragments were glycosylated to the same extent as gB, and their size was equivalent to the size of gB without the CT. It was hypothesized that the 98 kD and 93 kD fragments were generated by proteolytic cleavage after segment 3, and between segments 1 and 2 respectively, in agreement with the topologic model of Fig. 1.5.1a.

Although the model of Pellet et al. (1986) is now widely accepted, some criticisms were raised concerning its validity because segment 1 does not meet the criteria of a membrane anchoring domain (Rasile et al., 1993). According to Kyte and Doolittle (1982), 19 contiguous residues with an average hydropathy of 1.65 are sufficient to define a TM. Segment 1, which exhibits an average hydropathy of 0.8 in comparison with 1.7 and 2.4 for segments 2 and 3 (Rasile et al., 1993), contains three charged residues and thus should not be stable in the hydrophobic bilayer. A second model, in which gB is anchored solely by segment 3, was suggested by Rasile et al.

(1993), who demonstrated that segment 3 is both essential and sufficient for stable anchoring of gB (Fig. 1.5.1b).

The fact that segment 3 is essential and sufficient for stable anchoring does not necessarily refute the model of the triple TM. Furthermore, transmembrane protein with one ore more charged residues within their TM have been previously described (Bonifacino et al., 1991), and we cannot rule out that taken as a whole, segments 2 and 3 compensate for the hydrophilicity of segment 1. It was even proposed that the TM of gB could form an ion channel, in which the charged residues of segment 1 would face the aqueous pore (Pellet et al., 1985). Clearly, more experiments are required for a complete understanding of the topology of gB in the membrane.

Rate zonal centrifugation in sucrose gradients and electrophoresis on denaturing and nor-denaturing gels, have demonstrated that gB oligomerizes into dimers (Sarmiento and Spear, 1979; Ali, 1990; Highlander et al., 1991). The gB dimers can be isolated from purified virions and from the membranes of infected and transfected cells. The latter observation indicates that no other viral gene is required for dimerization of gB. Oligomerization occurs rapidly in the ER, because dimers are already present 7 to 10 min following synthesis, and contain the high mannose core oligosaccharides (Claesson-Welsh and Spear, 1986; Ali, 1990; Highlander et al., 1991).

The presence of oligosaccharides is not required for oligomerization of gB, because treatment with the glycosylation inhibitor tunicamycin did not impair dimerization (Claesson-Welsh and Spear, 1986; Highlander et al., 1991). The gB dimers

are relatively resistant to denaturation by SDS and by treatment with reducing agents such as β-mercaptoethanol and DTT (Sarmiento and Spear, 1979), suggesting that disulphide bonds are not involved, or are not essential for formation and maintenance of the dimers. The dimers however are readily dissociated in the presence of SDS when heated at 100°C.

The CT and the TM of gB are not required for oligomerization (Ali, 1990; Qadri et al., 1991; Navarro et al., 1993). A region essential for oligomerization has been mapped between residues 630 and 710, since proteins truncated before residues 630 remained monomeric, whereas proteins truncated after residue 720 formed dimers (Qadri et al., 1991; Navarro et al., 1993). A coprecipitation assay was used by Highlander et al. (1991) to map the region of oligomerization of gB. In this assay, cells were cotransfected with plasmids encoding two different deletion mutants of gB. The presence of dimers was monitored by immunoprecipitation using an antibody specific for only one of the mutants. If the two mutants were brought down by the antibody, it was inferred that they form dimers. Two regions sufficient for oligomerization were thus mapped: an upstream site encompassing residues 93-282 and a downstream site encompassing residues 596-711.

1.5.2 Processing and transport of gB

As pointed out in the previous section, gB is glycosylated at 5 of its 6 potential N-linked glycosylation sites (Cai et al., 1988b). The newly synthesized gB migrates on

SDS-PAGE with an apparent mol wt of 110 kD. This precursor form of gB (pgB) is associated with the high mannose core oligosaccharides and is completely sensitive to endo H, a glycosidase specific for this type of sugar. During transport to the cell surface, pgB is processed by the cellular machinery into the mature form, which has an apparent mol wt of 120 kD. The latter is found in the mature virions and on the plasma membrane (Eberle and Courtney, 1980b; Wenske et al., 1982).

Treatments of electrophoretically purified gB by mild alkaline borohydride liberated significant amounts of oligosaccharides, suggesting that gB is also O-linked glycosylated (Oloffson et al., 1981). The mature gB is reduced in size to 110 kD by digestion with α-D-N-acetylgalactosamine oligosaccharidase (Johnson and Spear, 1983), an enzyme specific for O-linked oligosaccharides (Huang and Aminoff, 1972). It was thus proposed that the size increment, when the mol wt of gB increases during maturation from 110 to 120 kD, is mainly a consequence of O-linked glycosylation (Johnson and Spear, 1983). Apart from glycosylation, no other post-translational modifications have been demonstrated for gB (Spear, 1984).

Glycoprotein B and other HSV-1 glycoproteins are transported and processed faster early in infection (4-7 h postinfection) than late in infection (Campadelli-Fiume et al., 1988; Sommer and Courtney, 1991). The HSV-1 glycoproteins are also transported faster in transfected than in infected cells (Johnson and Smiley, 1985; Campadelli-Fiume et al., 1988). Two hypothesis have been put forward to explain these observations. In one case the deleterious effects generated by the infection, such as the shut off of the host

macromolecular synthesis, which eventually lead to lysis and cell death, are believed to be directly responsible for the reduction of the rate of transport. In the second hypothesis, it is proposed that in early infection or in transfected cells the glycoproteins are transported freely and relatively rapidly by bulk flow to the cell surface. On the other hand in late infection, the glycoproteins are targeted to the NE where they are incorporated into the viral envelope before being transported, in association with the virus, to the cell surface. The whole assembly process is believed to reduce considerably the rate of transport (Johnson and Smiley, 1985; Campadelli-Fiume, 1988; Sommer and Courtney, 1991).

The effects produced by deletion, insertion and substitution of specific residues on the processing and transport of gB are well documented (Cai et al., 1988b; Raviprakash et al., 1990; Qadri et al., 1991; Navarro et al., 1993; Rasile et al., 1993). A total of more then 100 mutants were studied. The majority of the mutations slowed down or abolished the processing and transport of gB. The mutations affecting the processing and transport are distributed throughout the protein and no specific hot spot was mapped.

Dimerization, at least for the non-anchored gB, is not an absolute requirement for transport, since some truncation mutants of gB are secreted as monomers (Ali, 1990; Navarro et al., 1993). Dimerization of gB is neither sufficient for transport, since a mutant of gB (mutant LK479), constructed by inserting four residues in the EC, could form dimers but was unable to exit the ER (Qadri et al., 1991; Navarro et al., 1991).

The use of conformational antibodies has shown that Lk479 and other transport defective mutants do not fold properly (Qadri et al., 1991). The newly synthesized gB is transiently associated with the chaperons BiP and GRP94. Some transport defective mutants including LK479 on the other hand remain associated with BiP and GRP94 more strongly (Navarro et al., 1991). These observations indicate that proper folding is a key component determining the transport and processing of gB. The mechanism and requirements for efficient transport of gB are, therefore, very similar to other proteins (Section 1.2.9).

1.5.3 Distribution of gB in the NE

The presence of gB on the plasma membrane of infected and transfected cells has been demonstrated by cell fractionation and immunofluorescence (Spear, 1976; Spear and Roizman, 1972; Heine et al., 1972; Eberle and Courtney, 1980a; Koga et al., 1986; Ali et al., 1987, Pachl et al., 1987, Cai et al., 1988b). Immunofluorescence and cell fractionation have also been used to demonstrate a specific distribution of gB in the NE of infected and transfected cells (Compton and Courtney, 1984a; Ali et al., 1987; Raviprakash et al., 1990). On this cellular compartment, a recent immunoelectron microscopic study has presented evidence that gB is localized on both outer and inner membranes (Torrisi et al., 1992). Envelopment of HSV-1 occurs at the NE (Section 1.4.3) and it is generally conceded that gB is targeted to this compartment in order to be incorporated into the viral envelope. However, the mechanism of transport to the NE and

the determinants specifying NE localization of gB remain unknown. The fraction of gB present in the NE is associated only with the high mannose core oligosaccharides, indicating that transport to the NE is a pre-Golgi event (Compton and Courtney, 1984a; Torrisi et al., 1992).

Recent studies have been made to delineate the determinants specifying NE localization of gB. Raviprakash et al. (1990) have observed, by immunofluorescence and cell fractionation, that deletion of the CT of gB did not abolish NE localization. Using a similar approach, it was subsequently observed that membrane anchoring is a requirement for NE localization, because deletions in the TM of gB led to the production of secreted proteins that were not localized to the NE. In contrast, deletions in the TM of gB that did not impair membrane anchoring, did not abolish NE localization (Rasile et al., 1993).

1.5.4 Function of gB

Glycoprotein B is essential for virus growth and propagation. Virions made in the absence of gB possess an envelope and a structure similar to the wild type virions (Sarmiento et al., 1979). However, such virions are not infectious in vitro, unless the cell is treated with the fusogenic agent polyethylene glycol suggesting that one of the gB functions is to promote fusion with the plasma membrane (Sarmiento et al., 1979; Cai et al., 1988a). This is also in agreement with the observation that mutations within gB affect the fusogenic property of the protein (Cai et al., 1988a; Gage et al., 1993), and

that neutralizing antibodies specific to gB prevent entry and the spreading of the virus (Navarro et al., 1992). The gB-free virions are able to bind to the cell surface at the same rate as the wild type virions, indicating that the defect generated by the absence of gB is not a consequence of lack of adsorption (Cai et al., 1988a). The reports that gB can promote fusion in transfected cells following an acidic shift of extracellular PH, is another indication that gB is involved in membrane fusion (Ali et al., 1977; Butcher et al., 1990).

1.6 Glycoprotein G

1.6.1 Primary and secondary structure of G

Glycoprotein G (G) is one of the five proteins encoded by vesicular stomatitis virus (VSV), which is a small enveloped RNA virus (review in Emerson, 1985). In contrast to HSV-1, which possesses an envelope composed of a number of glycoproteins (section 1.4.4), G is the only transmembrane protein present in the mature envelope of VSV. Glycoprotein G, which is a typical Type I membrane protein has been the subject of intensive biochemical studies, and much of our present knowledge concerning synthesis, processing and transport of membrane proteins is derived from the study of G (Reviewed in Zilberstein et al., 1981; Ghosh, 1980).

The nucleotide sequence of the cDNA of the viral mRNA encoding G has been determined by Rose and Gallione (1981). The mRNA encodes a protein of 511 amino acids, composed of a typical signal sequence of 16 residues, an EC of 446 residues, a

20 amino acids long TM, and a short hydrophillic CT of 29 residues (Fig. 3.1.1). Two potential sites for N-linked glycosylation are present in the EC of G, and earlier studies have shown that they are both used for glycosylation (Etchison et al., 1977, Reading et al., 1978).

1.6.2 Post-translational modifications of G

Two major formes of G known as G_1 and G_2 are detected following infection with VSV (Knipe et al., 1977). G_1 (apparent mol wt of 65 to 67 kD) is the precursor of the 2 kD larger G_2, which is present on the plasma membrane and which is associated with the mature virion (Knipe et al., 1977). It has been demonstrated that G_1 corresponds to the newly glycosylated protein following cleavage of the signal sequence (Toneguzzo and Ghosh, 1977; Irving et al., 1979). G_1 is therefore associated with the high mannose core oligosaccharide, and the decrease in mobility observed when G_1 is transformed into G_2 is a consequence of the processing of the oligosaccharides into the complex type (Knipe et al., 1977; Zilberstein et al., 1981).

During its maturation, G of the Indiana serotype becomes acylated by the addition of palmitic acid (Schmidt and Schlesinger, 1979; Kotwal and Ghosh, 1984). Further characterization after partial proteolytic digestion of G, showed that the last 50-60 residues at the carboxy terminus contained the acylation site (Capone et al., 1982). The site was later located at the unique cysteine residue (C_{489}), which is 6 amino acids away from the TM, because truncation of the CT of G and replacement of C_{489} by serine,

abolished its acylation (Rose et al., 1984). Acylation of C_{489} has also been confirmed by chemical analysis (Mack and Kruppa, 1988).

The function of the covalently bound palmitate is presently not known. It is not involved in membrane anchoring and transport, since the non-acylated G is anchored in the membrane and is transported efficiently to the cell surface (Kotwal and Ghosh, 1984; Rose et al., 1984). Strains of VSV that synthesized a G protein lacking covalently bound palmitate residues have been described (Kotwal and Ghosh, 1984). Fatty acid acylation is not required for the fusogenic activity of G or for its incorporation into the virion, because cells treated with inhibitor of fatty acid acylation can produce infective VSV and because the non-acylated G can promote fusion and can complement a defective virus (Kotwal and Ghosh, 1984; Whitt and Rose, 1991). It was thus proposed that the main function of acylation is to prevent the formation of nonspecific disulphide bonds between C_{489} and other cysteines (Whitt and Rose, 1991).

A shorter form of G has been detected in the culture medium after infection with VSV (Kang and Prevec, 1970). This form, which is known as G_s, is about 10 to 12 kD smaller than G (Little and Huang, 1978; Irving and Ghosh, 1982). The amino acid sequence of the NH_2 terminus of G_s is identical to G and tryptic finger print analyses indicate that G_s lacks the normal carboxy terminus (Irving and Ghosh, 1982). It can thus be inferred that G_s is about 85 residues smaller than G and lacks both TM and CT.

It was earlier thought that G_s was produced by cleavage of G at the cell surface (Little and Huang, 1978). However, more recent studies have demonstrated the presence

of G_1 in the ER. It was also shown that G_1 is transported to the cell surface by formation of heterotrimers with the full length G, that are disassembled upon arrival to the cell surface (Graeve et al., 1986; Schmidt et al., 1992). The mechanism used to generate G_1 and its function remain obscure. It has been hypothesised that the release of G_1 in the extracellular medium inhibits the neutralizing effects of antibodies (Little and Huang, 1978).

1.6.3 Quaternary structure of G

Like many receptors and cell surface proteins, G is an oligomeric protein. It was shown by sedimentation analyses in sucrose density gradients and by chemical crosslinking, that G assembles into noncovalently homotrimers soon after synthesis (Doms et al., 1987). Proper oligomerization of G is essential for transport to the cell surface (Kreis and Lodish, 1986). The G trimers are very labile and they can be isolated only under mild acidic conditions. Kinetic studies have shown that G is first synthesized as monomers. The trimers are formed rapidly following synthesis (half time of 6-8 min) and oligomerization precedes exit from the ER. Sufficient information for oligomerization is encoded by the EC of G, because truncation of the CT and TM did not affect trimerization (Doms et al., 1988; Crise et al., 1989; Schmidt et al., 1992).

1.6.4 Distribution and transport of G

Rabdoviruses such as VSV acquire their envelope by budding at the plasma

membrane (reviewed in Emerson, 1985). In order to be incorporated into the virus, glycoprotein G must be transported efficiently to this cellular compartment. Studies by immunofluorescence and electron microscopy have shown that G is transported to the cell surface using the transport scheme depicted in Sections 1.2.7 and 1.2.8 (Bergmann et al., 1981; Bergeron et al., 1982; Bergmann and Singer, 1983). A small percentage of G has been found in association with the NE. The presence of G on this compartment is due to the fact that the outer membrane of the NE is involved in the synthesis of membrane proteins (Puddington et al., 1985; Section 1.3.1.1). Indeed the association of G with the NE is transient, and no evidence exists indicating that G specifically accumulates in this compartment.

The transport of G is efficient and rapid. In transfected cells for example, G reaches the Golgi complex within less than 30 min from the beginning of synthesis (Rose and Bergmann, 1983; Bergeron et al., 1982). Truncation of the CT of G or addition of foreign amino acids to this domain, greatly reduces the rate of transport, suggesting the involvement of the CT in transport (Rose and Bergmann, 1982; Rose and Bergmann, 1983). The reduction of the transport rate is not a consequence of misfolding, since the mutants were able to form trimers rapidly and efficiently (Doms et al., 1988). When the modified CT were attached to two reporter proteins, the rat growth hormone and the human chorionic gonadotropin-α subunit, similar reductions of the rate of transport were observed (Guan et al., 1988). The mechanism used by the CT of G to facilitate the transport is unknown. It has been suggested the CT of G acts by facilitating incorporation

into the transport vesicles. According to this view, proteins with the normal CT are incorporated more rapidly into these vesicles than proteins lacking this domain, resulting in more efficient transport (Doms et al., 1988; Guan et al., 1988; Rose and Doms, 1988). A peptide of 12 amino acids (NH_2-SRDRSRHDKIH-COOH) has been shown to inhibit the transport of G and other membrane proteins when appended to their carboxy termini (Rose and Bergmann, 1983; Guan et al., 1988). The mechanism of action of this so called "poison sequence" remained mysterious until recently, when it was observed that the last six residues of this peptide (RHDKIH) ressembles the consensus sequence of the retention signal of some ER membrane proteins (RXKXX; Section 1.2.10.2).

2.0 MATERIALS AND METHODS

2.1 Chemicals and reagents

All chemicals and reagents were of the purest grade available except when otherwise specified.

CHEMICAL	SUPPLIER
Acrylamide	Gibco/BRL
Adenosine 5'-triphosphate (ATP)	Pharmacia
Agar	Difco
Agarose	Gibco/BRL, Pharmacia
Ammonium sulfate	Baker Chemical
Ampicillin	Sigma
Aprotinin (10 000 U/ml)	Miles Canada Inc
Bactotryptone	Difco
5-bromo 4-chloro 3-indolyl β-D-galactoside (X-gal)	Gibco/BRL
Bromophenol blue	Sigma
Calf serum	Gibco/BRL
Cesium Chloride (99.9% pure)	Terochem Lab.
Chloramphenicol	Sigma
4-Chloro-1-Naphtol	Sigma
Citric acid (Monohydrate)	Baker Chemical
Cyanogen bromide-activated Sepharose 4B	Sigma
[α-^{35}S]-dATP (1000 Ci/mmol; 10 μCi/μl)	Dupont-New England Nuclear
Deoxynucleotides (dNTP)	Pharmacia
2,5 diphenyloxazole (PPO)	Fisher Scientific
Dithiotreitol (DTT)	Calbiochem
α-D-methylmanoside	Sigma
Ethidium bromide	Sigma
Fetal bovine serum (FBS)	Gibco/BRL
Ficoll (type 400)	Pharmacia
Glutaraldehyde (10%)	Marivac limited
Goat serum	Gibco/BRL
Gum arabic	Fluka
Hydroxyquinone (quinol)	BDH
8-Hydroxyquinoline	Sigma

Isopropyl β-D thiogalactopyranoside (IPTG)	Gibco/BRL
Lead nitrate	Baker Chemical
Lentil lectin-conjugated Sepharose 4B	Pharmacia
L-glutamine (200 mM)	Gibco/BRL
L-methionine	General Biochemicals
Lowicryl K4M	Polysciences
Low melting point agarose	Gibco/BRL
β-Mercaptoethanol	BDH, Baker Chemical
N-2-hydroxyethylpiperazine-N -2-ethanesulfonic acid (HEPES)	Boehringer Mannheim
2-[N-morpholino] ethanesulfonic acid (MES)	Sigma
3-[N-morpholino] propanesulfonic acid (MOPS)	Sigma
N,N-methylenebisacrylamide	Gibco/BRL
N,N,N,N,-tetramethylethylene-diamine (TEMED)	Gibco/BRL, Bio-Rad
Noble agar	Difco
Non-essential amino acids (100X)	Gibco/BRL
Nonidet P40	BDH
Osmium tetroxide	Marivac limited
Paraformaldehyde	Sigma
Penicillin (5000 U/ml), Streptomycin (5 mg/ml)	Gibco/BRL
Phenylmethylsulfonide fluoride	Sigma
Phenol (crystalline)	BDH
Piperazine-N,N-bis [2-ethanesulfonic acid] (PIPES)	Sigma
Polyethylene glycol-8000	Fisher Scientific
Protein A-Sepharose	Pharmacia
Rubidium chloride	Sigma
[^{35}S]-methionine (1100 Ci/mmol)	Dupont-New England Nuclear
Salicylic acid	Sigma
Silver lactate (lactic acid, silver salt)	Sigma
Sodium citrate (dihydrate)	Caledon Laboratories
Sodium carboxymethylcellulose (Aquacide II)	Calbiochem
Sodium deoxycholate	Calbiochem
Sodium dodecyl sulfate (SDS)	BDH, Bio-Rad
Spermidine	Sigma
Sucrose	Gibco/BRL
Tetracycline	Sigma

Thiamine hydrochloride	Sigma
Thimerosal	Sigma
Tris(hydroxymethyl)aminomethane (Tris)	Gibco/BRL, Boehringer Mannheim
Triton X-100	Sigma, Bio-Rad
Tween-20	Sigma
Uranyl acetate	Taab laboratories
Urea	Gibco/BRL
Vitamin solution (100X)	Gibco/BRL
Xylene cyanole FF	Baker Chemical
Yeast extract	Difco

2.2 Proteins and enzymes

Aldolase (rabbit muscle)	Sigma
Bovine serum albumin (BSA, frac V, 96-99% pure)	Sigma
Calf intestinal alkaline phosphatase (CIP)	Pharmacia, Boehringer Mannheim
DNA ligase (T4)	Gibco/BRL, Bio-Rad
DNA polymerase I (E.coli, Klenow fragment)	Gibco/BRL
DNA polymerase (T4)	Bio-Rad
DNA polymerase (T7, Sequenase™ Version 1.0 and 2.0)	United States Biochemical
β-Endoglycosidase H	Genzyme
Lysozyme	Sigma
Micrococcal nuclease	Sigma
Polynucleotide kinase (T4)	Gibco/BRL
Ribonuclease A	Sigma
Restriction endonucleases	Gibco/BRL, Boehringer Mannheim, New England Biolabs, Pharmacia
Rabbit IgG	Sigma
Trypsin (0.5%), 5.3 mM EDTA	Gibco/BRL
T4 gene 32 protein	Bio-Rad

2.3 Antibodies

The rabbit anti-HSV-1 antibody was bought from Dakopatts (Denmark). The horseradish peroxidase- and the fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies were bought from Cappel Lab. The 1 nm gold-conjugated goat anti-rabbit antibody was from Amersham International. The anti-VSV antibody was made by Mr. M. Butcher (McMaster University) by inoculating rabbits with purified and detergent treated VSV (serotype Indiana). The anti-CT antibody was made by Mr. D. Snoddy by inoculating rabbits with the synthetic peptide: NH_2-Y T Q V P N K D G D A D E D D L-COOH, which corresponds to the last 16 amino acids of the carboxy terminus of gB.

2.4 Oligodeoxyribonucleotides

The oligodeoxyribonucleotides used for DNA sequencing and in vitro mutagenesis are shown in Table 2.4.1. The M13/pUC universal primer was purchased from United States Biochemical. The primer Prm5 was synthesised by Dalton Chemical Laboratories Inc, North York, Ont. All the other primers were synthesized by the Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton.

2.5 Multicomponent systems

In vitro mutagenesis was performed by using the protocols and reagents provided by the Muta-Gene^R M13 In Vitro Mutagenesis Kit of Bio-Rad. DNA sequencing was performed using the protocols and reagents provided by the Sequenase^R Version 1.0 and

2.0 Kit of United States Biochemical. The protocols and reagents used to isolate and purify specific DNA fragments from agarose gels were provided by the GeneecleanII^R Kit of Bio 101 Inc.

Table 2.4.1 Oligodeoxyribonucleotides

Name	Oligodeoxyribonucleotide sequence (region of homology)	Purpose
M13/PUC Universal primer	5'-GTTTTCCCAGTCACGAC-3' (M13mP18: 6205-6221)	Sequencing spliced regions of gB-G
M13/PUC Reverse primer	5'-CAGGAAACAGCTATGAC-3' (M13mp18: 6205-6221)	Sequencing spliced regions of G-gB-2, Gm6 and gB-G
Prm1	5'-CACGACCTGCGCTTC-3' (gB: 2681-2695)	Sequencing spliced regions of gB-tmG and Δ12CT799
Prm2	5'-CACGCCGACGCCGCGGTCGGCAA-3' (gB: 2717-2728 and 2789-2799)	Sequencing spliced regions of G-tmgB
Prm3	5'-GCCTTCTTCGCCTTTCGAGTTGGTATCCATCTT-3' (gB:2918-2932 and G:1476-1493)	Construction of G-tmgB
Prm4	5'-GTCATCCACGCCGACAACCCCTTTGGGGCGCT-3' (gB:2711-2725 and 2864-2880)	Construction of Δ12CT799
Prm5	5'-TCTTGGTTCTCCGATACGTCATGCGGCT-3' (G:1465-1478 and gB:2936-2949)	Construction of gB-tmG

2.6 Plasmids, cDNAs and bacteriophages

The plasmids pGEM-4Z and pUC118 were purchased from Promega (Fisher Scientific) and Gibco/BRL respectively. The eukaryotic expression vectors pXM and p91023 were kindly provided by Dr. G. G. Wong (Genetics Institute Inc, Cambridge MA) and Dr. R.J. Kaufman (Genetics Institute Inc, Cambridge MA). The vector pSVGL

encoding the VSV G cDNA was a generous gift of Dr J. K. Rose (Yale University, School of Medicine, New Haven CT). The fragment from HSV-1 KOS strain, encoding glycoprotein B, was kindly provided by Dr. J. Smiley (McMaster University). Phage M13mp18 double stranded DNA (replicative form I (RFI)) was purchased from Gibco/BRL and Bio-Rad. Phage M13mp18 single stranded DNA was purchased from United States Biochemical. Phage lambda DNA (Clind1ts857 Sam7) was from Gibco/BRL.

2.7 Molecular weight (mol wt) markers

The DNA mol wt marker, One kilobase ladder, was purchased from Gibco/BRL. Three kinds of protein mol wt markers were used for SDS-PAGE. The most commonly used was bought from Sigma and was composed of the following proteins: bovine erythrocyte carbonic anhydrase (29 000 D), ovalbumin (45 000 D), bovine plasma albumin (66 000 D), rabbit muscle phosphorylase B (97 000 D), E. coli β-galactosidase (116 000 D), rabbit muscle myosin (205 000 D). This marker was used to estimate the mol wt of the mutant proteins by SDS-PAGE.

The second protein mol wt marker was from Gibco/BRL and was composed of the following proteins: lysozyme (14 300 D), β-lactoglobulin (18 400 D), bovine erythrocyte carbonic anhydrase (29 000 D), ovalbumin (43 000 D), bovine serum albumin (68 000 D), rabbit muscle phosphorylase B (97 000 D), myosin H-chain (200 000 D).

The third protein mol wt marker consisted of detergent treated VSV (serotype Indiana or Cocal) and labeled with [^{35}S]-methionine. This marker was prepared by Mrs M. Butcher and E. Sat (McMaster University). The following proteins are observed after SDS-PAGE and fluorography: M protein (29 000 D), NS and N proteins (49 000 and 50 000 D), G protein (69 000 (Indiana), 71 000 (Cocal)), L protein (190 000 D), (Toneguzzo, 1977; Capone et al., 1983).

2.8 Growth media and buffers

The composition of frequently used growth media and buffers is presented in Table 2.8.2.

2.9 Bacterial strains and culture conditions

Escherichia coli (E. coli) DH5α (supE44, ΔlacU169 (ϕ80lacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1) was purchased from Gibco/BRL.

E. coli Dm1 (dam-13::Tn9(Cm^R), dcm$^-$mcrB, hsdR$^-$M$^+$, gal1, gal2, ara$^-$, lac$^-$, thr$^-$, leu$^-$, ton^R, tsx^R, Su^O) was purchased from Gibco/BRL.

E. coli MV1190 (Δlac-pro AB), thi, supE, Δ(srl-recA)306::Tn10 (tet^r) [F':traD36, pro AB, lacI^{q}ZΔM15]) was purchased from Bio-Rad.

E. coli CJ236 (dut, ung, thi, relA; PCJ105(cam^{r}F1) was purchased from Bio-Rad.

The bacteria, unless otherwise specified, were grown in LB at 37°C with vigourous agitation in an incubator shaker Model G25 from New Brunswick Scientific.

Stocks of bacteria were kept frozen at -70°C in LB containing 15% glycerol. The liquid media, the plates and the top agar used to propagate CJ236 were supplemented with 30 μg/ml of chloramphenicol. The chloramphenicol was diluted from a stock solution of 30 mg/ml in absolute ethanol, that was kept at -20°C in the dark.

Table 2.8.2 Growth media and buffers

Buffer/ Medium	Composition
SOB	2% bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 10 mM $MgSO_4$
LB	1% bactotryptone, 0.5% yeast extract, 0.5% NaCl
2xYT	1.6% bactotryptone, 1.0% yeast extract, 0.5% NaCl
H	1.0% bactotryptone, 86 mM NaCl
Minimal	42.3 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl, 1 mM $MgSO_4$, 0.001% thiamine HCl, 0.2% glucose
TE	10 mM Tris-Cl(pH 8), 1 mM EDTA(pH 8)
TBE	89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3
PBS	137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4

2.10 Mammalian cells and culture conditions

COS-1 cells (Gluzman, 1981), were grown in high glucose Dulbecco's modified Eagle's Medium (D-MEM) supplemented with 7% calf serum or 7% fetal bovine serum

(FBS). CV-1 cells were grown in D-MEM supplemented with 7% (FBS). Baby Hamster Kidney 21 (BHK) cells were grown in D-MEM supplemented with 7% (FBS).

The D-MEM was made by diluting a packet of D-MEM powder (high glucose, Gibco/BRL) in 9.6 litre of distilled water. 100 ml of vitamin (100X), 100 ml of non-essential amino acids (100X), and 200 ml of 400 mM L-glutamine were added and the medium was sterilized by filtration. 450 ml were transferred into 500 ml glass bottles, and the following were added into each bottle: 35 ml of serum, 5 ml Penicillin (5000 U/ml)/Streptomycin (5 mg/ml), 5 ml of 1 M HEPES(pH 7.3), and 10 ml of 0.89 M $NaHCO_3$.

Monolayer cultures of cells were propagated in 10 cm diameter plates at 37°C under an atmosphere of 5% CO_2. The cells were split when they reached confluence as follows: the medium was removed, the monolayer was washed with PBS, and 1 ml of 0.05% trypsin, 0.53 mM EDTA (diluted in PBS) was added. After 1 to 3 min, the trypsin was removed, the cells were detached by knocking the plate several times, and they were resuspended in 10 ml of medium. Aliquots of cells were then dispersed into plates containing fresh medium.

Cell stocks were kept frozen in medium supplemented with 10% dimethyl sulfoxide (DMS0) in liquid nitrogen.

2.11 Viruses

The recombinant adenoviruses expressing gB-1 (AdgB-2, Johnson et al., 1988b)

or VSV G (AdG12, Schneider et al., 1989) were kindly provided by Dr. D. Johnson (McMaster University) and Dr. L. Prevec (McMaster University) respectively.

Herpes simplex virus type 1 (KOS Strain) was provided by Dr J. Smiley (McMaster University). VSV (serotype Indiana) was kindly provided by Dr. L. Prevec (McMaster University).

2.12 Recombinant DNA technology

Basic recombinant DNA techniques were employed to construct chimeric proteins, synthesize DNA, and subclone DNA fragments into specific shuttle or expression vectors. These techniques are described in Sambrook et al., (1989), and unless indicated, the protocols used are essentially those appearing in this excellent laboratory manual. Detail flow charts of the approach used to construct the chimeric proteins are presented in Section 3.3.

2.13 Restriction enzyme digestions

Restriction enzymes were used at the temperatures and with the buffers recommended by the manufacturers. The DNA (300 ng to 40 μg) was digested in the presence of a two to five fold excess of enzyme, at a concentration ranging from 30 to 400 ng/μl for 1 to 18 h. When more than one enzyme was used, the enzymes requiring similar buffers were used simultaneously, while the enzymes requiring different buffers were used consecutively. Following the first digestion, heat sensitive enzymes were

denatured by incubation for 10 min at 65°C, while heat resistant enzymes were denatured by extraction with phenol/chloroform and chloroform (Section 2.17.3). The DNA was then recovered by ethanol precipitation (Section 2.18) and resuspended in the appropriate buffer for digestion with the next enzyme.

2.14 Analytical agarose gel electrophoresis

Restriction digests were analyzed by electrophoresis in 0.6 to 1.5% agarose gels in TBE containing 0.5 μg/ml ethidium bromide. The agarose gel apparatuses from Bio-Rad (Mini-subTM and Wide mini-subTM DNA Cell) and from Hoefer Scientific instruments (Minnie and Max submarine units, model HE 33 and 99) were used for electrophoresis. Before electrophoresis, 1/5 vol of loading buffer 6X was added to the DNA samples (6X loading buffer is: 0.25% bromophenol blue, 0.25% xylene cyanol, 15% ficoll (type 400)) and 5 to 20 μl of samples were loaded on each lane. Phage lambda DNA, digested with HindIII (2 μg/lane), or 1 kb DNA Ladder (2 μg/lane), were used as mol wt markers. The DNA was visualized by exposing the gels to short waves of ultraviolet light, and pictures were taken with a Polaroid MP-4 Lad Camera, using black and white Polaroid films no 667.

2.15 Dephosphorylation of DNA

To reduce recircularization of the vector DNA during ligation, opened vectors with cohesive or blunt ends were dephosphorylated using calf intestinal alkaline

phosphatase (CIP). One to 5 μg of opened plasmid were incubated with 1 to 4 U of CIP in 50 mM Tris-Cl(pH 9), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine, or in One Phor All Buffer (10 mM Tris-acetate(pH 7.5), 10 mM magnesium acetate, 50 mM potassium acetate; Pharmacia). Protruding 5' termini were dephosphorylated by incubation at 37°C for 30 min, while blunt ends and recessed 5' termini were incubated for an extra 30 min at 56°C. After dephosphorylation, CIP was heat inactivated for 15 min at 68°C in the presence of 0.5% SDS and 1 mM EDTA. The solution was then extracted with phenol/chloroform and chloroform (Section 2.17.3), and the DNA was recovered by ethanol precipitation (Section 2.18).

2.16 Production of blunt ends

Recessed 3' termini were filled in by the 5'→ 3' polymerase activity of the large fragment of E. coli DNA polymerase I (Klenow fragment), while protruding 3'termini were digested by the 3'→ 5' exonuclease activity of this enzyme. One to 20 μg of DNA were incubated with 0.1 U/μl of Klenow fragment in the same buffer used for restriction digestion, or in One Phor all Buffer (10 mM Tris-acetate(pH 7.5), 10 mM magnesium acetate, 50 mM potassium acetate; Pharmacia) supplemented with 0.1 mM of the four dNTP for 30 min at room temperature. The enzyme was then heat inactivated for 10 min at 65°C in the presence of 25 mM EDTA, and extracted with phenol/chloroform and chloroform (Section 2.17.3). The DNA was recovered by ethanol precipitation (Section 2.18).

2.17 Extraction of DNA

2.17.1 Preparation of phenol

Crystalline phenol was distilled and stored at -20°C. One mg/ml of 8-hydroxyquinoline was diluted into melted phenol at 65°C. The phenol was then extracted once with 1 vol of 1 M Tris-Cl(pH 8) and several times with 1 vol of 0.1 M Tris-Cl(pH 8) containing 0.2% β-mercaptoethanol until the pH of the aqueous phase reached 7.4. The equilibrated phenol was kept in the cold room under one vol of 0.1 M Tris-Cl(pH 8) containing 0.2% β-mercaptoethanol.

2.17.2 Phenol extraction

The extractions were done in 1.5 ml microcentrifuge tubes. One vol of solution was mixed by vortexing with an equal vol of equilibrated phenol. The organic and aqueous phases were separated by centrifugation for 30 sec at 16 000 x *g*, and the aqueous phase (the top layer) was then transferred to a fresh tube.

2.17.3 Phenol/chloroform extraction

The extractions were done as described above, except an equal vol of phenol:chloroform:isoamyl alcohol (1:1:1/48) or an equal vol of chloroform:isoamyl alcohol (1:1/48) was used.

2.18 Ethanol Precipitation

One tenth vol of 2.5 M sodium acetate and two vol of absolute ethanol were mixed with one vol of DNA. After 10 to 20 min incubation at room temperature or at 4°C, the DNA was recovered by centrifugation at 16 000 x *g* for 15 min at 4°C. The supernatant was carefully removed, the DNA pellet was resuspended in 500 μl of ice-cold 70% ethanol and centrifuged at 16 000 x *g* for 5 min. As much ethanol as possible was removed and the DNA pellet was dried in a desiccator.

2.19 Preparative agarose gel electrophoresis

The DNA fragments (insert and vector) utilised for the ligation were first purified by preparative agarose gel electrophoresis. The DNA was recovered from the agarose using low melting point agarose or adsorption to silica.

2.19.1 Low melting point agarose

Following digestion with restriction enzymes, DNA fragments were separated by agarose gel electrophoresis as described in Section 2.14, except that the gel was made with low melting point agarose. At the end of the electrophoresis, the DNA was visualized in long wave lenght ultraviolet light and the gel portions containing the required DNA fragments were excised with a razer blade and transferred into 1.5 ml microcentrifuge tubes. One vol of TE was added, and the agarose was melted by incubation at 65°C for 15 min. The agarose was removed by two consecutive phenol

extractions, followed by phenol/chloroform, and chloroform extraction (Section 2.17). The DNA was recovered by ethanol precipitation (Section 2.18) and resuspended in 10 to 20 μl of TE. An aliquot was used to estimate the DNA concentration by analytical agarose gel electrophoresis using known amounts of DNA as standard (Section 2.14).

2.19.2 Adsorption to silica

The protocols and reagents were provided by the GenecleanII^R Kit of Bio 101 Inc. Following digestion with restriction enzymes, DNA fragments were separated by agarose gel electrophoresis as described in Section 2.14. The DNA was visualized in long wave lenght ultraviolet light and the bands containing the required DNA fragments were excised with a razor blade and transferred into 1.5 ml microcentrifuge tubes. Half a vol of TBE modifier (Bio 101) and 4.5 vol of 6 M sodium iodide (Bio 101) were added to the agarose which was dissolved by heating at 55° for 5 min. Five μl of silica matrix (Glassmilk^R, Bio 101) were added, and the tubes were incubated at room temperature for 5 min with frequent agitation. The complex DNA-silica was recovered by centrifugation at 16 000 x *g* for 15 sec and washed three times with 700 μl of an ice-cold solution of salt and ethanol (New Wash, Bio 101). The complex DNA-silica was resuspended in 5 μl of TE and the DNA was eluted by incubation at 55°C for 5 min. The tubes were centrifuged at 16 000 x *g* and the supernatants were transferred to fresh microcentrifuge tubes. The elution was repeated a second time with 5 μl of TE and the supernatants were pooled. An aliquot was used to estimate the DNA concentration by analytical agarose gel

electrophoresis using known amounts of DNA as standard (Section 2.14).

2.20 Ligation

A total mass of about 200 ng of DNA (insert and vector), diluted in a final vol of 10 μl, were used for the ligation. Two moles of vector for one mole of insert were usually used. For ligation of cohesive termini, the DNA was incubated for 18 h at 16°C in 25 mM Tris-Cl(pH 7.8), 10 mM $MgCl_2$, 0.4 mM ATP, 0.03% β-mercaptoethanol containing 0.1 U/μl of T4 DNA ligase. For ligation of blunt ends, the DNA was incubated for 2 h at room temperature in 50 mM Tris-Cl(pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 25% (wt/vol) polyethylene glycol-8000 containing 0.1 U/μl of T4 DNA ligase.

2.21 Transformation of bacteria

Aliquots of frozen competent cells (200 μl) in 1.5 ml microcentrifuge tubes were thawed on ice (see Section 2.2.22 for preparation of competent cells). When the cells were liquid, 5 μl of ligation mixture or 1 to 10 ng of supercoiled plasmid were added and mixed. The cells were incubated on ice for 10 to 30 min, heat shocked by incubation for 90 sec in a 42°C water bath, and returned back on ice for 5 min. Eight hundred μl of LB at room temperature were added, and the cells were incubated at 37°C with moderate agitation for 40 min. At the end of the recovery period, 10 to 200 μl of bacteria were

plated on ampicillin plates (LB containing 1.5% agar (wt/vol) and 50 μg/ml of ampicillin) or on tetracycline plates (LB containing 1.5% agar (wt/vol) and 15 μg/ml of tetracycline), and incubated upside down at 37°C for 18 h. The antibiotics were diluted from stock solutions of 25 mg/ml ampicillin in water and 12.5 mg/ml tetracycline in 50% ethanol, that were stored in the dark at -20°C.

2.22 Preparation of competent cells

2.22.1 Rubidium chloride method

Competent cells were prepared using the rubidium chloride procedure described by Hanahan (1985). Frozen E. coli DH5α or Dm1 were streaked on a SOB plate (SOB containing 1.5% agar) and incubated for 18 to 24 h at 37°C. Several 1 to 2 mm colonies were picked with a wooden applicator stick and dispersed into 1 ml of SOB that was used to inoculate 100 ml of SOB in a 1 litre flask. The culture was incubated at 37°C with vigourous agitation until the OD_{550} reached 0.4. The culture was cooled down on ice and transferred into two sterile 50 ml co-polymer tubes and centrifuged at 1000 x *g* for 15 min at 4°C. All subsequent manipulations were done at this temperature. The supernatants were removed carefully and the cells were resuspended in a total vol of 30 ml of filter sterilized Solution I (100 mM rubidium chloride, 50 mM $MnCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$, 15% glycerol (wt/vol), pH adjusted to 5.8 with 0.2 M acetic acid). The cells were incubated for 15 min on ice, and they were recovered by centrifugation at 1000 x *g* for 15 min. The supernatants were removed carefully and the

cells were resuspended in a total vol of 8 ml of filter sterilized Solution II (10 mM MOPS(pH 6.8), 10 mM rubidium chloride, 75 mM $CaCl_2$, 15% glycerol (wt/vol). The cell suspension was incubated for 30 min on ice, aliquots (200 μl) were dispensed into chilled 1.5 ml microcentrifuge tubes and flash frozen in liquid nitrogen. The competent cells were stored at -70°C. The efficiency varied from 10^6 to 10^7 transformed colonies/μg of DNA.

2.22.2 Calcium Chloride method

This method is described in Sambrook et al. (1989) and it is a modification of the procedure of Cohen et al. (1972). One ml of an overnight grown culture of DH5α in LB was used to inoculate 100 ml of LB in a 1 litre flask. The cell culture was grown at 37°C with vigorous agitation until the OD_{550} reached 0.4. The culture was then cooled down on ice and transferred into two sterile 50 ml co-polymer tubes. The bacteria were recovered by centrifugation at 1000 x *g* for 15 min at 4°C. All subsequent manipulations were done at this temperature. The supernatants were removed carefully. The cells were resuspended in a total vol of 20 ml of ice-cold 0.1 M $CaCl_2$ and centrifuged at 1000 x *g* for 15 min. The supernatants were removed and the cells were resuspended in a total vol of 4 ml of ice-cold 0.1 M $CaCl_2$. An aliquot of 140 μl of DMSO was added and mixed gently. After 15 min on ice, a second 140 μl aliquot of DMSO was added and 200 μl of cells were transferred into chilled 1.5 ml microcentrifuge tubes and flash frozen in liquid nitrogen. The competent cells were stored at -70°C. The efficiency varied from

10^5 to 10^6 transformed colonies/μg of DNA

2.23 Small-Scale preparation of plasmid DNA

Two different procedures, lysis by alkali and lysis by boiling, were used to isolate plasmid from E. coli. Both methods are described in Sambrook et al. (1989).

2.23.1 Lysis by alkali

This method is a modification of the procedure of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981). Well isolated colonies of transformed E. coli were picked with a sterile wooden applicator stick and transferred into 1.5 x 12.5 cm glass tubes containing 5 ml of LB supplemented with the appropriate antibiotic (50 μg/ml ampicillin or 15 μg/ml tetracycline). The cultures were grown overnight at 37°C with vigorous agitation. Aliquots of 1.5 ml were then transferred into 1.5 ml microcentrifuge tubes, and the cells were recovered by centrifugation at 16 000 x *g* for 30 sec at 4°C.

The supernatants were carefully removed and each pellet was resuspended in 100 μl of ice-cold solution I (50 mM glucose, 25 mM Tris-Cl(pH 8), 10 mM EDTA(pH 8). Aliquots of 200 μl of freshly prepared solution II at room temperature (0.2 N NaOH, 1% SDS) were added to the tubes, which were mixed by inversion followed by incubation on ice for 5. Aliquots of 150 μl of an ice-cold Solution III (60% 5 M potassium acetate, 11.5% acetic acid) were then added to the tubes, which were vortexed in an inverted position for 10 sec and incubated on ice for 5 min. The tubes were then centrifuged at

16 000 x *g* for 5 min at 4°C. The supernatants were transferred into fresh tubes and extracted once with phenol/chloroform (Section 2.17.3). The DNA was recovered by ethanol precipitation as described in Section 2.18, except that no extra salt was added. The DNA pellets were resuspended in 50 μl of TE buffer containing 20 μg/ml of ribonuclease A (Section 2.2.24). The plasmids were analyzed by digestion with restriction enzymes as described in Section 2.13 using 4 μl of DNA per digestion.

2.23.2 Lysis by boiling

This method is a modification of the procedure described by Holmes and Quigley (1981). Bacteria were grown and harvested as described in Section 2.23.1. The pellets were resuspended at room temperature in 300 μl of STET (0.1 M NaCl, 10 mM Tris-Cl(pH 8.0), 1 mM EDTA (pH 8.0), 5% Triton-100). Aliquots of 25 μl of lysozyme (10 mg/ml in 10 mM Tris-Cl(pH 8.0)) were added and mixed by vortexing. The tubes were immediately boiled for 90 sec in a water bath, centrifuged at room temperature for 10 min at 16 000 x *g*, and the pellets were removed using wooden applicator sticks. The DNA was precipitated by the addition of 40 μl of 2.5 M sodium acetate(pH 5.2) and 420 μl of isopropanol followed by 5 min incubation at room temperature. The DNA was recovered by centrifugation at 16 000 x *g* for 5 min at 4°C. The DNA pellets were washed with 70% ethanol and dried as described in Section 2.18. The DNA was then resuspended in 50 μl of TE containing 20 μg/ml of ribonuclease A (Section 2.24) and aliquots of 4 μl were analyzed by digestion with restriction enzymes as described in

Section 2.13.

2.24 Preparation of ribonuclease A

A stock solution of ribonuclease A was prepared by incubating 10 mg/ml of ribonuclease A diluted in 10 mM Tris-Cl(pH 7.5), 15 mM NaCl, in a boiling water bath for 10 min. The solution was cooled down slowly at room temperature, and aliquots of 5 μl were dispensed into 1.5 ml microcentrifuge tubes and stored at -20°C.

2.25 Large scale preparation of plasmid DNA

This method, which can be used to isolate mg quantities of plasmid DNA, is similar to the one described in Section 2.23.1. Six ml of LB in a 1.5 x 12.5 cm glass tube supplemented with the appropriate antibiotic (50 μg/ml ampicillin or 15 μg/ml tetracycline) were inoculated with a well isolated colony of bacteria. The culture was incubated for 18 h at 37°C with vigorous agitation, and 1 ml was used to inoculate 250 or 500 ml of LB supplemented with the appropriate antibiotic (50 μg/ml ampicillin or 15 μg/ml tetracycline). After incubation with vigorous agitation for 18 h at 37°C, the culture was cooled down on ice, transferred into 250 ml bottles and centrifuged at 2 500 x *g* using a Sorval rotor type GSA for 15 min at 4°C.

The supernatants were removed. The cell pellets were resuspended using 5 ml of ice-cold solution I (50 mM glucose, 25 mM Tris-Cl(pH 8.0), 10 mM EDTA) for each 250 ml of culture by vortexing and by scraping with a sterile wooden applicator stick.

The cells were then transferred into 50 ml centrifuge tubes (5 ml/tube) and 10 ml of fresh Solution II (0.2 N NaOH, 1% SDS) at room temperature were added. The solutions were mixed by inversion, incubated 10 min on ice, and 7.5 ml of an ice-cold solution III (60% 5 M potassium acetate, 11.5% acetic acid) were added to each tube. The solutions were mixed by inversion, incubated 10 min on ice, and centrifuged at 17 000 x *g* using a Sorval rotor type SS-34 for 20 min at 4°C.

The supernatants were transferred and filtered with two layers of cheesecloth into 50 ml centrifuge tubes. The DNA was precipitated by the addition of 0.6 vol of isopropanol (about 12 ml) followed by incubation for 15 min at room temperature. The DNA was then recovered by centrifugation at 17 000 x *g* using a Sorval rotor type SS-34 for 20 min at room temperature. The supernatants were discarded, the DNA pellets were washed with 5 ml of ice-cold 70% ethanol, dried in a desiccator, and resuspended in 4 ml of TE.

2.26 Centrifugation in cesium chloride gradients

The DNA isolated in the previous section was further purified by equilibrium centrifugation in cesium chloride gradients. A mass of 4.4 g of cesium chloride was added to each 4 ml of DNA. After complete dissolution, 0.8 ml of ethidium bromide (10 mg/ml) was added to every 10 ml of solution, which was then transferred into 13 x 51 mm Quick-Seal Beckman centrifuge tubes using a 5 ml syringe. The tubes were then centrifuged with a Beckman vertical rotor type VTi 65 at 55 000 RPM (275 000 x *g*) for

16 to 18 h at 25°C.

The closed circular plasmid, which formed a thick red band visible under day light, was removed by sucking the band with a 5 ml syringe. The DNA (0.5 to 1 ml) was transferred into 1.7 10 cm polypropylene tubes and extracted several times with an equal vol of 1-butanol equilibrated with water. The extractions were repeated until the organic and aqueous phases became colourless. The DNA was diluted five times (according to the original vol before extraction) with TE and recovered by ethanol precipitation as described in Section 2.18, except the centrifugation was done in 1.7 x 10 cm polypropylene tubes using a Sorval rotor type SS-34 at 8000 x *g*. The DNA was washed with 5 ml of 70% ice-cold ethanol, centrifuged for 10 min as described above, dried and resuspended in 500 μl of TE.

The DNA concentration was estimated by measuring the OD_{260} of aliquots diluted in TE using quartz cuvettes. The DNA concentration was calculated using an absorbance ($A^{1\%1cm}$) of 200 for double stranded DNA (OD_{260} of 1 = 50 μg/ml).

2.27 DNA sequencing

The nucleotide sequence of the mutated regions was determined by the chain termination method developed by Sanger et al. (1977). The protocols, the chemicals and the modified T7 DNA polymerase (Sequenase version 1.0 or 2.0) used for sequencing, except for the radioisotopes, were purchased from United States Biochemical. Single stranded DNA and double stranded DNA were sequenced. In order to sequence double

stranded DNA, the two complementary strands were first separated to allow the hybridization of the primer as described below.

2.27.1 Double stranded DNA preparation

This protocol is a modification of the procedure described by Zhang et al. (1988). Three μg of cesium chloride purified plasmid (Section 2.26) were resuspended in 18 μl of water in a 1.5 ml microcentrifuge tube. One μl of 4 N NaOH and 1 μl of 4 mM EDTA were added and the tube was incubated for 5 min at room temperature. The solution was neutralized by the addition of 2 μl of 2 M ammonium acetate(pH 4.6). The tube was transferred on ice, and the DNA was recovered by ethanol precipitation as described in Section 2.18, except that no extra salt was added.

2.27.2 Single stranded DNA preparation

A culture of E.coli MV1190 was infected with M13 and incubated for 5 to 6 h as described in Section 2.28.2. One ml of cell culture was transferred into a 1.5 ml microcentrifuge tube and centrifuged at 16 000 x *g* for 10 min at 4°C. The supernatant was transferred to a fresh tube and the phages were precipitated by the addition of 1/9 vol of 40% polyethylene glycol 8000 and 1/9 vol of 5 M sodium acetate(pH 7). The tube was incubated on ice for 15 min and the phage precipitate was recovered by centrifugation at 16 000 x *g* for 10 min at 4°C. The supernatant was removed carefully and the pellet was resuspended in 200 μl of TE.

The solution was extracted with phenol, phenol/chloroform and chloroform (Section 2.17). The single stranded DNA was precipitated by the addition of 0.1 vol of 3 M sodium acetate and 2.5 vol of absolute ethanol. After 30 min incubation at -70°C, the single stranded DNA was recovered by centrifugation at 16 000 x *g* for 15 min at 4°C. The supernatant was removed, the pellet was resuspended with 500 µl of 70% ethanol and centrifuged again for 10 min. The ethanol was removed, the pellet was dried in a desiccator and resuspended in 20 µl of TE. An aliquot was used to estimate the DNA concentration by analytical agarose gel electrophoresis using known amounts of single stranded DNA as standard (Section 2.14).

2.27.3 Sequencing reaction

In a 1.5 ml microcentrifuge tube, 1 µg of single stranded DNA or 3 µg of denatured double stranded DNA were diluted with 0.5 pmol of primer in 10 µl of 40 mM Tris-Cl(pH 7.7), 20 mM $MgCl_2$, 50 mM NaCl. The tube was heated for 2 min at 65°C, then slowly cooled down over a period of 30 min until the temperature reached 30°C. The tube was then centrifuged for 30 sec at 16 000 x *g* and transferred on ice.

To the annealed template-primer, 1.0 µl of 0.1 M DTT, 2.0 µl of diluted labeling mix (1.5 µM of each dGTP, dCTP and dTTP), 0.5 µl of [α-^{35}S]dATP and 2 µl of modified T7 DNA polymerase (Sequenase, 1.6 U/µl) were added. The solution was incubated for 5 min at room temperature and transferred into 4 tubes labeled G, A, T, C, (3.5 µl per tube) containing 2.5 µl of termination mixture (80 µM of each of the four

dNTP, 50 mM NaCl and 8 μM of one of the four ddNTP: tube G contained ddGTP, tube A contained ddATP etc...). The four tubes were incubated for 5 min at 37°C and the reaction was stopped by the addition of 4 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05%ylene cyanol FF). The tubes were kept at -20°C.

The above protocol was developed for reading sequences up to 300-400 bp from the primer. When the sequence of interest was less then 100 bp away from the primer, an aliquot of 1 μl of 0.15 M sodium isocitrate, 0.1 M $MnCl_2$ was added to the reaction mixture before adding the enzyme.

Compression problems were observed when sequencing the TM of gB. In order to eliminate these artifacts, the reaction was done by replacing the dGTP of the diluted labeling mix with 3 μM of dITP, and 80 μM dITP was used instead of 80 μM dGTP in the four termination mixtures.

2.27.4 Electrophoresis of sequencing products

Before electrophoresis, the samples were heated for 2 min at 80°C and centrifuged for 30 sec. Two to three μl were analyzed by electrophoresis in 6% polyacrylamide gels (5.7% acrylamide (wt/vol), 0.3% N,N-methylene bisacrylamide (wt/vol), 7 M Urea) made in TBE. The samples were separated for 2 to 6 h at a power of 60 Watts (1 500 volt and 30 mA) using the electrophoresis apparatus Model S2 from BRL. The gels were fixed for 15 min in 5% acetic acid, 15% methanol, blotted on filter

papers (Whatman no 1), and dried at 80°C for 30 to 45 min with a slab gel dryer Model 483 from Bio-Rad. The gels were then exposed at room temperature for 16 to 72 h to Kodak films (XAR-5).

2.28 Site directed mutagenesis

Deletion of specific gene portions were generated by site directed mutagenesis using the method of Kunkel et al. (1987). The protocols, chemicals, enzymes and bacteria were obtained from the Bio-Rad M13 In Vitro Mutagenesis Kit.

2.28.1 Subcloning into phage M13

The bacteriophage M13mp18 was used as a vector to produce the single stranded DNA essential to carry the mutagenesis reaction. The DNA fragments encompassing the region to be mutated were isolated and inserted into the multiple cloning site of the circular double stranded DNA (RFI) of M13mp18 as described in Sections 2.2.12 to 2.2.20. When the ligation reaction was completed, 5 μl were used to transform 250 μl of competent cells (E. coli MV1190) as described in Section 2.21, except that the cells were heat shocked for 3 min at 42°C. After 5 min on ice, 10, 50 and 100 μl of cells were transferred into 1.5 x 12.5 cm glass tubes containing 300 μl of overnight culture of MV1190 (Section 2.28.3). Aliquots of 2.5 ml of melted H top agar (H medium containing 0.7% agar (wt/vol) at 55°C, supplemented with 400 μg/ml Xgal and 0.8 mM IPTG, were then added into each tube. After a brief agitation, the contents of the tubes

were poured quickly on top of H plates (H medium containing 1.5% agar (wt/vol). The plates were incubated 10 min at room temperature, then for 12 to 16 h at 37°C in an inverted position.

2.28.2 Phage and double stranded DNA isolation

White plaques, produced by recombinant bacteriophages, were transferred using plugs made with sterile pasteur pipettes into 1.5 x 12.5 cm glass tubes containing 2 ml of LB and 50 μl of overnight culture of MV1190 (Section 2.28.3). The tubes were then incubated with vigorous agitation for 5 to 6 h at 37°C. One ml of culture was transferred into a 1.5 ml microcentrifuge tube and centrifuged at 16 000 x *g* for 3 min at 4°C. The supernatant containing the phage was transferred into a fresh 1.5 ml microcentrifuge tube and stored at 4°C for further studies.

The circular double stranded DNA of recombinant M13 (RFI) was isolated from the cell pellet using the alkaline lysis method as described in Section 2.23.1. After ethanol precipitation, the DNA was resuspended into 20 μl of TE containing 20 μg/ml of ribonuclease A (Section 2.24). Aliquots of 4 μl were analyzed by digestion with restriction enzymes as described in Section 2.13. The undigested DNA was stored at -20°C.

2.28.3 Overnight culture of MV1190

Frozen E. coli, strain MV1190, were streaked on a Minimal agar plate (Minimal

medium containing 1.5% agar (wt/vol)). After incubation for two days at 37°C, well isolated colonies were transferred into 20 ml of LB and incubated for 16 h at 37°C with vigorous agitation. After a week, the culture of MV1190 was discarded and a fresh batch was prepared again.

2.28.4 Preparation of competent cells

In a 2 litter flask, 250 ml of LB were inoculated to an OD_{600} of 0.1 with an overnight culture of MV1190 (Section 2.28.3). The cells were incubated at 37°C with vigorous agitation until the OD_{600} reached 0.9. The culture was cooled down on ice, transferred into two sterile 250 ml bottles and centrifuged in a Sorval rotor type GSA at 1000 x *g* for 10 min at 4°C. All subsequent manipulations were done at this temperature. The supernatant was removed carefully and the cells were resuspended in a total vol of 50 ml of ice-cold 0.1 M $MgCl_2$. The cells were centrifuged as described above, and the pellet was resuspended in 100 ml of ice-cold 0.1 M $CaCl_2$. After 30 min on ice, the cells were recovered by centrifugation as described above and resuspended in a total vol of 12.5 ml of 85 mM $CaCl_2$, 15% glycerol. Aliquots of 0.6 ml were transferred into 1.5 ml microcentrifuge tubes and flash frozen in liquid nitrogen. The competent cells were stored at -70°C.

2.28.5 Titration of the phages

Stocks of recombinant phages containing the proper insert, prepared and isolated

as described in Section 2.28.2, were titered. Aliquots of 300 μl of overnight culture of MV1190 in 1.5 x 12.5 cm glass tubes, were infected with 100 μl of phage stock diluted 10^4, 10^6, 10^8, and 10^{10} in LB. After addition of the phages, the tubes were vortexed briefly and incubated 5 min at room temperature. Aliquots of 2.5 ml of melted LB top agar (LB containing 0.7% agar (wt/vol)) at 55°C were added to each tube. After a brief agitation, the contents were poured on top of LB plates (LB containing 1.5% agar (wt/vol)). The plates were incubated for 10 min at room temperature and then for 16 to 18 h at 37°C in an inverted position. The number of plaques on each plate was determined and the titer of the phage stock was calculated.

2.28.6 Growth of uracil-containing phages

Five ml of LB supplemented with 30 μg/ml of chloramphenicol were inoculated with one well isolated colony of E. coli CJ236 from a chloramphenicol LB plate (LB containing 1.5% agar (wt/vol) and 30 μg/ml chloramphenicol). The culture was incubated for 16 to 18 h at 37°C with vigorous agitation, and one ml was used to inoculate 50 ml of 2xYT supplemented with 30 μg/ml of chloramphenicol. The culture was grown with vigorous agitation until an OD_{600} of 0.3 was reached, which corresponds approximately to 1 x 10^7 CFU/ml. The bacteria were infected with the recombinant phage at a MOI of 0.1, and the culture was grown for 5 to 6 h.

The culture was cooled down on ice. Forty ml were transferred into a 50 ml tube, which was centrifuged in a Sorval rotor type SS-34 at 17 000 x *g* for 15 min at 4°C. The

supernatant containing the recombinant phages was transferred into a fresh 50 ml tube and the centrifugation was repeated. The supernatant was transferred to a fresh tube and the phages were titered on E. coli MV1190 and CJ236. The titration was done as described in Section 2.28.5, except that the plates and the top agar were supplemented with 30 μg/ml of chloramphenicol and an overnight culture of CJ326 was used when the titration was done on CJ326. A good batch of uracil-containing phages had a titer at least 10^4 higher on CJ326.

2.28.7 Isolation of the template

The solution of uracil-containing phage (about 40 ml) was incubated at room temperature for 30 min with 150 μg of ribonuclease A (Section 2.24). The phages were precipitated by the addition of 0.25 vol of 3.5 M ammonium acetate containing 20% (wt/vol) of polyethylene glycol 8000. After 30 min incubation on ice, the phages were recovered by centrifugation in a Sorval rotor type SS-34 at 17 000 x *g* for 15 min at 4°C. The supernatant was removed. The small whitish phage pellet was resuspended in 200 μl of high salt buffer (300 mM NaCl, 100 mM Tris-Cl(pH 8.0), 1 mM EDTA) and transferred into a 1.5 ml microcentrifuge tube. After a 30 min incubation on ice, the tube was centrifuged for 2 min at 16 000 x *g* and the supernatant was transferred into a fresh tube and kept at 4°C.

The 200 μl phage solution was extracted twice with phenol, once with phenol/chloroform, and several times with chloroform (Section 2.17). The solutions were

back extracted with 100 μl of TE and the supernatants were pooled. The single stranded DNA was precipitated by the the addition of 0.1 vol of 7.8 M ammonium acetate and 2.5 vol of ethanol, followed by incubation for 30 min at -70°C. The DNA was recovered by centrifugation at 16 000 x *g* for 15 min at 4°C. The supernatant was carefully removed. The DNA pellet was washed with 500 μl of ice-cold 90% ethanol, centrifuged for 10 min, dried in a desiccator and resuspended in 20 μl of TE. Aliquots were used to estimate the DNA concentration by analytical agarose gel electrophoresis using known amounts of single stranded DNA as standard (Section 2.14). About 20 μg of DNA were usually recovered from 50 ml of culture.

2.28.8 Phosphorylation of the primer

The oligodeoxyribonucleotides used as primer for the mutagenesis reaction were phosphorylated by T4 polynucleotide kinase. Two hundred pmol of primer were diluted in 30 μl of 100 mM Tris-Cl(pH 8) containing 10 mM $MgCl_2$, 5 mM DTT and 0.4 mM ATP in a 500 μl microcentrifuge tube. Five units of T4 polynucleotide kinase were added, and the solution was incubated at 37°C for 45 min. The reaction was stopped by heating at 65°C for 10 min. The primer was diluted with TE to a final concentration of 6 pmol/μl and the solution was stored at -20°C.

2.28.9 Annealing of the primer

In a 500 μl microcentrifuge tube, 200 ng of uracil-containing DNA (Section

2.28.7) and 2-3 pmol of phosphorylated primer were mixed in a final vol of 10 μl of annealing buffer (20 mM Tris-Cl(pH 7.4), 2 mM $MgCl_2$, 50 mM NaCl, Bio-RAd). The solution was incubated for 5 min in a 70°C water bath and then slowly cooled down over a period of 40 to 60 min until the temperature reached 30°C. The solution was then centrifuged for 30 sec at 16 000 x *g* and transferred on ice.

2.28.10 Synthesis of the complementary strand

To the ice-cold primer-template solution, 1 μl of 10x synthesis buffer (10X buffer is: 5 mM each dNTP, 10 mM ATP, 100 mM Tris-Cl(pH 7.4), 50 mM $MgCl_2$, 20 mM DTT; Bio-Rad), 1 μl of T4 DNA ligase (2-5 U/μl) and 1 μl of T4 DNA polymerase (1 U/μl) were added. In some cases to enhance the mutagenesis efficiency, 1 μl of T4 gene 32 protein (1 μg/μl) was added just before the the addition of the ligase and the polymerase. The reaction was subsequently incubated 5 min on ice, 5 min at room temperature, and 90 min at 37°C. At the end of the incubation period, 90 μl of stop buffer (10 mM Tris-Cl(pH 8), 10 mM EDTA) were added and the solution was stored frozen at -20°C.

Aliquots (9 μl) were analyzed by agarose gel electrophoresis as described in Section 2.14, except that TAE (40 mM Tris-acetate(pH 7.9), 2 mM EDTA) instead of TBE was used as buffer. Before electrophoresis, 1μl of 10X SDS-loading buffer (1% SDS, 0.25% bromophenol blue, 50% glycerol) was added. As controls, 20 ng of double stranded DNA (RFI) and 9 μl of mutagenesis reaction (without primer) were analyzed

simultaneously.

Competent MV1190 cells were then transformed with 10 to 40 μl of mutagenic reaction (Section 2.28.1). The next day, well isolated plaques were picked and grown, and the RFI DNA was isolated and analyzed for proper deletion by restriction enzymes as described in Sections 2.13 and 2.14. The nucleotide sequences of the spliced regions of the mutated cDNAs were confirmed by DNA sequencing using single stranded DNAs isolated as described in Section 2.27.2.

2.29 Preparation of anti-gB and anti-G antibodies

Polyclonal antibodies specific to glycoprotein B of HSV-1 and glycoprotein G of VSV were made by inoculating rabbits with recombinant adenoviruses expressing gB (ADgB-2 Johnson et al., 1988) and glycoprotein G (AdG12, Schneider et al., 1989). Rabbits (young white male, New Zealand) were injected subcutaneously with 0.5 ml of 10^8 PFU/ml of AdgB-2 or AdG12 diluted in PBS. The rabbits were injected three times, and the injections were separated by an interval of one to two weeks. One week after the last injection, the rabbits were killed and bled.

The immunoglobulin fraction of the blood was fractionated and concentrated by ammonium sulfate precipitation (Harlow and Lane, 1988). The blood was transferred into 50 ml co-polymer tubes and incubated at 4°C for 1 to 3 h until coagulation. The tubes were centrifuged at 2 000 x *g* for 10 min at 4°C and the clarified serum was transferred into a glass flask. All the subsequent manipulations were done at 4°C. Thirty-five ml of

serum were diluted with an equal vol of ice-cold PBS, and the immunoglobulins were precipitated by slow addition of 70 ml ice-cold saturated ammonium sulfate (700 mg/ml) while constantly stirring the mixture. After 30 min, the solution was transferred into two 250 ml bottles and the protein precipitate was recovered by centrifugation in a Sorval rotor type GSA at 2000 x *g* for 30 min. The supernatants were carefully removed. Each pellet was resuspended with 50 ml of ice-cold 50% saturate ammonium sulfate and centrifuged as described above. The protein precipitate was washed a second time with a total vol of 60 ml of ice-cold 50% saturated ammonium sulfate. The pellets were pooled, resuspended in 14 ml of ice-cold PBS and dialysed for 24 h against the same buffer at 4°C.

The protein concentration was determined as described in Section 2.32. The protein concentration of the anti-gB and anti-G antibodies were 1 mg/ml and 7 mg/ml respectively. The specificity of the two antibodies was checked by immunoprecipitation (Section 2.35) using lysate of cells infected with HSV-1 or VSV (Section 2.33), or lysate of cells transfected with pXM gB or pXM G (Section 2.34). The specificity of anti-gB was also verified by immunoblot (Section 2.38).

2.30 Affinity purification of anti-gB antibody

2.30.1 Preparation of the affinity column

The anti-gB antibody was affinity purified using a column made with a mixture of HSV-1 glycoproteins isolated by a modification of the procedure described by Pachl

et al. (1987). Ten plates (10 cm diameter) of subconfluent BHK cells were washed with PBS, and each plate was infected with 1 ml of D-MEM containing HSV-1 (KOS strain) at a MOI of 0.1 for 1 h at 37°C. The medium was removed, 10 ml of fresh D-MEM supplemented with 7% FBS were added to each plate, and the cells were incubated for 40 h at 37°C. The cells were detached by scraping gently with a policeman in 10 ml of medium. They were transferred into 50 ml co-polymer tubes, and recovered by centrifugation at 1 500 x *g* for 15 min at 4°C.

The pellets were washed with 5 ml of ice-cold PBS containing 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$ and centrifuged as described above. The pellets were pooled, resuspended into 5 ml of lysis buffer (20 mM Tris-Cl(pH 8), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml micrococcal nuclease, 1.0 mM PMSF, 100 U/ml aprotinin), and incubated at room temperature for 30 min with gentle rotation. The cell lysate was transferred into a 13 ml polycarbonate tube and centrifuged at 12 000 x *g* with a Beckman rotor type 70.1Ti for 30 min at 4°C. The supernatant was then applied to a 5 ml column of lentil lectin-Sepharose 4B. The column was washed with 15 ml of buffer A (0.25 M NaCl, 20 mM Tris-Cl(pH 8), 0.5% Nonidet P-40, 0.1 mM $MnCl_2$, 0.1mM $CaCl_2$). The glycoproteins were eluted with 20 ml of buffer A containing 0.2 M α-D-methylmannoside and collected into 1.5 ml microcentrifuge tubes. The presence of glycoproteins in the fractions was verified by SDS-PAGE and Coomassie blue staining (Section 2.36). The fractions containing the glycoproteins were pooled. The protein concentration was determined (Section 2.32), and

3 mg of protein were coupled to activated CNBr-Sepharose 4B (Harlow and Lane, 1988).

For coupling to Sepharose, 5.5 ml of HSV-1 glycoproteins (0.55 mg/ml) were dialysed at 4°C for 18 h against 0.1 M $NaHCO_3$(pH 8.3), 0.5 M NaCl, 0.5% Nonidet-P40. Meanwhile, 0.5 g of activated CNBr-Sepharose 4B was resuspended into 10 ml of 3 mM HCl in a 1.7 x 10 cm polypropylene tube and centrifuged at 1000 x *g* for 5 min. The supernatant was removed, the beads were washed a second time with the same vol of 3 mM HCl, and then two times with 10 ml of coupling buffer (0.1 M $NaHCO_3$(pH 8.3), 0.5 M NaCl). The HSV-1 glycoproteins were then combined with the beads, and incubated with constant rotation at room temperature for 2 h. The beads were then centrifuged at 1000 x *g* for 5 min. The supernatant was removed. The beads were resuspended in coupling buffer containing 0.2 M glycine, and incubated overnight at 4°C with constant rotation. The next day, the beads were washed once with 10 ml of coupling buffer, twice with 10 ml of 0.1 M acetate buffer(pH 4), 0.5 M NaCl, and once with 10 ml of PBS. The complex HSV-1 glycoprotein-Sepharose was kept at 4°C in PBS supplemented with 0.01% thimerosal.

2.30.2 Purification of anti-gB antibody

The antibody was affinity purified by combining in a 1.7 x 10 cm co-polymer tube, 1 ml of anti-gB, 4 ml of ice-cold PBS and the glycoprotein-Sepharose complex. The solution was incubated with constant rotation at 4°C for 45 min. The beads were then packed into a 5 ml column and washed with 20 ml of PBS. The bound antibodies

were then eluted with 6 ml of 0.2 M glycine-HCl(pH 2.6), and fractions of 600 μl were collected into 1.5 ml microcentrifuge tubes containing 200 μl of 1 M Tris-HCl(pH 8). The fractions were pooled and dialysed against PBS at 4°C for 24 h. The antibody was concentrated by covering the dialysis bag with sodium carboxymethylcellulose (Aquacide II, Calbiochem) to a vol of 500 μl. The antibody was transferred to a fresh dialysis bag and dialysed against PBS for 24 h at 4°C. The protein concentration of the affinity purified anti-gB was 0.4 μg/μl (Section 2.32). Two μl of 1% thimerosal were added, and the antibody was kept frozen at -20°C. The specificity of the affinity purified antibody was verified by immunoprecipitation using lysate of cells infected with HSV-1 (Sections 2.33 and 2.35).

2.31 Affinity purification of anti-G antibody

2.31.1 Preparation of the affinity column

The anti-G antibody was affinity purified using a column made with the purified glycoprotein G. The method described by Kelley et al. (1972) was used to prepare and purify G. Ten plates (15 cm diameter) of BHK cells were washed with PBS and each plate was incubated for 30 min at 37°C with 4 ml of D-MEM containing VSV at a MOI of 5. The medium was removed and replaced with fresh D-MEM containing 7% FBS, and the cells were incubated at 37°C for 17 h. All the subsequent manipulations were done at 4°C. The medium was carefully transferred into 50 ml co-polymer tubes and clarified by centrifugation at 600 x *g* for 10 min. The supernatant was transferred into

four 95 ml polycarbonate tubes, and centrifuged at 50 000 x *g* for 1 h 30 in a Beckman rotor type 45ti. The viral pellets were resuspended in a total vol of 1 ml of NTE (0.13 M NaCl, 0.05 M Tris-Cl(pH 7.8), 1 mM EDTA).

The viruses were purified by rate zonal centrifugation by loading them onto two 12.6 ml continuous 5 to 30% sucrose gradients made in NTE. The gradients were centrifuged at 4°C in a Beckman rotor type SW 41ti for 90 min at 25 000 RPM (50 000 x *g*). The two white viral bands were collected by perforating the tubes and by sucking the bands with a 5 ml syringe. The viruses were resuspended in a total vol of 22 ml of NTE, transferred into two 13 ml Ultra-clear™ tubes and centrifuged in a Beckman rotor type SW41 at 78 000 x *g* for 90 min. The two viral pellets were resuspended in a total vol of 0.5 ml of 0.01 M HEPES(pH 7.3) and frozen at -20°C.

The viruses were thawed, sonicated for 2 min, and resuspended in 5 ml of 0.01 M HEPES(pH 7.3) containing 2% Triton X100. The solution was transferred into a 13 ml polycarbonate tube, and centrifuged using a Beckman rotor type 70.1Ti at 140 000 x *g* for 90 min. The supernatant, which contained the purified G, was dialysed for 24 h against 0.1 M $NaHCO_3$(pH 8.3), 0.5 M NaCl, 0.5% Nonidet P40. The protein concentration was determined as described in Section 2.32 and the purity of G was assessed by SDS-PAGE and coomassie blue staining (Section 2.36). About 300 μg of G was coupled to 300 mg of cyanogen bromide-activated Sepharose 4B as described in Section 2.30.1.

2.31.2 Purification of anti-G antibody

Two ml of anti-G antibody were affinity purified using the glycoprotein G conjugated-Sepharose 4b as described in Section 2.30.2. The eluate containing the affinity purified antibody was pooled, dialysed and concentrated as described in Section 2.30.2. The final vol of the affinity purified antibody was 500 μl and the protein concentration was 0.1 μg/μl. BSA and thimerosal, at a final concentrations of 1 and 0.01%, were added and the antibody was dispensed into 1.5 ml microcentrifuge tubes which were frozen at -20°C. The specificity of the affinity purified anti-G was verified by immunoprecipitation using lysate of cells infected with VSV (Sections 2.2.33 and 2.2.35).

2.32 Protein determination (Bradford)

The protein concentration was determined using the protein assay of Bio-Rad, based on the procedure of Bradford (1976). Aliquots of unknown samples were diluted in a final vol of 800 μl of TE in a microcentrifuge tube. Two hundred μl of Dye Reagent Concentrate (Bio-Rad) were added, mixed by vortexing, and after 5 min, the OD_{595} was measured. A calibration curve made with 1 to 20 μg of rabbit IgG was used to estimate the protein concentration of the unknown samples.

2.33 Infection and cell labeling

For infection with HSV-1, subconfluent plates (10 cm diameter) of CV-1 or COS-

l cells were washed with PBS and incubated for 1 h at 37°C with 1 ml of D-MEM without serum containing the virus at a MOI of 10. The medium was removed and replaced with fresh D-MEM supplemented with 2% FBS. The cells were incubated for 16 to 18 h at 37°C. They were washed with PBS and starved for 1 h with 1 ml of D-MEM without methionine at 37°C. One hundred μCi of [^{35}S]-methionine were then added and the cells were labeled for 2 to 3 h at 37°C.

For infection with VSV, subconfluent plates (10 cm diameter) of COS-1 cells were washed with PBS and incubated for 30 min at 37°C with 1 ml of D-MEM without serum containing the virus (serotype Indiana) at a MOI of 10. The medium was removed and replaced with fresh D-MEM containing 7% FBS and the cells were incubated for 3 h at 37°C. The cells were washed with PBS and starved for 1 h with 1 ml of D-MEM without methionine at 37°C. One hundred μCi of [^{35}S]-methionine were added and the cells were labeled for 1 h at 37°C.

2.34 Transfection and labeling of COS-1 cells

For transient expression of wild types and mutants, subconfluent monolayers of COS-1 cells (6 cm diameter plates) were transfected using the $Ca_3(PO_4)_2$ method (Graham and Van Der Eb, 1973) followed by a glycerol shock (Frost and Williams, 1978). Before transfection, the medium was replaced with fresh D-MEM containing 7% calf serum. In a 1.5 ml microcentrifuge tube, 20 μg of plasmid were diluted in a total vol of 250 μl of 0.25 M $CaCl_2$. The DNA solution was then added slowly, with constant agitation, to 250

μl of HEBS buffer (1% Hepes, 1.6% NaCl, 0.076% KCl, 0.02% Na_2HPO_4, 0.2% dextrose, pH adjusted to 7.1) in a 1.7 x 1.2 polystyrene tube. The DNA solution was incubated at room temperature without agitation for 30 min, and then slowly transferred to the plates of COS-1 cells. The cells were incubated for 4 h at 37°C, the medium was removed, and 0.5 ml of D-MEM containing 15% glycerol was added. After 2 min, the glycerol was diluted by the addition of 5 ml of D-MEM. The medium was removed. The cells were washed with 5 ml of D-MEM and incubated with D-MEM containing 7% FBS at 37°C for 18 h. For labeling, the cells were washed with PBS and starved for 1 h with 0.5 ml D-MEM without methionine at 37°C. Fifty μCi of [^{35}S]-methionine were added and the cells were incubated for 2 to 3 h at 37°C.

2.35 Immunoprecipitation

At the end of the labeling period, the medium was removed, the cells were washed with ice-cold PBS, and scraped into 0.5 ml to 1 ml of ice-cold lysis buffer (1% Nonidet P40, 0.4% sodium deoxycholate, 66 mM EDTA, 10 mM Tris-Cl(pH 7,4), 1 mM PMSF and 100 U/ml of aprotinin). All subsequent manipulations were carried out at 4°C. The cell lysates were transferred into 1.5 ml microcentrifuge tubes and clarified by centrifugation for 3 min at 16 000 x *g*. The supernatants were then transferred into fresh tubes.

One to 3 μl of antibody were added to the tubes, which were rotated for 30 to 60 min. Aliquots of 50 to 80 μl of a 10% (wt/vol) protein A-Sepharose suspension in lysis

buffer, were added and the tubes were rotated for an extra 30 to 60 min. The beads were recovered by centrifugation at 16 000 x g for 30 sec and washed 4 times with 1 ml of ice-cold lysis buffer containing 0.3% SDS. The beads were resuspended in 10 to 20 μl of lysis buffer containing 0.3% SDS, and an equal vol of 2X sample buffer (0.125 M Tris-Cl(pH 6.8), 4% SDS (wt/vol), 20% glycerol (vol/vol), 0.05% bromophenol blue (wt/vol), 10% β-mercaptoethanol (vol/vol)) was added. The samples were incubated for 3 min in a boiling water bath and centrifuged for 30 sec at 16 000 x g. They were then analyzed by SDS-PAGE (Section 2.36) followed by fluorography (Section 2.37).

2.36 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The proteins were analyzed by electrophoresis using the discontinuous system of Laemmli (1970), and the apparatus SE 400, Strudier SLAB Gel Electrophoresis Unit from Hoefer Scientific and Mini-Protean II Cell from Bio-Rad. The resolving gel was composed of 0.375 M Tris-Cl(pH 8.8), 0.1% SDS (wt/vol), 0.1% ammonium persulfate (wt/vol), 0.07% TEMED (vol/vol) and polyacrylamide. Depending on the size of the proteins analyzed, the polyacrylamide concentration (wt/vol) was either 7% (6.8% acrylamide, 0.19% bisacrylamide), 10% (9.7% acrylamide, 0.27% bisacrylamide) or 12% (11.7% acrylamide, 0.32% bisacrylamide). The stacking gel was composed of 0.125 M Tris-Cl(pH 6.8), 0.1% SDS (wt/vol), 0.05% ammonium persulfate (wt/vol), 0.05% TEMED (vol/vol), 3.89% acrylamide (wt/vol), 0.11% bisacrylamide (wt/vol). The electrode buffer (pH 8.3) was made of 0.02 M Tris base, 0.192 M glycine, 0.1%

SDS (wt/vol).

After electrophoresis, the gels were stained and fixed by incubation for 30 min at room temperature with gentle shaking in 0.125% coomassie blue, in 50% methanol and 10% acetic acid. The gels were destained by shaking for 1 to 3 h in 50% methanol, 10% acetic acid, and then up to 18 h in 7% acetic acid, 5% methanol. The gels were then processed for fluorography (Section 2.37), or they were blotted on a piece of filter paper (Whatman No 1) and dried for 1 to 2 h at 60°C with a Slab Dryer Model 483 from Bio-Rad.

2.37 Fluorography of polyacrylamide gels

Two fluorographic methods, impregnation with diphenyloxazole (PPO, Bonner and Laskey, 1974) or impregnation with sodium salicylate (Chamberlain, 1979), were used to enhance detection of radioactivity in polyacrylamide gels. The sensitivity of the two methods were very similar. The first method however, although more tedious than the second one, produced sharper radioactive gel bands. The reason for this is not known (Chamberlain, 1979).

2.37.1 Impregnation with 2,6 diphenyloxazole

Following staining and fixation, the gels were washed in water for 30 min, incubated with shaking in 250 ml of DMSO for 30 min. The DMSO was then replaced with a fresh one, and the incubation was carried on for an other 30 min. The gels were

incubated for at least 90 min with shaking in 250 ml of 22.2% PPO (wt/vol) dissolved in DMSO, and then washed with water for 1 h. The gels were blotted on a piece of filter paper and dried as described in Section 2.36. The gels were exposed to Kodak ray films (XAR-5) at -70°C.

2.37.2 Impregnation with sodium salicylate

After fixation, the gels were washed with water for 30 min and incubated with shaking for 30 min in 1 M salicylic acid, 1 M NaOH, pH 5 to 7. The gels were dried and exposed as described in Section 2.37.1.

2.38 Immunoblot

Plates of CV-1 cells (10 cm diameter) were infected for 16 h with HSV-1 at a MOI of 10 as described in Section 2.33. At 18 h postinfection, the cells were lysed with 1 ml of lysis buffer as described in Section 2.35. Fifty μl of cell lysate were mixed with an equal vol of 2X sample buffer (0.125 M Tris-Cl(pH 6.8), 4% SDS (wt/vol), 20% glycerol (vol/vol), 0.05% bromophenol blue (wt/vol) and 10% β-mercaptoethanol (vol/vol)). The samples were boiled for 3 min and analyzed by SDS-PAGE (Section 2.36). The proteins were blotted to a nitrocellulose sheet in Transfer Buffer (25 mM Tris-Cl(pH 8.3), 192 mM glycine, 20% methanol and 0.1% SDS Harlow and Lane, 1988) for 3 to 18 h at 110 mA using a Trans-Blot Apparatus from Bio-Rad. The nitrocellulose was then incubated for 2 h at room temperature in Blotto (5% Carnation

non-fat dry milk, 50 mM Tris-Cl(pH 8), 2 mM $CaCl_2$, 0.05% tween 20), and for 1 h 30 with anti-gB (diluted 1/300 in Blotto). The nitrocellulose was washed with Blotto for 30 min and incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit antibody (diluted 1/1000 in Blotto). The membrane was then washed with Blotto and PBS for 30 min each, and developed by incubation in PBS containing 20% methanol, 0.5 mg/ml 4-Chloro-1-Naphtol and 0.05% H_2O_2 (Hawkes, 1982).

2.39 Estimation of the molecular weight

2.39.1 Estimation by SDS-PAGE

The mol wt of the proteins was determined using a modification of the method of Laemmli (1970). The relative mobility (R_f) of each protein was determined by dividing its migration distance from the top of the separating gel by the migration distance of the bromophenol blue dye from the top of the separating gel: R_f = (distance of protein migration)/ (distance of dye). A calibration curve was made by plotting the R_f values of known standards against the logarithm of their mol wt. The mol wt of the wild types and mutants were estimated from this calibration curve.

The standards used were bovine erythrocyte carbonic anhydrase (29 000 D), ovalbumin (45 000 D), bovine plasma albumin (66 000 D), rabbit muscle phosphorylase B (97 000 D), E. coli β-galactosidase (116 000 D) and rabbit muscle myosin (205 000 D).

2.39.2 Theoretical calculation

The mol wt of the mutants were estimated from a combination of the predicted mobility of gB and G on SDS-PAGE and from the calculated mol wt of the segments used to make them. The mol wt of the protein segments were calculated according to their amino acid composition using the software PCGENE.

Example a: mol wt of gB-G.

The chimera gB-G, which becomes endo H resistant (Section 3.6.2), was constructed by fusing the EC of gB to the TM and CT of G (Figure 3.1.1). The mol wt of the endo H resistant gB on SDS-PAGE is 120 kD (Section 3.5.1). Thus, the mol wt of gB-G is: 120 kD - (mol wt of TM and CT of gB [19.31 kD]) + (mol wt of TM and CT of G (6.99 kD) = 107.7 kD.

Example b: Calculation of the mol wt of gB-tmG.

The chimera gB-tmG, which remains endo H sensitive (Section 3.6.2), was constructed by replacing the TM of gB with the TM of G (Fig. 3.1.1). The mol wt of the endo H sensitive gB on SDS-PAGE is 110 kD (Section 3.5.1). Hence, the mol wt of gB-tmG is 110 kD -(mol wt of TM of gB [7.28 kD]) + (mol wt of TM of G [3.52 kD]) = 106.3 kD.

2.40 Endoglycosidase H digestion

Plates of COS-1 cells (6 cm diameter) were transfected as described in Section .34. Eighteen to 24 h following transfection, the medium was removed, the cells were

washed with PBS and starved for 1 h with 0.5 ml of D-MEM without methionine at 37°C. Fifty μCi of [^{35}S]-methionine were added, and the cells were incubated at 37°C for 15 min. The cells were then washed with PBS and chased for 0, 30, 60 or 120 min with 5 ml of D-MEM supplemented with 7% calf serum and 2.5 mM methionine. At the end of the chase, the cells were lysed and immunoprecipitated as described in Section 2.35.

The immunoprecipitate was resuspended in 10 μl of 50 mM Tris-Cl(pH 6.8) containing 1% SDS and boiled for 3 min. Aliquots of 5 μl were diluted 6X with 50 mM Na citrate(pH 5.5) in a 1.5 ml microcentrifuge tube, and digested overnight with 1 mU of β-endoglycosidase H (endo H) at 37°C. As controls, aliquots of 5 μl were diluted 6X with 50 mM Na citrate(pH 5.5) and incubated in parallel without endo H. The samples were analyzed by SDS-PAGE and processed for fluorography as described in Sections 2.36 and 2.37. The percentage of acquisition of endo H resistance was quantified by scanning the bands with a GS300 Transmittance/reflectance Scanning Densitometer from Hoefer Scientific, using preflashed films (Section 2.41).

2.41 Preflashing of films

Kodak-ray films (XAR-5) were preflashed according to the method of Laskey and Mills (1975). The films were exposed to a brief flash of light (< 1 s) at a distance of one meter in order to increase the OD_{600} of the film by 0.1 to 0.3 above the background fog level of untreated films. The light intensity of the flash was attenuated with layers of

exposed-ray films and made more diffuse with a filter paper (Whatman No 1).

2.42 Sucrose gradient sedimentation

The oligomeric nature of the wild types and mutants were studied by centrifugation in a continuous sucrose gradient as described by Doms et al. (1987) and by Crise et al. (1989). Plates of COS-1 cells (60 mm diameter) were transfected, starved, labeled for 1 h, and chased for 90 min as described in Section 2.40. At the end of the chase, the cells were washed with ice-cold PBS and lysed by scraping the plate with a policeman in 300 μl of ice-cold 4x MNT buffer (80 mM MES, 400 mM NaCl, 120 mM Tris-Cl, 4 mM EDTA, 4 mM EGTA, 1 mM PMSF, 100 U/ml of aprotinin, final pH 5.7 or 7.4) containing 1% Triton X100. The lysates were transferred into 1.5 ml microcentrifuge tubes, incubated on ice for 30 min and clarified by centrifugation at 4°C for 2 min at 16 000 x *g*. As mol wt standards, 150 μg of aldolase from rabbit muscles (mol wt: 161 000 D, S_{20w}: 8.6 S, Kundu et al., 1991) and 150 μg of BSA (mol wt: 68 000, S_{20w}: 4.7 S, Persson and Pettersson, 1991) were added to the cell lysate, and 250 μl of lysate were loaded onto 12.6 ml continuous 5 to 20% sucrose gradients made in 2x MNT (pH 5.7 or 7.4) and containing 0.1% Triton X100.

The gradients were centrifuged at 4°C in a Beckman rotor type SW 41Ti for 20 to 22 h at 40 000 rpm. Eighteen fractions were collected from the bottom of the gradients, using a peristaltic pump connected to a fraction collector Model 2110 from Bio-Rad. Aliquots of 50 μl were set aside and used to determine the position of aldolase

and BSA by SDS-PAGE and coomassie blue staining (Section 2.36). One vol of lysis buffer (50 mM Tris-Cl(pH 8), 1% Nonidet P40 (vol/vol), 0.4% Na deoxycholate (wt/vol), 0.3% SDS (wt/vol), 20 mM EDTA) was added to each fraction, which were immunoprecipitated and analyzed by SDS-PAGE followed by fluorography (Sections 2.35 to 2.37).

2.43 Estimation of the mol wt by sucrose gradients

The procedure of Martin and Ames (1961) was used to estimate the mol wt of the proteins according to their sedimentation velocity in sucrose gradients, using BSA (mol wt: 68 000, S_{20w}: 4.7; Persson and Pettersson, 1991) and aldolase from rabbit muscles (mol wt: 161 000 D, S_{20w}: 8.6 S; Kundu et al., 1991) as standards. The S_{20w} of the wild types and mutants were estimated from their position in the gradient at the end of the centrifugation according to the relation: R = (S_{20w} of unknown)/(S_{20w} of standard), where R = (distance travelled from meniscus by unknown)/(distance travelled from meniscus by standard). The mol wt was then approximated according to the relation S_1/S_2 = (mol wt_1/mol wt_2)$^{2/3}$.

2.44 Indirect immunofluorescence

2.44.1 Absorption with cell extracts

The antibodies were preabsorbed with COS-1 cells before using them for immunofluorescence. Ten plates of confluent COS-1 cells (10 cm diameter) were washed

with ice-cold PBS, and scraped with a policeman into 1 ml of ice-cold PBS. The cells were transferred into a 1.7 x 12 cm polystyrene tube, and centrifuged at 4°C for 10 min at 2000 x *g*. The supernatant was removed, and the cells were frozen at -20°C with 1 mM PMSF and 100 U/ml of aprotinin. The cells were thawed, sonicated for 2 min, and one vol of antibody was added and mixed by vortexing. The suspension was dispensed into 1.5 ml microcentrifuge tubes and frozen at -20°C. The antibody was clarified by centrifugation for 3 min at 16 000 x *g* before its use.

2.44.2 Fixation and labeling

COS-1 cells grown on 18 mm x 18 mm glass coverslips in 6 cm diameter plates, were transfected as described in Section 2.34. Eighteen to 24 h after transfection, the cells were washed with PBS and fixed for 30 min at room temperature with a fresh solution of 4% paraformaldehyde (Section 2.44.3). The cells were washed with 5 ml of PBS. The coverslips were then transferred into vertical porcelain racks immersed in 100 ml of PBS, and washed by shaking the rack in the solution.

Before incubation with the primary antibody, cells processed for internal immunofluorescence were first permeabilized for 10 min at room temperature with 100 ml of 1% Triton X100 (vol/vol) diluted in PBS. They were then washed with PBS and incubated with the primary antibody as described below. For external immunofluorescence, the cells were directly incubated with the primary antibody after fixation and washing.

For incubation with the primary antibody, the fixed cells were washed with 100 ml of PBS containing 1% BSA (Buffer A). The coverslips were transferred into a humid chamber (10 cm plates containing wet paper towels), and 30 μl of primary antibody, preabsorbed with COS-1 cells (Section 2.44.1), were added on top of the coverslips, which were then incubated for 20 min at room temperature. The preabsorbed anti-gB and anti-G antibody were diluted 10 and 15 times with Buffer A respectively.

The coverslips were then washed with Buffer A, and incubated with 30 μl of fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (diluted 20X in Buffer A) as described above. The coverslips were then washed with Buffer A and with PBS. They were air dried and mounted in 50% glycerol/PBS. The cells were observed with a Zeiss epi-fluorescence microscope equipped with a high pressure mercury lamp. Exciter and barrier filters BP 450-490 and BP 515-565 were used, and pictures were taken with black and white Kodak Tmax 400 films.

2.44.3 Preparation of 4% paraformaldehyde

One g of paraformaldehyde was mixed with 10 ml of PBS. The solution was incubated at 65°C while slowly adding drops of 1 N NaOH until the paraformaldehyde was completely dissolved, making sure the pH was not higher than 8. The solution was then diluted to a final concentration of 4% with PBS.

2.45 Immunoelectron microscopy

2.45.1 Fixation and embedding

Plates of COS-1 cells were infected with HSV-1 or transfected with the different cDNAs as described in Sections 2.33 and 2.34. At 14 to 22 h postinfection, or 24 h after transfection, the cells were washed with PBS and fixed for 2 h at room temperature with 2% glutaraldehyde in 0.03M PIPES(pH 7.4). The cells were washed with three changes of 0.03 M PIPES(pH 7.4) for 30 min, and then gently scraped in one ml of 0.03 M PIPES(pH 7.4) with a razor blade. The cells were transferred into 1.5 ml microcentrifuge tubes and recovered by centrifugation for 3 min at 2000 x *g*. The supernatant was removed, the tubes were incubated for 3 min in a 45°C water bath, and the cells were resuspended in 10 to 30 μl of melted 2% noble agar at 45°C. The cells were rapidly transferred onto a glass slide. After solidification, the specimens were cut into small cubes ($1mm^3$), and transferred into small glass bottles containing 2 ml of ice-cold 0.03 M PIPES(pH 7.4).

The specimens were dehydrated with ethanol and embedded in Lowicryl K4M (Roth et al., 1981; Gilbert, 1988) by consecutive changes with two ml of 30, 50, 70, 80, and 90% ethanol. Each change lasted 20 min. The first change was done on ice, while the other ones were done at -20°C. The specimens were infiltrated at -20°C with a mixture of 90% ethanol and lowicryl K4M (5.2 ml Crosslinker A, 34.8 ml Monomer B, 200 mg Initiator C) as follows: 2 vol/1 vol (ethanol/K4M) for 2 h, 1 vol/2 vol (ethanol/K4M) for 2 h, pure K4M overnight and pure K4M for 1 h. The specimens were

transferred into gelatine capsules no 4 (Parke-Davis) filled with K4M, and they were polymerized for 24 h under ultraviolet light at -20°C.

2.45.2 Labeling and counterstaining

Ultrathin sections on nickel grids coated with a formvar film, were labeled by incubation on drops of buffer (15 μl) and antibody (8 μl) at room temperature. For incubation with the primary antibody, the sections were first incubated for 5 min on PBS, transferred for 5 min on PBS containing 1% BSA and 5% goat serum (Buffer A), and then incubated for 1 h on the primary antibody. The primary antibody consisted of the affinity purified anti-gB or the affinity purified anti-G diluted 5 and 10 times in buffer A respectively.

The sections were then transferred on several drops of buffer A, followed by drops of PBS for a total period of 10 min. They were then washed with a jet of PBS for 30 sec, incubated on several drops of PBS, followed by drops of buffer A for a total period of 10 min. The sections were incubated for 30 min on drops of 1 nm gold-conjugated goat anti-rabbit antibody (diluted 200 times in Buffer A). The grids were transferred on several drops of Buffer A, followed by drops of PBS for a total period of 10 min. They were then washed with a jet of PBS for 30 sec, transferred on drops of distilled water for 10 min, washed with a jet of distilled water for 30 sec and finally dried on a piece of filter paper (Whatman No 1).

The 1 nm gold was then silver enhanced according to a slight modification of the

method of Danscher and Nörgaard (1983). The grids were incubated on drops of developer solution (see below) in the dark at room temperature for 25 to 45 min. The reaction was stopped by incubating the grids at room temperature on drops of Kodak rapid fixative diluted 4 times with distilled water. The sections were then washed thoroughly by incubation on several drops of distilled water. They were then washed with a jet of distilled water and dried on a piece of filter paper (Whatman No. 1).

The developer was made fresh each time by mixing together 50 μl of citrate buffer, 300 μl of 50% gum arabic (wt/vol), 50 μl of 5.7% hydroquinone (Quinol, wt/vol) and 75 μl of 2.2% silver lactate (wt/vol). The citric buffer was made fresh each time by mixing 1 vol of 2.3 M citric acid and 1 vol of 1.6 M sodium citrate.

After silver enhancement, the sections were treated with vapours of osmium tetroxide. This was accomplished by incubation for 3 min at room temperature in a closed petri dish containing filter papers soaked with 2% osmium tetroxide (wt/vol). The sections were then counterstained by incubation for 5 min on drops of a saturate solution of uranyl acetate in 50% ethanol, followed by washing with a jet of distilled water. The sections were dried for 2 to 3 min. They were then incubated for 2 min on drops of a saturate solution of lead citrate, washed with a jet of distilled water and dried (Hayot, 1981). The specimens were observed with a JEOL electron microscope JEM 1200EX under an acceleration potential of 80 kilovolts.

2.45.3 Statistical analysis

Random pictures of cells were taken at a magnification of 5000 to 10 000. The negatives were magnified 9X with a MOP-Videoplam from Kontron, and the total length of NE, ER and PM for each cell was measured using the image analysis programm Version 5.41 of Kontron. The total number of silver grains over each membrane type was determined, and the average grain density over the NE, ER and PM was calculated for each cell. In cells infected with HSV-1, the average number of silver grains over viral particles in the nucleoplasm and in the cytoplasm, or associated with the NE and the plasma membrane, was also determined for each cell.

The data was analyzed by an analysis of variance (ANOVA) at a $F(\nu 1,\nu 2)$ of 5%. When the means were different, the comparisons were done with the test of Scheffé with a $F(\nu 1,\nu 2)$ of 5% (Woolson, 1987). The silver density of the NE, ER, and PM of infected and transfected cells, was also compared with the density of the similar membrane type of mock infected and mock transfected cells using the test of Student (α= 5%, Woolson, 1987). The softwares Parametric Statistics IBM PC Version 1.01 from Lundon Software Inc. and T-ease version 2.0 from ISI Software were used for the statistical analysis.

The percentage of silver grains associated with the NE was also determined. Only micrographs of sections showing a complete cell and crossing the cell centre (such as Fig. 3.10.3b) were used for this calculation. For each cell, the total number of grains associated with the NE was compared with the total number of grains in the cell. The

means in percentage were computed and analyzed by an analysis of variance as described above. To reduce the variation between the samples, the square roots of the means ($\sqrt{}$[mean]) were used for the analysis.

2.46 Subcellular fractionation

Cells were fractionated according to the procedure of Bos et al. (1989). Plates of COS-1 cells (10 cm diameter) were transfected as described in Section 2.34, except that twice the amount of DNA and buffer were used. 24 h after transfection, the medium was removed, the cells were washed with PBS and they were starved for 1 h with 1.0 ml of D-MEM without methionine at 37°C. The cells were labeled with 100 μCi of [^{35}S]-methionine for 30 min at 37°C. They were then washed with PBS and chased for 1 h 30 min with 5 ml of D-MEM supplemented with 7% calf serum and 2.5 mM methionine.

At the end of the chase, the cells were scraped in 2 ml of ice-cold PBS, transferred into 1.7 x 12 cm polystyrene tubes, and centrifuged at 1000 x *g* for 5 min at 4°C. All subsequent manipulations were done at this temperature. The supernatants were removed, the cells were gently resuspended into 1 ml of ice-cold hypotonic buffer (25 mM Tris-Cl(pH 7.8), 1 mM $MgCl_2$, 5 mM KCl, 1 mM PMSF and 100 U/ml of aprotinin) and incubated on ice for 5 min. One ml of hypotonic buffer containing 1% Nonidet P40 was added, and after an additional 5 min on ice, the nuclei were recovered by centrifugation at 1000 x *g* for 5 min. The supernatants were transferred into fresh tubes (postnuclear fraction), and the nuclei were washed by vigorous vortexing in one

ml of hypotonic buffer containing 0.5% Nonidet P40, and centrifuged as described above. The nuclei were washed a second time, and the supernatants of the washes were combined with the postnuclear fraction.

The nuclei were resuspended in 1 ml of lysis buffer (25 mM Tris-Cl(pH 7.8), 150 mM NaCl, 0.5% Nonidet P40, 0.5% Na deoxycholate, 0.5% SDS, 1 mM PMSF and 100 U/ml of aprotinin) and sheared several times through a 25-gauge needle. The detergent concentration of the postnuclear fractions were adjusted to that of the lysis buffer. Nuclear and postnuclear fractions were clarified by centrifugation for 10 min at 2000 x *g*. The supernatants were transferred to fresh tubes, and analyzed by immunoprecipitation and SDS-PAGE as described in Sections 2.35 to 2.37. The amount of protein found in each fraction was quantified by scanning the bands with a GS 300 Transmittance/Reflectance Scanning Densitometer using preflashed films (Section 2.41).

3.0 RESULTS

3.1 Description of the mutants made for this study

Glycoprotein B, a membrane protein with a long carboxy terminal hydrophobic domain is specifically localized to the NE (Section 1.6.3). Although disagreements exist concerning the number of residues embedded within the membrane (Section 1.6.1), in this thesis, to simplify the presentation of the data, the TM of gB is considered to be composed of 69 amino acids as defined by Pellet et al. (1985) (See Fig. 3.1.1). In order to find which portion of gB encodes the information essential for NE localization, the transport and distribution by light and electron microscopy of chimeras and deletion mutants were studied. The chimeric proteins were made by fusing different domains of gB with domains of the glycoprotein G of VSV. Glycoprotein G is also a membrane protein, but it is not specifically localized to the NE (Section 1.7.4). The amino acid sequence of the TM regions of gB and G and the structure of the chimeras and mutants constructed in the present study are presented in Fig. 3.1.1.

Chimera gB-G was made by fusing the EC of gB with the TM and CT of G. This protein was constructed by coupling the first 721 residues of gB to the last 11 amino acids of the EC of G. The mutants G-gB-1 and G-gB-2 were made by fusing the EC of G to the TM and CT of gB. The chimera G-gB-1 was constructed by coupling the first 363 residues of G to the last 32 amino acids of the EC of gB. Consequently, the last 99

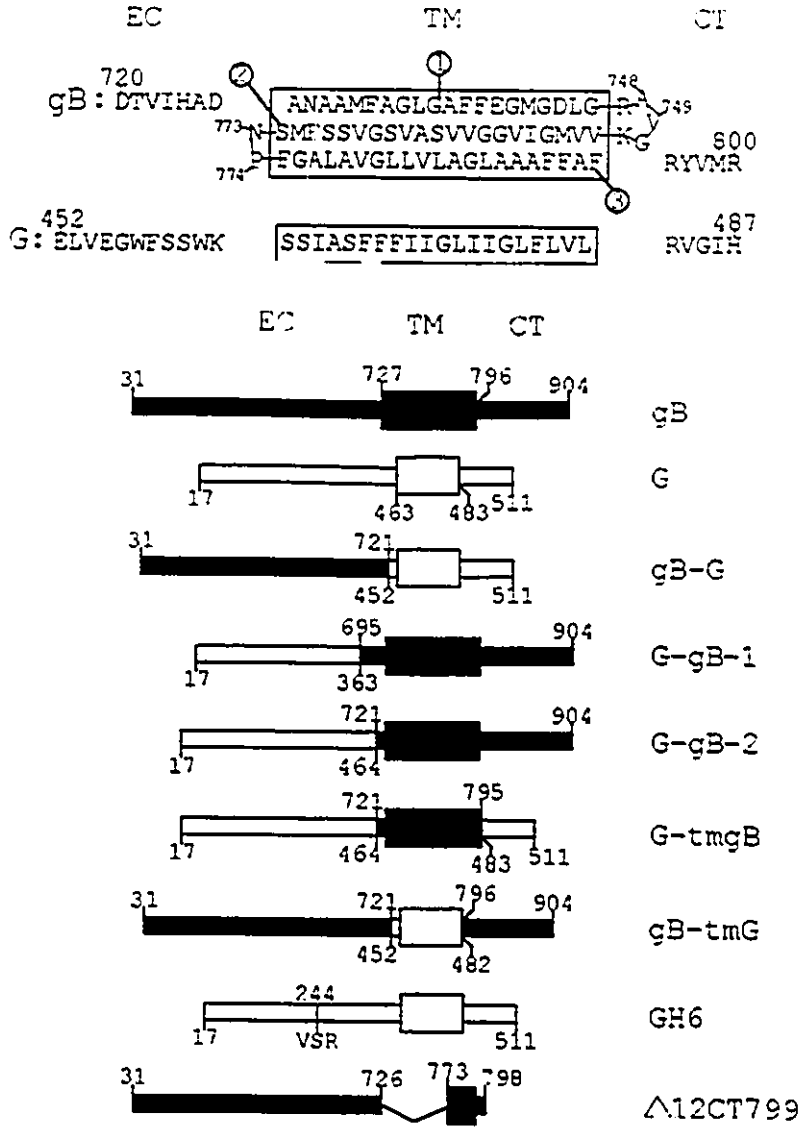

Figure 3.1.1 Transmembrane domains and structure of the mutants. a) Amino acid sequences of the TM and adjacent regions of gB and G. The amino terminus is on the left, while the carboxy terminus is on the right. The residues believed to be embedded within the membrane are boxed (Bzik et al., 1986; Pellet et al., 1985; Rose and Gallione, 1981). For gB, residues 720 to 800 possess an α-helix conformation except for V^{749}, G^{750}, N^{773} and P^{774} which have a β-turn conformation (Bzik et al.,1986). The position of segments 1, 2 and 3 of the TM of gB are also indicated. b) Structure of the mutants constructed for this study. The residues belonging to gB (black) and to G (white) are above and below the bars respectively. For gB and G, the first residue of each domain (excluding the signal sequence) and the last residue of the protein are indicated. The numbers above and below the mutants correspond to the first and last residues of the protein segments used to make them. Mutant GH6 was made by inserting the tripeptide VAL, SER, ARG after residue 244. EC: ectodomain; TM transmembrane domain; CT: Cytoplasmic domain.

amino acids of the EC of G are missing. Biochemical studies have shown that chimera G-gB-1 forms aggregates that are not transported out of the ER (Sections 3.6 and 3.7). In an attempt to make a similar chimera that would fold properly and that would be transported out of the ER, G-gB-2 was constructed. This protein contains the entire EC of G including 2 residues of the TM of G, fused to the last 6 amino acids of the EC of gB (Fig. 3.1.1).

Chimeras G-tmgB and gB-tmG were constructed by swapping the TM of gB and G. To make G-tmgB, the TM of G was replaced with the TM of gB and 6 residues of the EC of gB. To make gB-tmG, the TM of gB was replaced with the TM of G and 11 residues of the EC of G. The last five residues of the EC of gB are missing in gB-tmG. Mutant Δ12CT799 contains the EC of gB and segment 3 of the TM of gB. This protein was constructed by deleting segment 1 and segment 2 of the TM of gB, as well as the entire CT except for 3 residues.

In contrast with gB and G, some mutants were not transported to the cell surface when expressed in COS-1 cells. As a control for proteins that do not move to the cell surface and lack a specific NE localization signal, the distribution of GH6, a transport defective mutant of G, was also studied. Mutant GH6, a generous gift of Dr. Yun Li (McMaster University), was made by inserting three amino acids in the EC of G resulting in a protein that forms aggregates and that remains in the ER (Sections 3.6 and 3.7).

3.2 Eukaryotic expression vectors

Wild type and mutant proteins were expressed in COS-1 cells using the eukaryotic vectors p91023 or pXM (Fig 3.2.1). These two vectors possess an SV40 origin of replication allowing plasmid replication in COS-1 cells. The transcription of the different cDNAs is under the control of the adenovirus major late promoter (MLP) a constitutive eukaryotic promoter in COS-1 cells (Kaufman, 1985). The mRNA produced by these plasmids are flanked at their 5' ends by the tripartite leader of adenovirus late RNAs (TPL) and at their 3' ends by the mouse dihydrofolate reductase (DHFR) coding sequence, which appears to increase the mRNA stability (Wong et al., 1985). An SV40 early cleavage and polyadenylation signal was also inserted at the 3' end of the DHFR coding region for proper cleavage of mRNA and addition of the poly (A) tail. These two plasmids also encode the VA genes of adenovirus. These genes, which are transcribed by RNA polymerase III, produce small RNAs of about 160 nucleotides. The translation of some mRNA flanked at their 5' end by the tripartite leader is increased by a factor of 5 to 10 in the presence of the VA genes (Kaufman, 1985; Schneider et al., 1984; Logan and Shenk, 1984). The VA genes stimulate translation by preventing phosphorylation of the eukaryote initiation factor 2 (EIF-2) which inhibits translation (Kaufman, 1987; Kaufman, 1990).

The cDNAs of this work were expressed in COS-1 by using the vector pXM, except for mutants CT799, CT796, Δ3 and gB-gC (Fig. 3.10.5) which were studied by using p91023 vector.

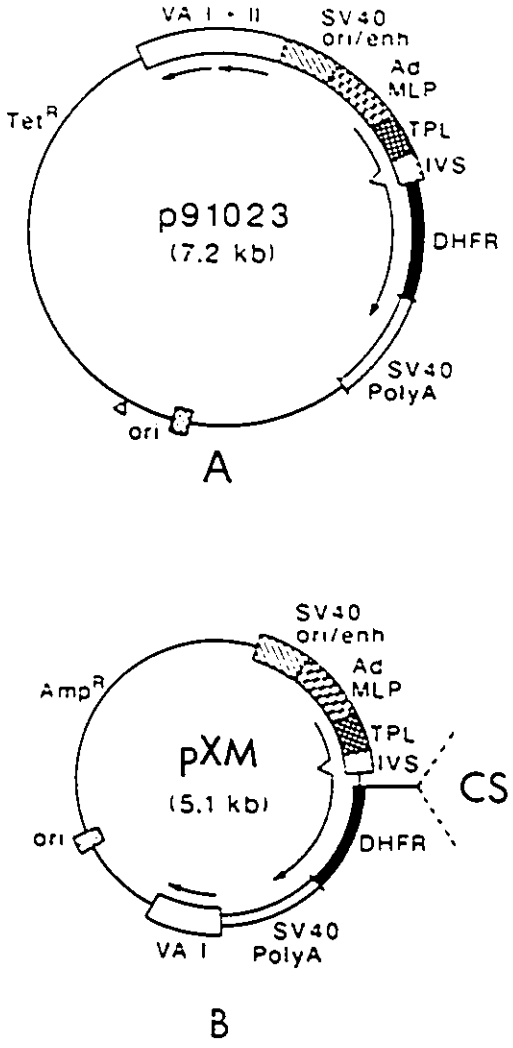

Figure 3.2.1 Expression plasmids. The plasmid pXM was constructed by inserting the eukaryotic transcription unit of p91023 into pUC 18 that had been previously modified to include a SV40 origin of replication and the adenovirus VA I gene. Ad MLP: adenovirus major late promoter; AmpR ampicillin resistance gene; DHFR: dihydrofolate reductase coding sequence; IVS: intervening sequence; CS: cloning site; ori: pBR322 origin of replication; SV40 ori/enh: SV40 origin of replication and enhancer; SV40 polyA: SV40 early polyadenylation signal; Tet^R: tetracycline resistance gene; VA I + II: adenovirus VA I and II genes. Adapted from Kaufman (1990), see text for more details.

3.3 Construction of the mutants

Some mutants were constructed exclusively by isolation and ligation of specific DNA fragments generated by digestion with restriction enzymes using standard molecular biology techniques (Sambrook et al., 1989). Other mutants however were made by using a combination of standard molecular biology techniques and site directed mutagenesis according to the method of Kunkel et al. (1987). Each specific step, as well as the plasmids and enzymes used to generate the mutants are schematically presented in Figs 3.3.1 to 3.3.6. The amino acid and nucleotide numbers correspond to those assigned by Rose and Gallione (1981) and by Bzik et al. (1986) (see Appendices for a complete amino acid sequence of G and gB). The primers used for site direct mutagenesis and for DNA sequencing are presented in Table 2.4.1. The predicted amino acid and nucleotide sequences of the mutated regions are found in Table 3.3.1. The nucleotide sequences of the mutated cDNA's were confirmed by DNA sequencing using the chain termination method (Sanger et al., 1977; Fig. 3.3.7).

3.3.1 Construction of gB-G

The chimera gB-G was made by first subcloning the 2.1 kb SalI-EcoRI fragment of p9 gB containing a portion of the EC of gB into pUC 118 (Fig. 3.3.1). The 0.65 kb KpnI-BamHI fragment of pSVGL encompassing a portion of the G cDNA was isolated. This fragment was then further digested with TaqI and EcoRI in order to isolate the DNA encoding the TM and CT of G. The resulting 0.3 kb TaqI-EcoRI fragment was subcloned

into the Tth111I-EcoRI sites of pUC gB. Before the ligation, to keep the reading frame intact, the Tth111I ends of gB and the TaqI ends of G were filled in with Klenow fragment of DNA polymerase. The 1.5 kb SalI-EcoRI fragment of pUC gB-G encoding the incomplete gB-G cDNA was then subcloned into the SalI-EcoRI site of pXM gB.

The spliced region of gB-G was confirmed by DNA sequencing. This was done by deleting a portion of gB by digestion of pUC gB-G with MluI and SalI, and by using the M13/pUC Universal primer for the sequencing reaction (Table 2.4.1 and Fig. 3.3.7). The opposite strand was also sequenced using pUC gB-G and the M13/pUC Reverse primer (Table 2.4.1, data not shown). In both cases the sequences obtained were identical to the expected one.

3.3.2 Construction of G-gB-1

This construct was made by first subcloning the 1.7 kb BamHI fragment containing the G cDNA into the BamHI site of pGEM -4Z (Fig 3.3.2). The 2.1 kb SalI-EcoRI fragment encoding portion of the gB gene was isolated and the ends were filled in with klenow fragment of DNA polymerase. This fragment was further digested with Sau3a, in order to isolate the TM and CT of gB, and then subcloned into the BclI and XbaI sites of pGEM G. The 2.1 kb HindIII-XhoI fragment of pGEM G-gB-1 encoding G-gB-1, was then subcloned into the XhoI-EcoRI sites of pXM.

The BclI and the Sau3a ends at the junction of gB and G fragments were cohesive and the original reading frame was not modified by the ligation. The spliced region of

G-gB-1 was not sequenced, because no extra nucleotide could have been removed or added during the construction of this chimera. Biochemical characterization of the product of G-gB-1 has shown that it corresponds to the expected protein (Section 3.5). The enzyme BclI does not cut methylated DNA when the 5'A residue is N^6-methylated. For this reason, the plasmid used for digestion with BclI was previously grown in E. coli Dm1 which does not methylate these residues.

3.3.3 Construction of G-gB-2

In order to make G-gB-2, the 0.47 kb Tth111I fragment of pXM gB encoding the TM and a portion of the EC of gB was isolated (Fig. 3.3.3). This fragment was then subcloned into the XhoI site of pXM Gm6. Before the ligation, to maintain the original reading frame, the XhoI and Tth111I ends were filled in with Klenow fragment of DNA polymerase. The 1.7 kb BglII fragment of pXM G-gB-G encoding portion of the G-gB-2 gene was then subcloned into the BglII MluI site of pXM gB.

Plasmid pXM Gm6 (a generous gift of Mr. C. Drone, McMaster University) was constructed by introducing an XhoI site using linker 5'-CTC GAG CTC GAG-3' into the AluI site (position 1416) at the junction of the EC and TM of G (Table 3.3.1). The mutated region of Gm6 was confirmed by DNA sequencing (Fig. 3.3.7). The nucleotide sequence was determined by subcloning the 0.8 kb PstI-StuI fragment encoding the TM of Gm6 into the PstI-SmaI site of pUC118. The M13/pUC reverse primer (Table 2.4.1) was used for the sequencing reaction.

The spliced region of G-gB-2 was also confirmed by DNA sequencing (Fig. 3.3.7). The 0.8 kb PstI fragment of pXM G-gB-2 encoding the TM was subcloned into the PstI site of pUC 118 and the junction of G-gB-2 was sequenced using the M13/pUC Reverse primer (Table 2.4.1).

3.3.4 Construction of G-tmgB

In order to make G-tmgB, the chimera G-gB-gB-G was first constructed by fusing the 1.7 kb BglII-MluI fragment of pXM G-gB-2 encoding the EC and TM of G-gB-2 into the BglII and XhoI sites of pXM Gm6 (Fig. 3.3.4). The XhoI ends of Gm6 and the MluI ends of G-gB-2 were filled in with Klenow fragment of DNA polymerase before the ligation. The 2 kb EcoRI fragment encoding G-gB-gB-G was then subcloned into M13mp18. The primer prm3 (Table 2.4.1) was then used to delete the EC of gB and the TM of G by site directed mutagenesis according to the method of Kunkel et al. (1987). The 1.8 kb EcoRI fragment of M13 G-tmgB encoding G-tmgB was then subcloned into the EcoRI site of pXM.

The nucleotide sequence of the spliced region of G-tmgB was confirmed by DNA sequencing using single stranded DNA produced by M13 G-tmgB and primer prm2 (Table 2.4.1 and Fig. 3.3.7).

3.3.5 Construction of gB-tmG

Chimera gB-tmG was made by first constructing gB-G-G-gB (Fig. 3.3.5). This

was done by fusing the 0.7 kb BglI-EcoRI fragment of pXM G-gB-2 encoding the TM and CT of gB into the StuI-EcoRI site of pUC gB-G (Fig. 3.3.1). Before the ligation, the BglI ends of gB were filled in with Klenow fragment of DNA polymerase. The 2.2 kb SalI-EcoRI fragment encoding gB-G-G-gB was then subcloned into M13mp18. The Primer prm5 (Table 2.4.1) was then used to delete the CT of G and the TM of gB by site directed mutagenesis according to the method of Kunkel et al. (1987). The 2.0 kb SalI-EcoRI fragment of M13 gB-tmG was then subcloned into the SalI-EcoRI sites of pXM gB.

The nucleotide sequence of the spliced region of gB-tmG was confirmed by DNA sequencing using single stranded DNA produced by M13 G-tmgB and primer prm1 (Table 2.4.1 and Fig. 3.3.7).

3.3.6 Construction of Δ12CT799

Mutant Δ12CT799 was made by subcloning the 2.1 kb SalI-EcoRI fragment of p9 CT799 into M13mp18 (Fig.3.3.6). Primer prm4 (Table 2.4.1) was then used to delete segments 1 and 2 of the TM of gB by site directed mutagenesis according to the method of Kunkel et al. (1987). The 2.0 kb SalI-EcoRI fragment of M13 Δ12CT799 encoding Δ12CT799 was then subcloned into the SalI-EcoRI sites of pXM gB. Mutant CT799, a generous gift of Mr. L. Rasile (McMaster University), is a truncation mutant of gB in which a termination codon was inserted at amino acid 799 resulting in a protein lacking the CT except for three residues (Raviprakash et al., 1990; see also Fig. 3.10.5).

The nucleotide sequence of the spliced region of Δ12CT799 was confirmed by DNA sequencing using single stranded DNA produced by M13 Δ12CT799 and primer prm1 (Table 2.4.1 and Fig. 3.3.7). Because of the high GC content of the TM of gB, artifacts such as compressions were observed during the sequencing of this domain (Bzik et al., 1986 and Fig 3.3.7). Some of the ambiguities generated by these artifacts, such as two G instead of four at nucleotides 2873-2876, were resolved when the reaction was repeated by replacing dGTP with dITP.

3.3.7 Construction of GH6

This mutant was a generous gift of Dr. Yun Li (McMaster university). GH6 was made by introducing an XhoI site using the linker 5'-CTC GAG CTC GAG-3' into an HinfI restriction site in the EC of G (position 760) resulting in the insertion of VAL, SER and ARG after GLY 244.

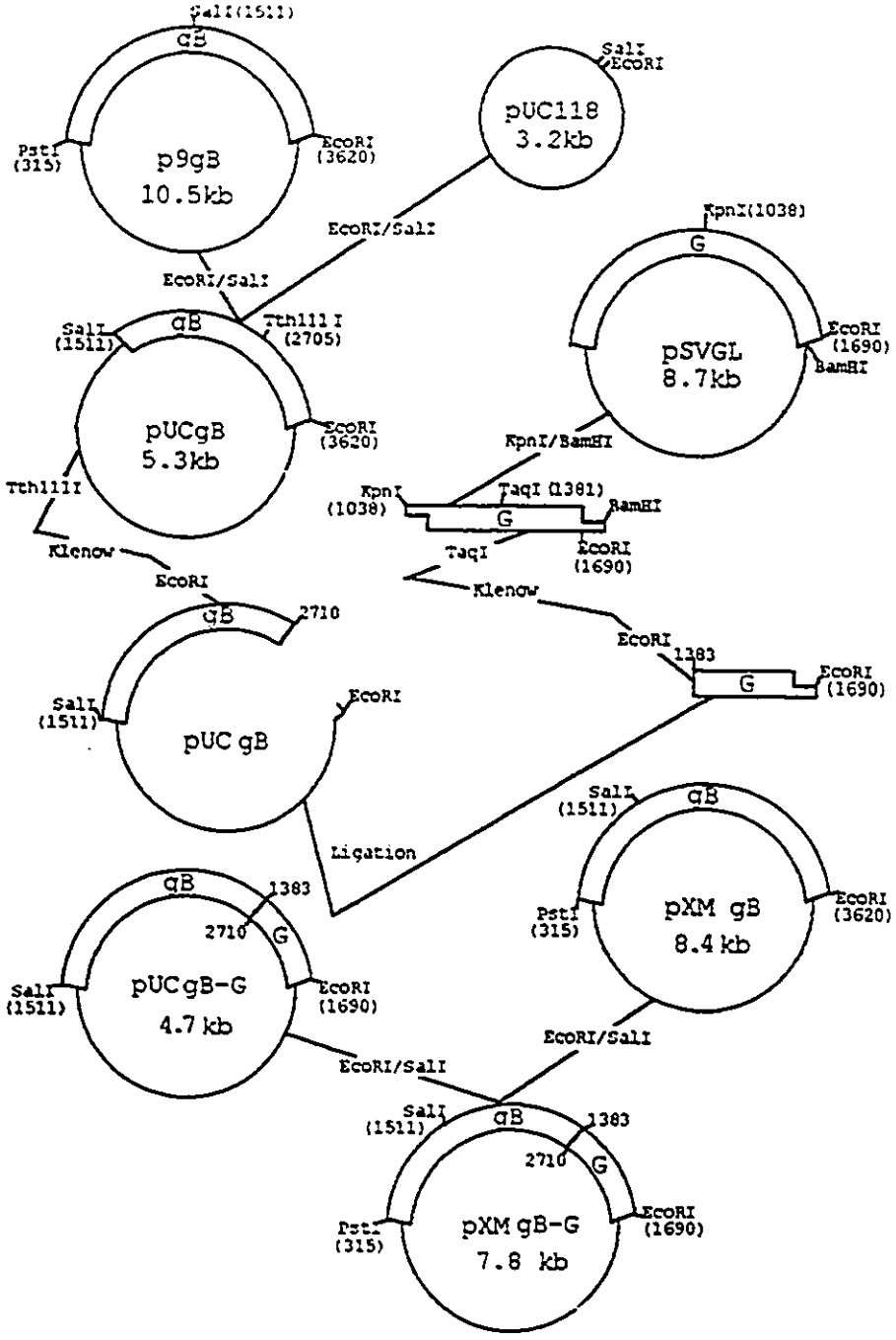

Figure 3.3.1 Strategy to construct gB-G. See text for details.

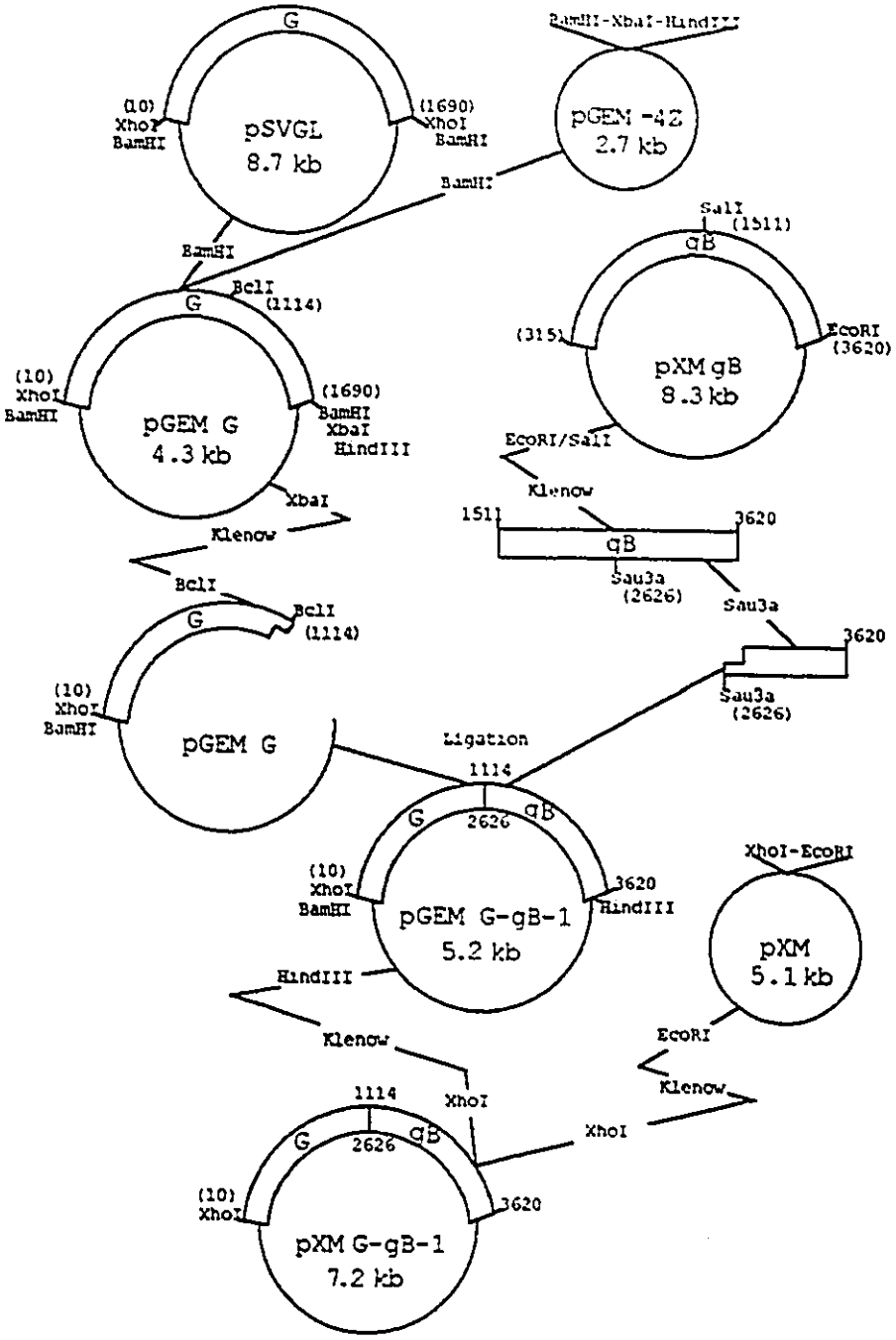

Figure 3.3.2 Strategy to construct G-gB-1. See text for details.

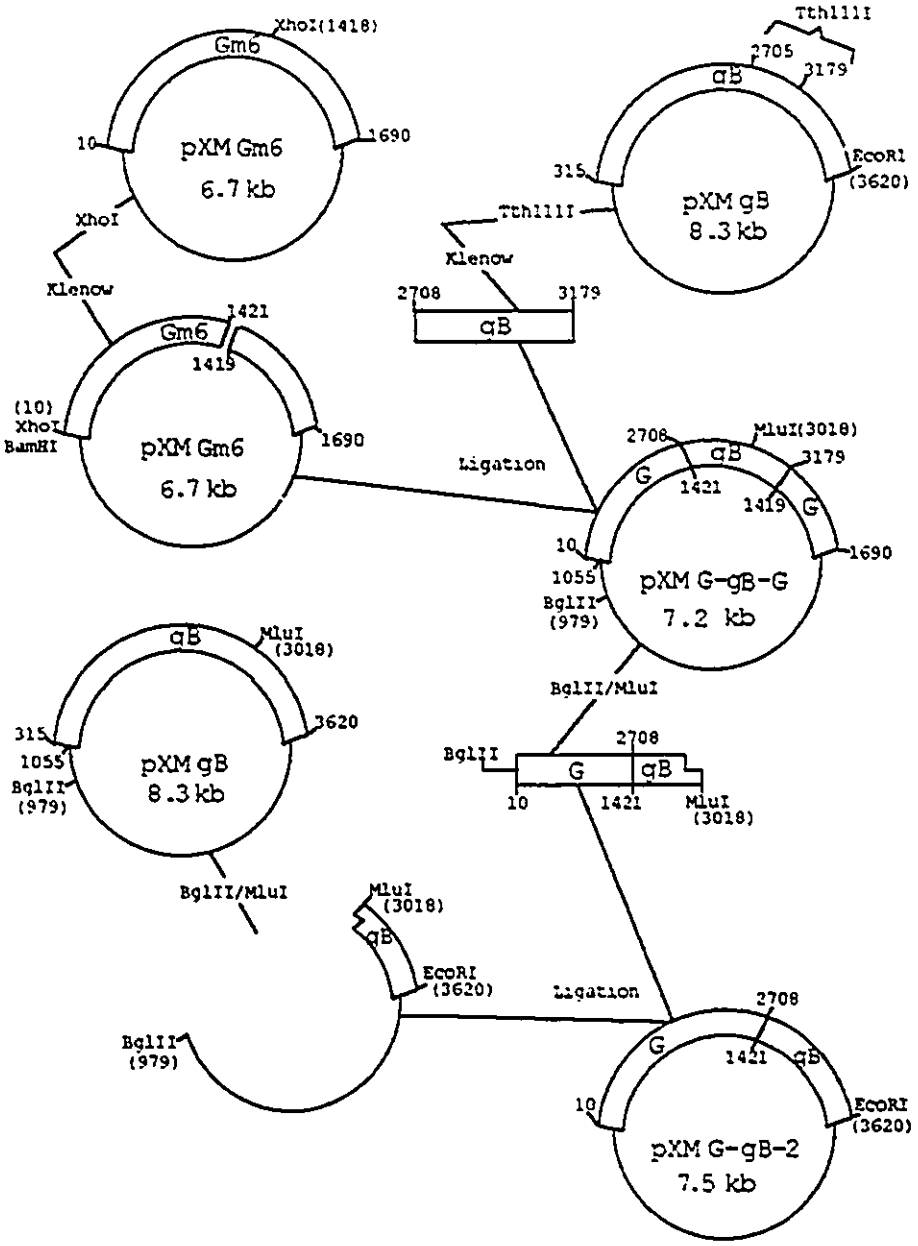

Figure 3.3.3 Strategy to construct G-gB-2. See text for details.

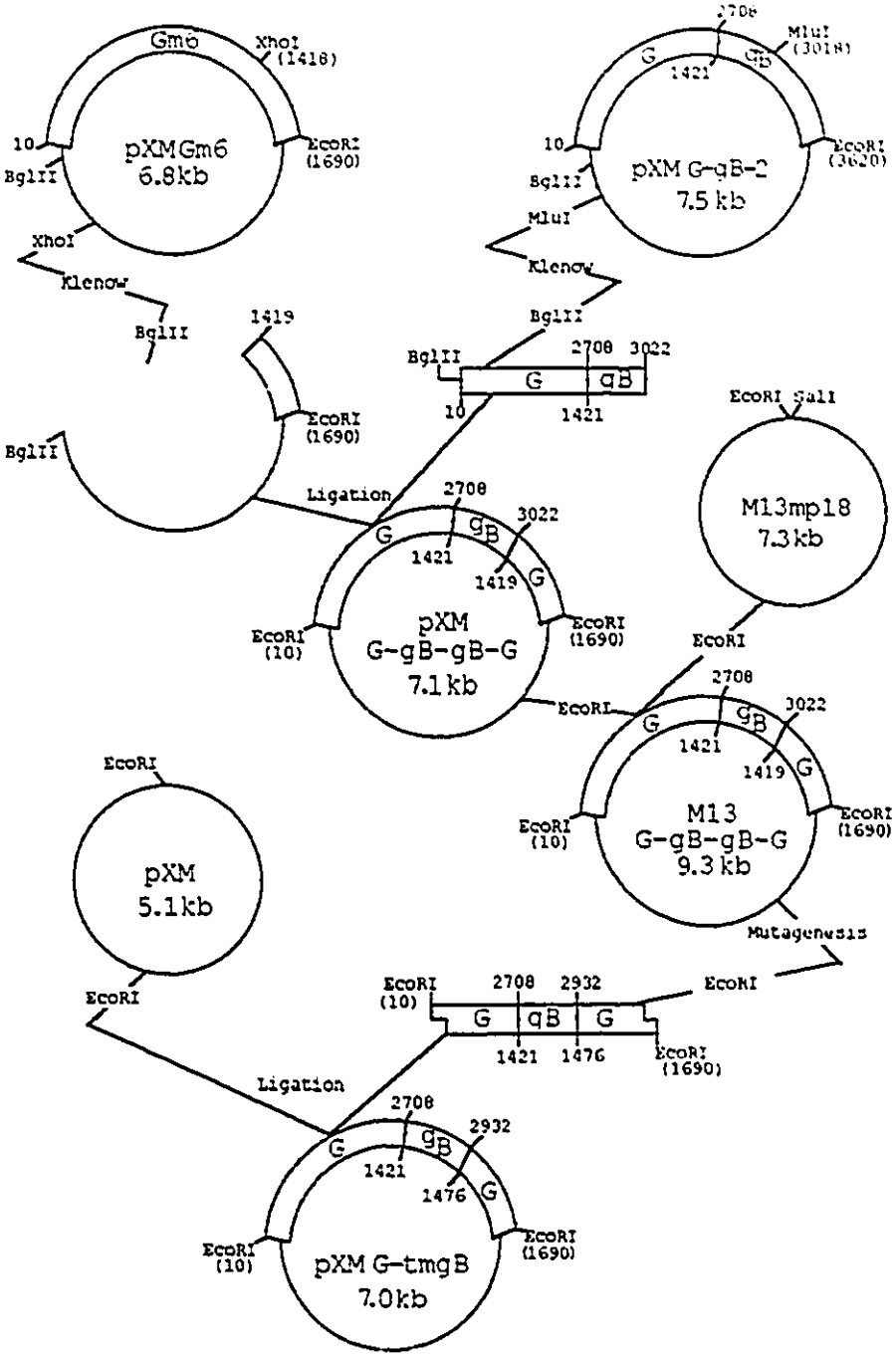

Figure 3.3.4 Strategy to construct G-tmgB. See text for details.

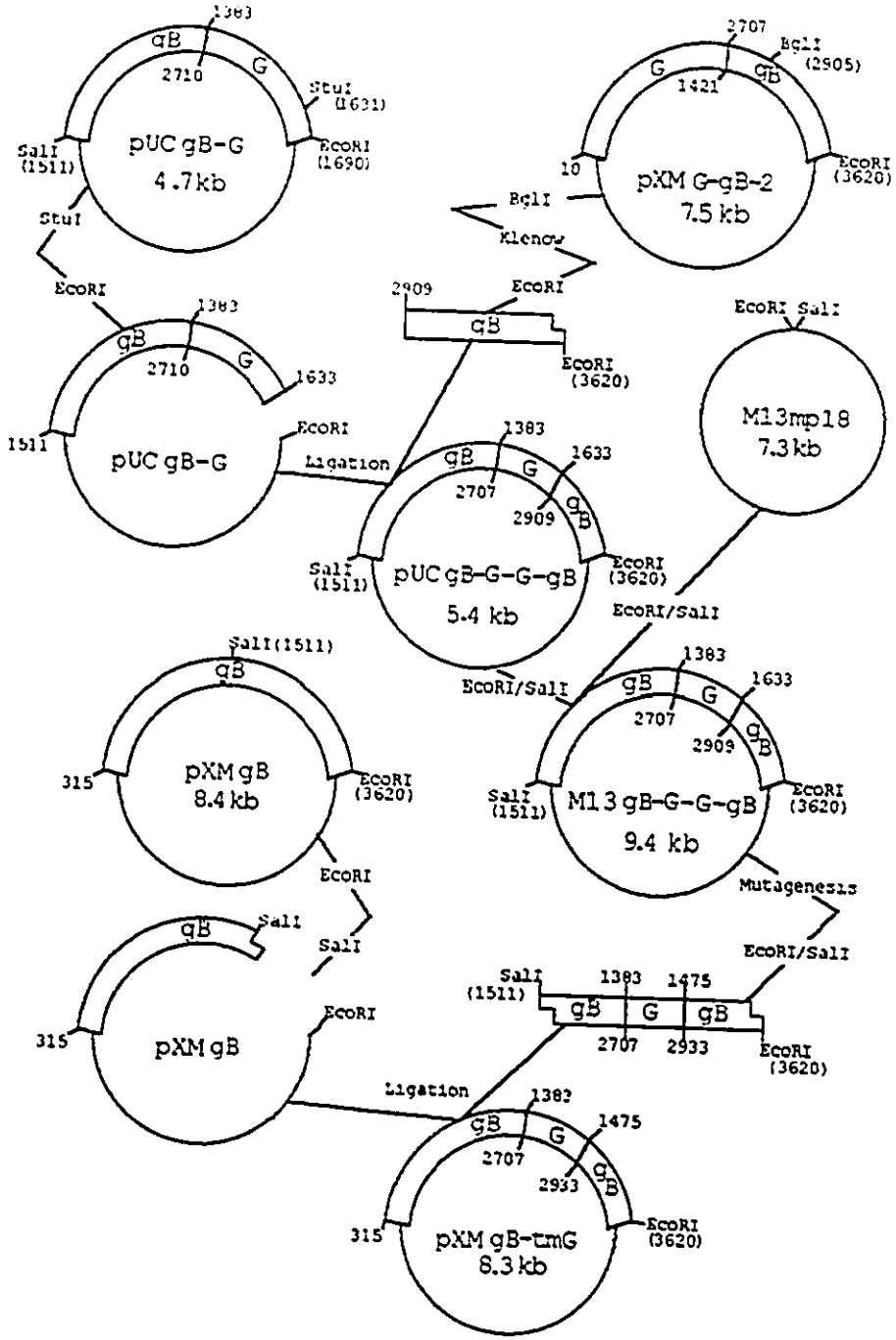

Figure 3.3.5 Strategy to construct gB-tmG. See text for details.

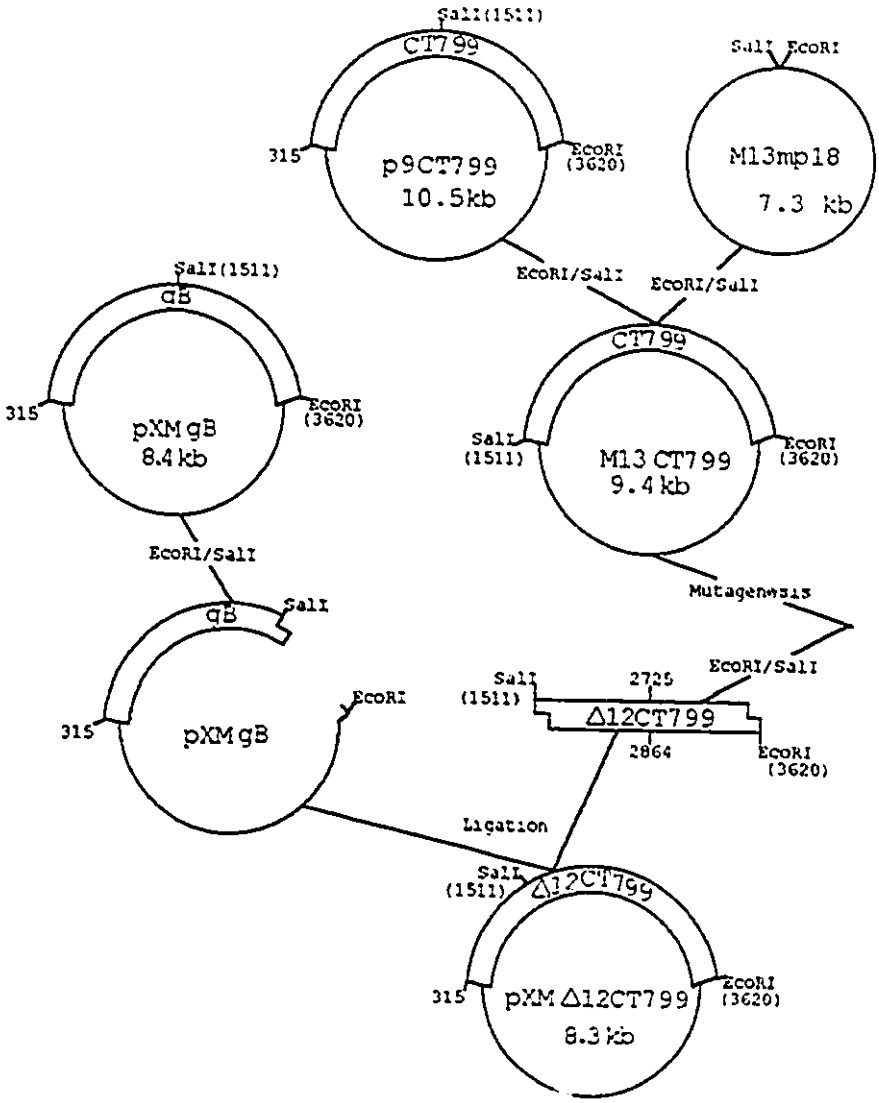

Figure 3.3.6 Strategy to construct Δ12CT799. See text for details.

Table 3.3.1 Predicted sequences of the mutated regions. Numbers above and below each sequence correspond to the nucleotide and amino acid numbers respectively (Bzik et al., 1986; Rose and Gallione, 1981). Residues inserted in Gm6 and belonging to gB are bold.

```
                                                                  1433
      1404
Gm6       AGT AGT TGG AAA AGC TCG AGC TCT ATT GCC TCT TTT
          ser ser trp lys ser ser ser ser ile ala ser phe
          459                                             468
      2693          EC (gB)             EC (G)              1400
gB-G      TTC GCC GAC ATC GAC ACC-GAG CTT GTA GAA GGT TGG
          phe ala asp ile asp thr-glu leu val glu gly trp
          716                                             457
      1101          EC (G)              EC (gB)             2647
G-gB-1    AGA ATG GTC GGA ATG ATC-AAG GAC AGC GGC CTG CTG
          arg met val gly met ile-lys asp ser gly leu leu
          358                                             700
      1404          EC (G)              EC (gB)             2725
G-gB-2    AGT AGT TGG AAA AGC TCG-ACG GTC ATC CAC GCC GAC
          ser ser trp lys ser ser-thr val ile his ala asp
          459                                             726
      2915          TM (gB)             CT (G)              1493
G-tmgB    GCG GCC TTC TTC GCC TTT-CGA GTT GGT ATC CAT CTT
          ala ala phe phe ala phe-arg val gly ile his leu
          790                                             488
      1458          TM (G)              CT (gB)             2950
gB-tmG    GGA CTA TTC TTG GTT CTC-CGA TAC GTC ATG CGG CTG
          gly leu phe leu val leu-arg tyr val met arg leu
          477                                             801
      2708          EC (gB)             3rd TM (gB)         2881
Δ12CT799  ACG GTC ATC CAC GCC GAC-AAC CCC TTT GGG GCG CTG
          thr val ile his ala asp-asn pro phe gly ala leu
          721                                             778
```

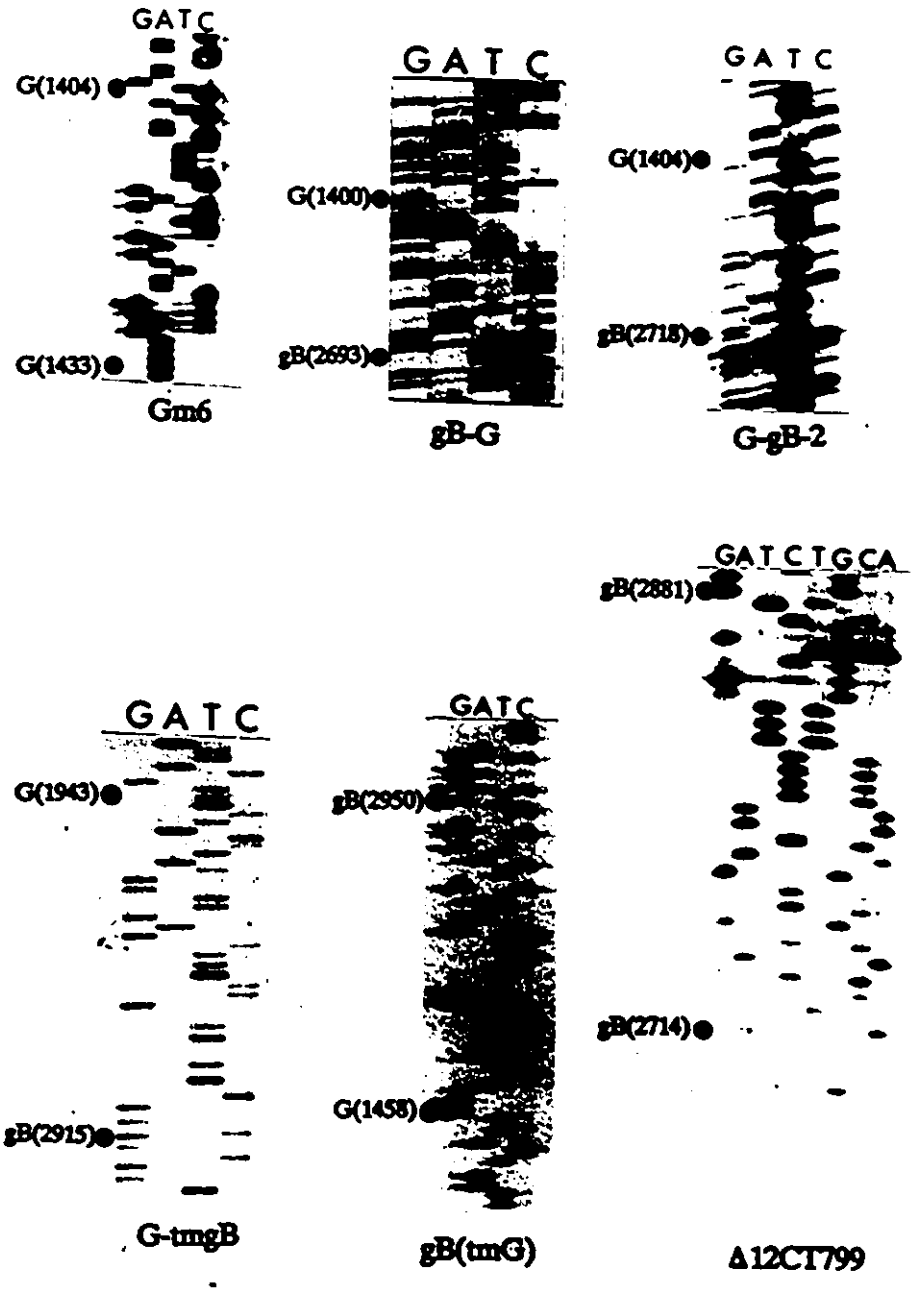

Figure 3.3.7 Sequence analysis of the mutated regions. The nucleotide sequences of the mutated and spliced regions of Gm6, gB-G, G-gB-2, G-tmgB gB-tmG and Δ12CT799 were determined by the chain termination method. For Gm6 and G-gB-2 the strand opposite to the coding sequence was read. The sequence of Δ12CT799 was determined by using dGTP (four left lanes) and dITP (four right lanes).

3.4. Preparation of antibodies against gB and G

In order to study the biochemical properties and the distribution of wild types and mutants, specific polyclonal antibodies against gB and G were prepared. The antibodies were made by inoculating rabbits with recombinant adenoviruses expressing gB or G (Johnson et al., 1988a; Schneider et al., 1989). As a first purification step, the immunoglobulins were isolated from the antiserum by ammonium sulfate precipitation.

3.4.1 Specificity and purification of anti-gB antibody

The specificity of the antibody raised against gB (anti-gB) was established by immunoprecipitation using lysates of cells transfected with gB or lysates of cells infected with HSV-1 (Fig. 3.4.1a). In cells transfected with gB, anti-gB antibody recognised two proteins with apparent molecular weights (mol wt) of 110 kD and 120 kD (Fig. 3.4.1a arrowheads; see also Fig. 3.5.2). No band was observed in cells transfected with the expression vector (pXM). The 110 kD protein corresponds to the precursor of gB (pgB) which is associated with the unprocessed high mannose core oligosaccharides, while the 120 kD protein represents the mature and fully processed gB (Wenske et al., 1982).

When the immunoprecipitation was done using HSV-1 infected cells, pgB was detected but not the mature gB (Fig 3.4.1a). Other proteins with apparent mol wt of 140, 65 and 48 kD were also detected. The mature form of gB was not observed with the HSV-1 lysate, because in late HSV-1 infection, as it was the case in this experiment, viral glycoproteins are processed and transported to the cell surface at a slower rate

(Johnson and Smiley, 1985; Campadelli-Fiume et al., 1988; Sommer and Courtney, 1991). The 65 kD band corresponds to the IgG Fc receptor of HSV-1, composed of glycoprotein gE and gI (Johnson et al., 1988a), whereas the 48 kD band represents most likely the gI precursor (pgI) or a degradation product. The nature of the 140 kD protein, which was not present all the time (compare Figs 3.4.1a and 3.4.1b) is not known. The 65 and 48 kD proteins were the only ones observed after immunoprecipitation of HSV-1 infected cells using the antibody raised against G (anti-G), indicating that they bind indifferently to any kind of rabbit IgG (Fig. 3.4.3). When the specificity of anti-gB antibody was analyzed by immunobloting using HSV-1 infected cells, only pgB and gB were detected confirming the specificity of the antibody (Fig. 3.4.1b).

Anti-gB antibody was further purified using an affinity column that was made with a mixture of HSV-1 glycoproteins. The glycoproteins were obtained by isolating the glycoproteins of cells infected with HSV-1 using a lentil lectin-conjugated sepharose column as described by Pachl et al. (1987). The bound glycoproteins were eluted with α-D-methylmanoside, collected and analyzed by SDS-PAGE (Fig. 3.4.2A). The fractions containing the glycoproteins were pooled and used to make the column (see Materials and Methods).

A band at 110 kD corresponding to pgB was observed after immunoprecipitation of HSV-1 infected cells with the affinity purified anti-gB antibody (Figs. 3.4.1C and 3.4.3). The 65 kD contaminant was detected only after longer exposure of the gel, indicating that the affinity purification has improved the specificity of the antibody.

3.4.2 Specificity and purification of anti-G antibody

The specificity of the antibody raised against G (anti-G) was verified by immunoprecipitation using lysates of VSV infected cells (Fig. 3.4.4A). A major band with an apparent molecular weight of 63 kD was detected (large arrow). This band corresponds to G, because the same band was detected after immunoprecipitation with an antibody raised against VSV. A less intense band just below the mature G (small arrows, Figs 3.4.4A and 3.4.4B) was also detected after immunoprecipitation with the anti-G antibody. This faster migrating band corresponds most likely to the soluble G synthesized during VSV infection (Little and Huang, 1978, Irving and Ghosh, 1982; Graeve et al., 1986; See also Section 1.7.2). No band was observed when the immunoprecipitation was done using mock infected cells (Fig. 3.4.4A). In COS-1 cells transfected with pXM G, only one band corresponding in size with G was detected after immunoprecipitation (Fig. 3.5.1).

Anti-G antibody was also affinity purified using a column made with G, that was purified according to the method of Kelley et al. (1972). Briefly, VSV virions were isolated by centrifugation of media of infected cells. The virions were then purified by rate zonal centrifugation in a linear sucrose gradient and G was released from the viral envelope by incubation in 2% Triton. The G depleted virions were then removed by centrifugation and G was recovered in a pure form in the supernatant (Fig. 3.4.2B).

The specificity of the affinity purified anti-G was confirmed by immunoprecipitation using lysate of VSV infected cells (Fig. 3.4.4B).

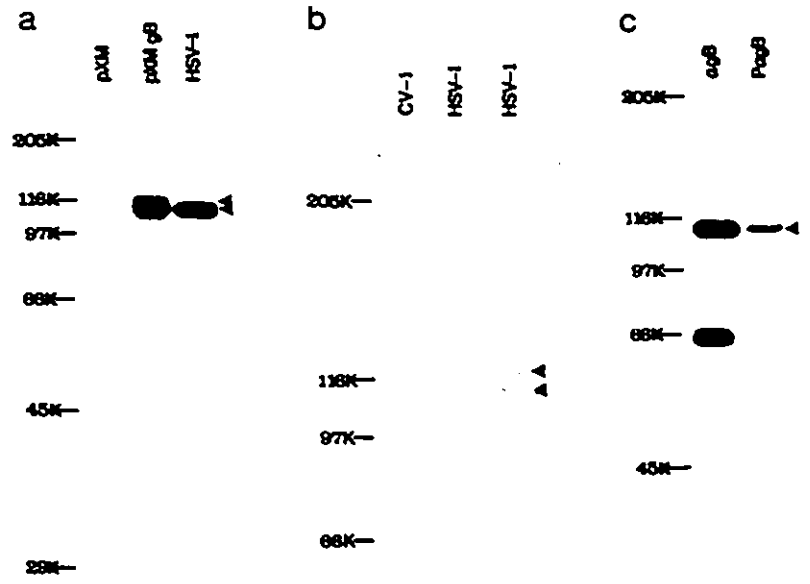

Figure 3.4.1 Specificity of anti-gB antibody. a) COS-1 cells transfected with the expression vector (pXM) or with the expression vector containing the gB gene (pXM gB), and CV-1 cells infected with HSV-1 (HSV-1) were pulse labeled with [^{35}S]-methionine and lysed. The lysate was immunoprecipitated with anti-gB antibody and analyzed by SDS-PAGE followed by fluorography. b) Lysate of CV-1 cells (CV-1) or CV-1 cells infected with HSV-1 (HSV-1) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then reacted with anti-gB antibody followed by a horseradish peroxidase-conjugated goat anti-rabbit antibody. c) COS-1 cells infected with HSV-1 were labeled with [^{35}S]-methionine and lysed. Half of the lysate was immunoprecipitated with anti-gB antibody (αgB) or with the affinity purified anti-gB antibody (PαgB). The immunoprecipitate was analyzed by SDS-PAGE followed by fluorography. Arrowheads: position of pgB and gB. The position of the mol wt marker in kD is indicated.

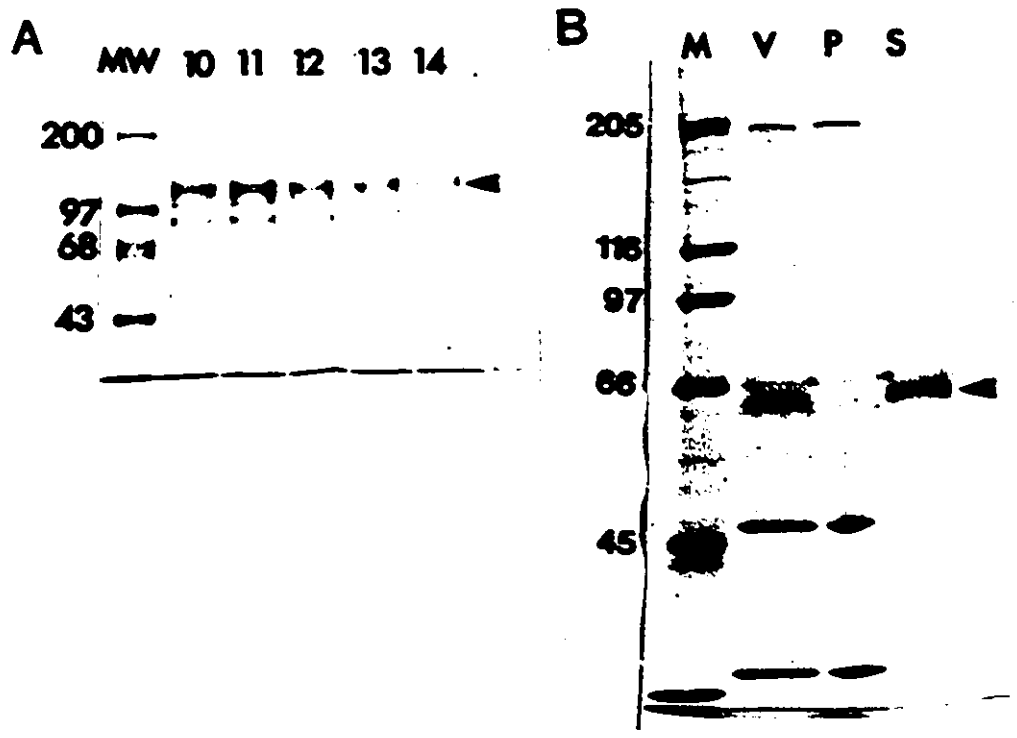

Figure 3.4.2 Purification of HSV-1 glycoproteins and glycoprotein G. A) Lysates of HSV-1 infected cells were passed through a lentil lectin conjugated-Sepharose column. The bound glycoproteins were eluted with α-D-methylmannoside, and aliquots of fractions 10 to 14 were analyzed by SDS-PAGE and Coomassie blue staining. B) Purified VSV virions were resuspended in 0.0.1 M HEPES(pH 7.3) containing 2% Triton. The solution was then centrifuged to separate glycoprotein G from the other viral components. The pellet (P), the supernatant containing G (S), and the virus before detergent extraction, were analyzed by SDS-PAGE and Coomassie blue staining. MW and M: mol wt marker (kD); Arrowheads: position of gB (A) or G (B).

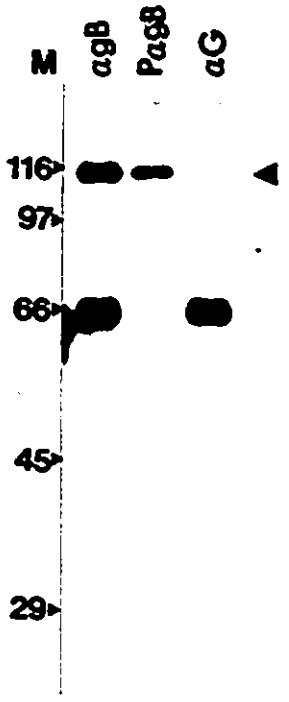

Figure 3.4.3 Specificity of the affinity purified anti-gB antibody. COS-1 cells infected with HSV-1 were pulse labeled with [^{35}S]-methionine and lysed. One third of the lysate was immunoprecipitated with anti-gB (αgB), with the affinity purified anti-gB (PαgB), or with anti-G (αG) antibodies. The immunoprecipitate was then analyzed by SDS-PAGE followed by fluorography. Arrowhead: position of pgB; M: Mol wt marker (kD).

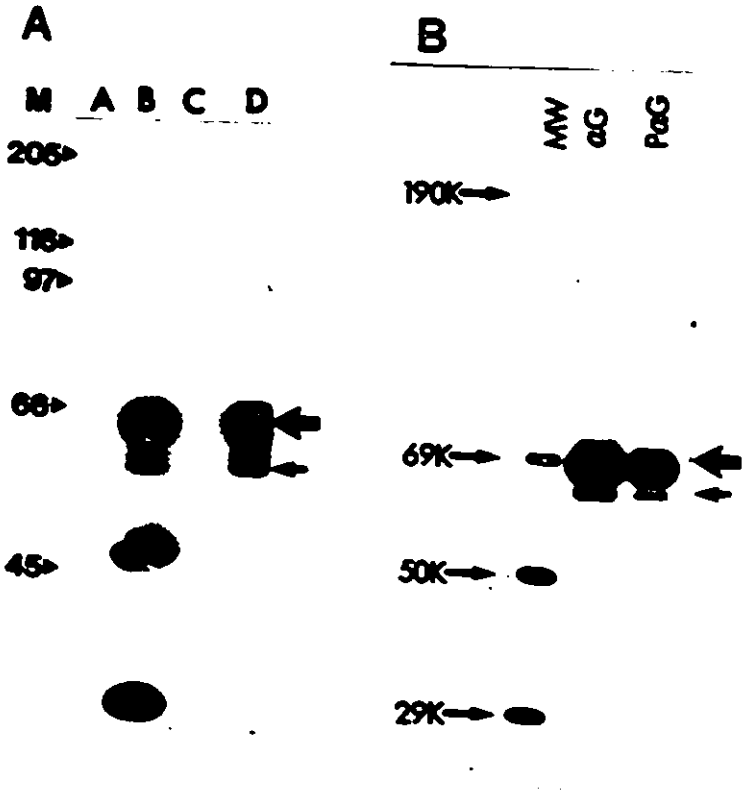

Figure 3.4.4 Specificity of the anti-G antibody. A) Mock infected cells (A,C) and cells infected with VSV (B,D) were labeled with [^{35}S]-methionine and lysed. Half of the lysate was immunoprecipitated with an antibody specific to VSV (A,B) or with anti-G (C,D). The immunoprecipitate was analyzed by SDS-PAGE followed by fluorography. B) Cells infected with VSV were labeled with [^{35}S]-methionine and lysed. Half of the lysate was immunoprecipitated with anti-G (αG) or the affinity purified anti-G (PαG). The immunoprecipitate was analyzed by SDS-PAGE followed by fluorography. M: Mol wt marker (kD); MW: labeled VSV (Indiana) was used as mol wt marker (kD); Small arrow: position of the soluble G; Large arrow: position of G.

3.5 Analysis of mutants by immunoprecipitation

The chimeric and mutant proteins of Fig. 3.1.1 and deletion mutant CT799 (Fig 3.10.5) were studied by transient expression in COS-1 cells. One day after transfection, the cells were labeled for 2 to 3 h with [^{35}S]-methionine and the proteins were analyzed by immunoprecipitation using anti-gB or anti-G (Fig 3.5.1 to 3.5.4). The mol wt of each protein was determined by comparing their mobility with the mobility of known standards as described by Laemmli (1970) (See Section 2.39.1). When a mutant migrated as a doublet (see below), it was the slower migrating band that was used for the determination of the experimental mol wt. The experimental mol wt of the mutants were in agreement with theoretical values (Table 3.5.1).

The proteins made with the EC of gB were recognized only by anti-gB antibody, whereas the proteins made with the EC of G were recognized only by anti-G antibody, suggesting the antibodies were raised against the EC of gB and G (Figs 3.5.1 to 3.5.3). Although G-gB-1 and G-gB-2 were not immunoprecipitated with anti-gB antibody, both proteins were recognized by an antibody (anti-CT) raised against the last 16 amino acids of the carboxy terminus of gB (Fig 3.5.2 and 3.5.3). Anti-CT antibody, a generous gift of Mr. D. Snoddy (McMaster University), recognized gB, and gB-tmG, but not mutant Δ12CT799 which lacks the CT of gB (Fig. 3.5.4).

Anti-CT antibody had a stronger affinity for gB, gB-tmG and G-gB-1 than for G-gB-2 (compare Figs 3.5.2, 3.5.3 and 3.5.4), which suggests that the carboxy terminus of G-gB-2 was not accessible to the antibody and may consequently fold differently than

gB.

Glycopotein B, gB-G and Δ12CT799 migrated as doublets (Figs 3.5.2, 3.5.4 and 3.6.1). As discussed in Section 3.6, the faster migrating bands of these doublets correspond to the precursor which is associated with the high mannose core oligosaccharides (endo H sensitive), while the slower migrating bands correspond to the proteins associated with the complex type of oligosaccharides (endo H resistant).

In some experiments, the chimera G-gB-1 migrated as a unique band of 70 kD corresponding in size with the expected mol wt of the glycosylated protein (Table 3.5.1 and Fig. 3.5.2). In other experiments however, a faster migrating band was also observed after immunoprecipitation (Figs 3.11.1). G-gB-1 possesses two potential glycosylation sites and the faster migrating band could be an incomplete glycosylation form or a degradation product.

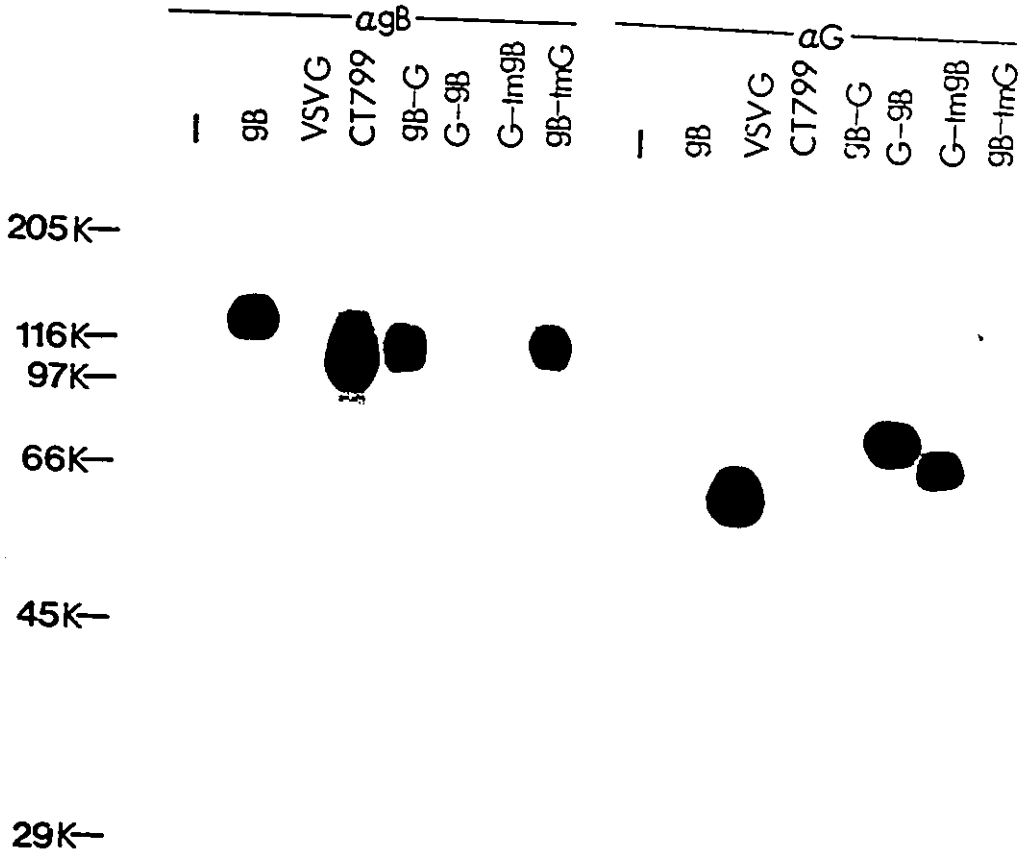

Figure 3.5.1 Immunoprecipitation of the chimeras. Mock transfected cells (-) or COS-1 cells transfected with gB, G, CT799, gB-G, G-gB, G-tmgB and gB-tmG were labeled with [^{35}S]-methionine and lysed. Half of the lysate was immunoprecipitated with anti-gB (αgB) or anti-G (αG). The immunoprecipitate was analyzed by SDS-PAGE followed by fluorography. The position of the mol wt marker in kD is indicated.

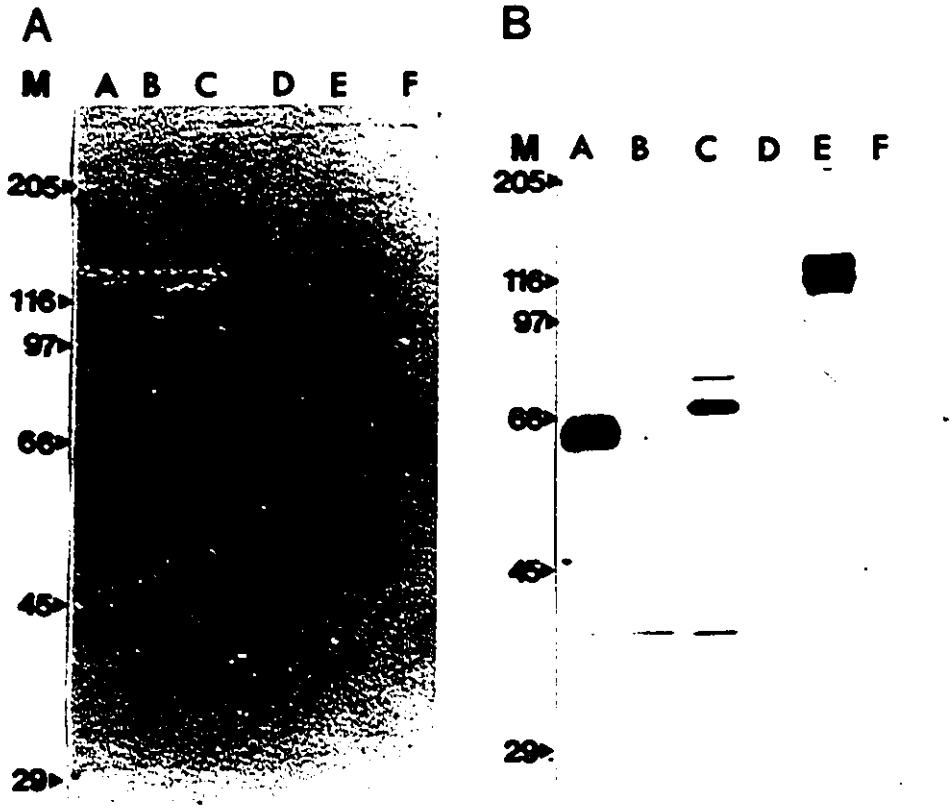

Figure 3.5.2 Immunoprecipitation of G-gB-1. A) COS-1 cells transfected with G (A,D), gB (B,E) and G-gB-1 (C,F) were labeled with [^{35}S]-methionine and lysed. Half of the lysate was immunoprecipitated using anti-G (A,B,C) or anti-CT (D,E,F). The immunoprecipitate was then analyzed by SDS-PAGE followed by fluorography. B) COS-1 cells transfected with G (A,D), gB (B,E) and G-gB-1 (C,F) were labeled with [^{35}S]-methionine and lysed. Half of the lysate was immunoprecipitated using anti-G (A,B,C) or anti-gB (D,E,F). The immunoprecipitate was then analyzed by SDS-PAGE followed by fluorography. M: Mol wt marker (kD).

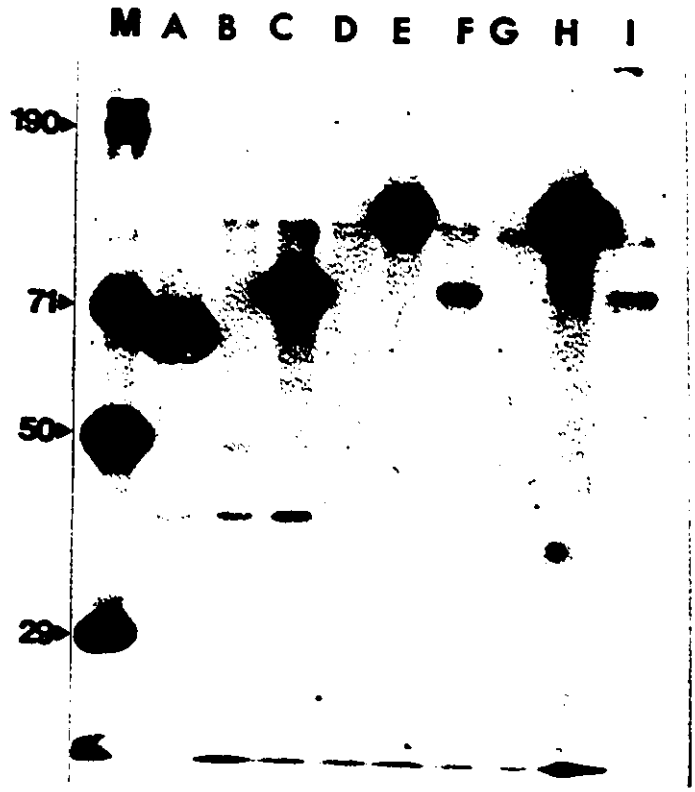

Figure 3.5.3 Immunoprecipitation of G-gB-2. COS-1 cells transfected with G (A,D,G), gB (B,E,H) and G-gB-2 (C,F,I) were labeled with [^{35}S]-methionine and lysed. One third of the lysate was immunoprecipitated with anti-G (A,B,C), anti-CT (D,E,F) or an antibody specific to HSV-1 (G,H,I). The immunoprecipitate was analyzed by SDS-PAGE followed by fluorography. The gel was overexposed to show the faint G-gB-2 bands (lane F and I). M: labeled VSV (coccal) was used as mol wt marker (kD).

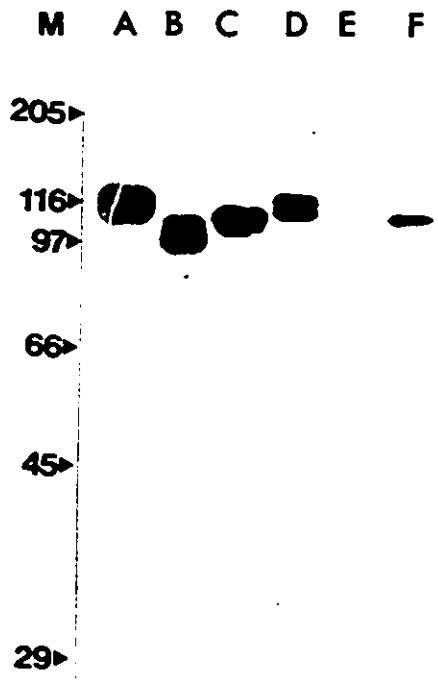

Figure 3.5.4 Immunoprecipitation of G-tmgB and Δ12CT799. COS-1 cells transfected with gB (A,D), Δ12CT799 (B,E), and gB-tmG (C,F) were labeled with [^{35}S]-methionine and lysed. Half of the lysate was immunoprecipitated with the anti-gB (A,B,C) or anti-CT (D,E,F). The immunoprecipitate was analyzed by SDS-PAGE followed by fluorography. M: Mol wt marker (kD).

Table 3.5.1 Molecular weight and acquisition of endo H resistance

Protein	Theoretical[a] Molecular Weight (kD)	Experimental[b,c] Molecular Weight (kD)	Time For Acquisition of 50% Endo H Resistance (min)
gB	-	120	54 (3)[d]
G	-	63	22 (3)
gB-G	108	110	52 (3)
G-gB-1	67	70	Sensitive (2)
G-gB-2	77	80	Sensitive (2)
GH6	63	63	Sensitive (2)
G-tmgB	68	68	33 (2)
gB-tmG	106	110	Sensitive (2)
Δ12CT799	104	110	42 (2)

a: Calculated from the experimental values of gB and G (see Materials and Methods)
b: Estimated by SDS-PAGE using the mobility of known standards (Laemmli, 1970; see Materials and Methods)
c: Value of the fully processed protein
d: Number of independent experiments
Sensitive: Remained completely sensitive to endo H after a chase of 2 h

3.6 Rate of transport to the Golgi complex

Membrane glycoproteins, such as gB and G, are glycosylated at specific asparagine residues in the ER by addition of high mannose oligosaccharides (Section 1.2.6) that can be cleaved by endoglycosidase H (endo H). Cleavage by endo H increases the mobility of the target protein on SDS-PAGE (Fig 3.6.1). During transport to the plasma membrane, the oligosaccharides are modified by removal and addition of specific residues by Golgi enzymes. Some of these modifications abolish the sensitivity to endo H (Tarentino et al., 1974). The rate of acquisition of endo H resistance, which is monitored on SDS-PAGE by a band shift, can therefore be used to determine the rate of transport of the glycoproteins from the ER to the Golgi complex.

3.6.1 Transport of gB and G

The processing and glycosylation of the proteins were studied in transfected COS-1 cells after labeling for 15 min with [^{35}S]-MET followed by a chase with cold MET. Glycoprotein B was first synthesized as a completely endo H sensitive form (pgB) that migrated with an apparent mol wt of 110 kD (Fig 3.6.1). Post-translational modifications occurring in the Golgi complex, such as O-linked glycosylation (Johnson and Spear, 1983) and processing of the N-linked high mannose oligosaccharides, result in an increase of the protein size of about 10 kD (Fig. 3.6.1, arrow). The mature form of gB was partly resistant to endo H, because gB is N-linked glycosylated at 5 different sites (Cai et al., 1988b) and some of these carbohydrates are not completely modified by

Golgi enzymes and therefore, remain endo H sensitive (Wenske et al., 1982). The half time of acquisition of endo H resistance by gB was 54 min (Table 3.5.1). After a chase of 2 h, about 25% of the protein was still completely endo H sensitive.

Glycoprotein G possesses two N-linked glycosylation sites (Rose and Gallione, 1981). The conversion of the precursor of G (endo H sensitive) to the mature form of G (endo H resistant) should have increased the size of the protein by about 2 kD (Knipe et al., 1977). However, this slight shift of mobility was not detected in the present study (Fig 3.6.1). Glycoprotein G was transported more rapidly than gB to the Golgi complex, because G was completely endo H resistant after a chase of 1 h (Fig. 3.6.1 and Table 3.5.1).

3.6.2 Transport of mutants

Just after labeling, all the mutants were completely sensitive to endo H digestion indicating that they were glycosylated and translocated into the lumen of the ER (Fig 3.6.1). Mutants gB-G, G-tmgB and Δ12CT799 became endo H resistant, an indication that they were transported to the Golgi complex. On the other hand, G-gB-1, G-gB-2, GH6 and gB-tmG remained completely endo H sensitive which suggested that they did not reach the Golgi complex and that they remained in the ER.

The mature form of gB-G and Δ12CT799 (arrows Fig. 3.6.1) migrated more slowly than their precursors, suggesting that they were O-linked glycosylated as in the

case of gB. The rates of acquisition of endo H resistance of gB-G and Δ12CT799 were similar to those observed in the case of gB (Table 3.5.1). After a chase of 2 h, about 15% of Δ12CT799 was still completely endo H sensitive. It was difficult to measure the rate of acquisition of endo H resistance of G-tmgB, because the endo H resistant form disappeared with time (Fig 3.6.1). After a chase of 2 h, no G-tmgB was detected in the medium suggesting the endo H resistant form of G-tmgB was not secreted, but was degraded intracellularly. The chimera G-tmgB was transported efficiently out of the ER, because after a chase of 1h, 60% of the protein was endo H resistant (Fig 3.6.1 and Table 3.5.1). As observed for gB and Δ12CT799, a fraction of G-tmgB remained completely sensitive to endo H after a chase of 2 h.

In well resolved gels, G-gB-2 appeared sometimes as a doublet; the faster migrating band being about 3 kD smaller (Fig 3.6.1). The doublet was present just after the pulse, one band was not chased into the other one, and they were both sensitive to endo H. The exact nature of the faster migrating band is not known but it could correspond to a degradation product of G-gB-2, or to premature termination of translation.

Mutants, such as G-gB-1 and GH6 that were made with the EC of G, possess two potential glycosylation sites. Digestion of G-gB-1 and GH6 with endo H generated two closely migrating bands, suggesting that only one of the two oligosaccharide chains was sometimes accessible and digested by endo H, most likely because G-gB-1 and GH6 aggregate (Section 3.7). Mutant GH6 was degraded (Fig 3.6.1). A quantitative analysis

of the gels showed that GH6 was degraded with a half life of about 30 min.

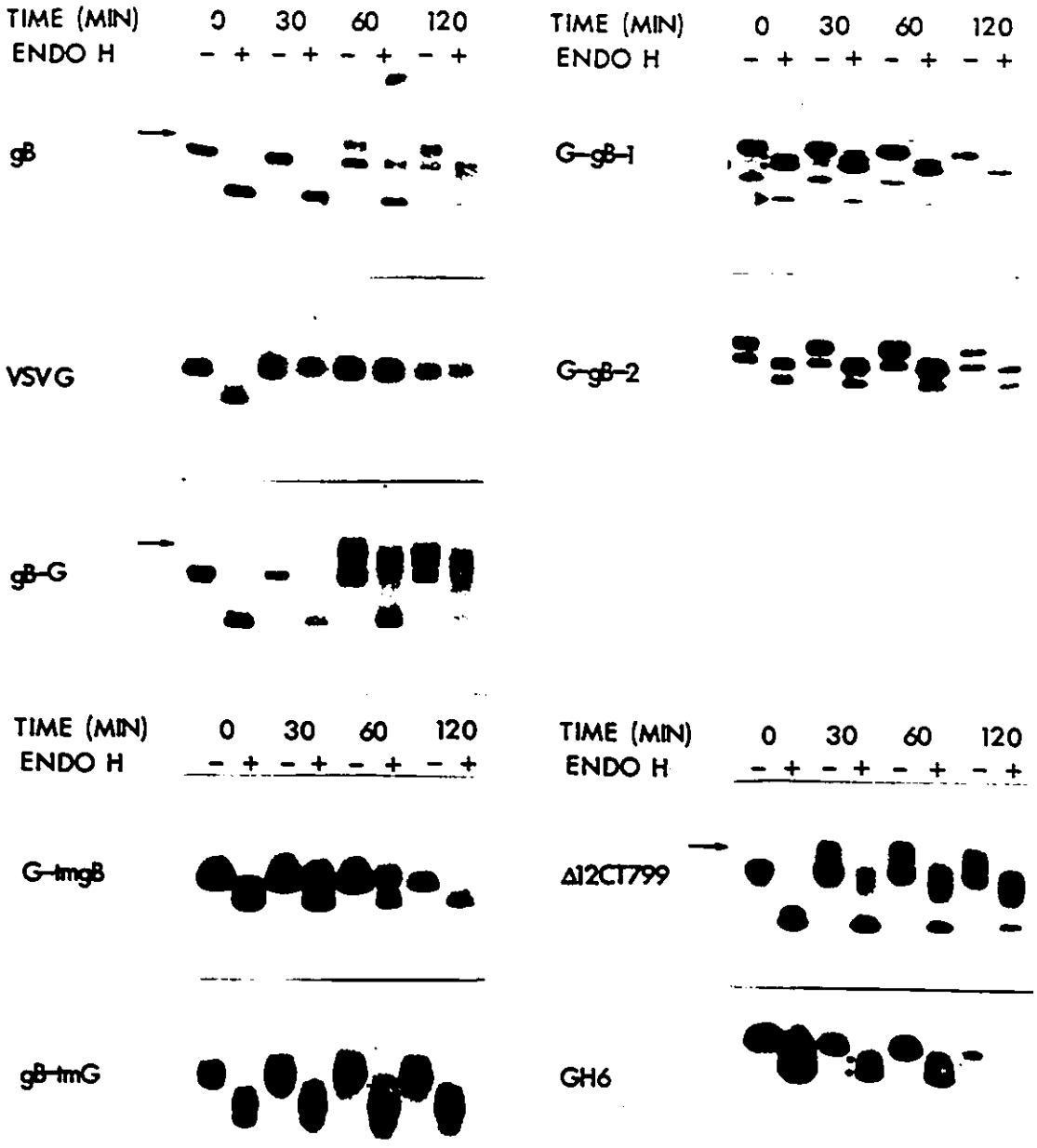

Figure 3.6.1 Processing and endo H digestion. COS-1 cells transfected with the different cDNAs were pulse labeled with [^{35}S]-methionine for 15 min and then chase for 0, 30, 60 or 120 min. The cells were lysed and the proteins were immunoprecipitated. Half of the immunoprecipitate was digested (+) or not (-) with endo H, and then analyzed by SDS-PAGE followed by fluorography. Arrows: position of the fully processed form of gB, gB-G and Δ12CT799; Dots: position of the completely and partially digested products of G-gB-1 and GH6; arrowhead: position of a likely degradation product of G-gB-1.

3.7 Oligomeric nature

Soon after their synthesis, gB and G oligomerize into dimers and trimers respectively (Sections 1.5.1 and 1.6.3). Proper folding and oligomerization are a requirement for most proteins to exit the ER and to reach the cell surface. It is also well known that many mutants do not fold or oligomerize properly and form aggregates that are unable to exit the ER (Section 1.2.10.1).

In order to determine the oligomeric nature of the mutants made during this study, transfected COS-1 cells were labeled for 30 min with [^{35}S]-methionine, chased for 1.5 h, lysed, and centrifuged on continuous 5-20% sucrose gradients as previously described (Doms et al., 1987). Eighteen fractions were collected from the bottom of the tubes and the odd fractions were analyzed by immunoprecipitation (Fig 3.7.1A). Aldolase (mol wt: 161 kD; S_{w20}: 8.6S) and BSA (mol wt: 67 kD; S_{w20}: 4.7S) were loaded simultaneously with each sample, and were used as standards to estimate the mol wt of the mutants according to the method of Martin and Ames (1961).

In agreement with previous studies, gB and G sedimented as dimers and trimers respectively (Fig. 3.7.1A and Table 3.7.1). The chimeras gB-G sedimented as dimers. The chimera G-gB-2 cosedimented with G, whereas G-tmgB sedimented slightly faster because a higher proportion of protein was found in the adjacent fraction closest to the bottom. This suggests that the oligomers formed by G-tmgB are larger than those formed by G-gB-2. The oligomeric natures of G-tmgB and G-gB-2 thus correspond to trimers and dimers respectively. The chimeras G-gB-1 and GH6 were found at the bottom of the

gradient and were consequently associated into oligomeric structures larger than 400 kD. Aggregation of G-gB-1 and GH6 would explain why they remained completely sensitive to endo H (Section 3.6), since protein aggregates are unable to exit the ER.

Mutants gB-tmG and Δ12CT799 sedimented as monomers, dimers, trimers as well as aggregates found at the bottom of the gradients. However, in both cases the proportion of dimeric proteins was more important indicating that indeed these proteins formed dimers, but that dimerization is not very efficient (Fig. 3.7.1B). A certain proportion of gB was also found at the bottom of the gradient, but the relative amount of aggregates was much higher with Δ12CT799 and with gB-tmG. The presence of aggregates suggests that a subpopulation of Δ12CT799 and gB-tmG encountered folding or oligomerization problems. This observation could explain the lack of transport of gB-tmG, since it is well known that proper folding is a requirement for most proteins to be transported efficiently. On the other hand, the problems encountered during the folding of Δ12CT799 seemed to have no obvious effect on its transport, since Δ12CT799 was transported quite rapidly (Table 3.5.1).

The previous oligomerization studies were done at pH 5.7, a value known to stabilize the G trimers during centrifugation, since at neutral pH the G trimers dissociate into monomers (Doms et al., 1987). In order to see if G-gB-2 was also sensitive to similar fluctuations of pH, the oligomerization assay was repeated at neutral pH. When the pH of the lysis buffer and gradients were raised from 5.7 to 7.4, the G-gB-2 oligomers behaved exactly as the G and dissociated into monomers (Fig. 3.7.1C).

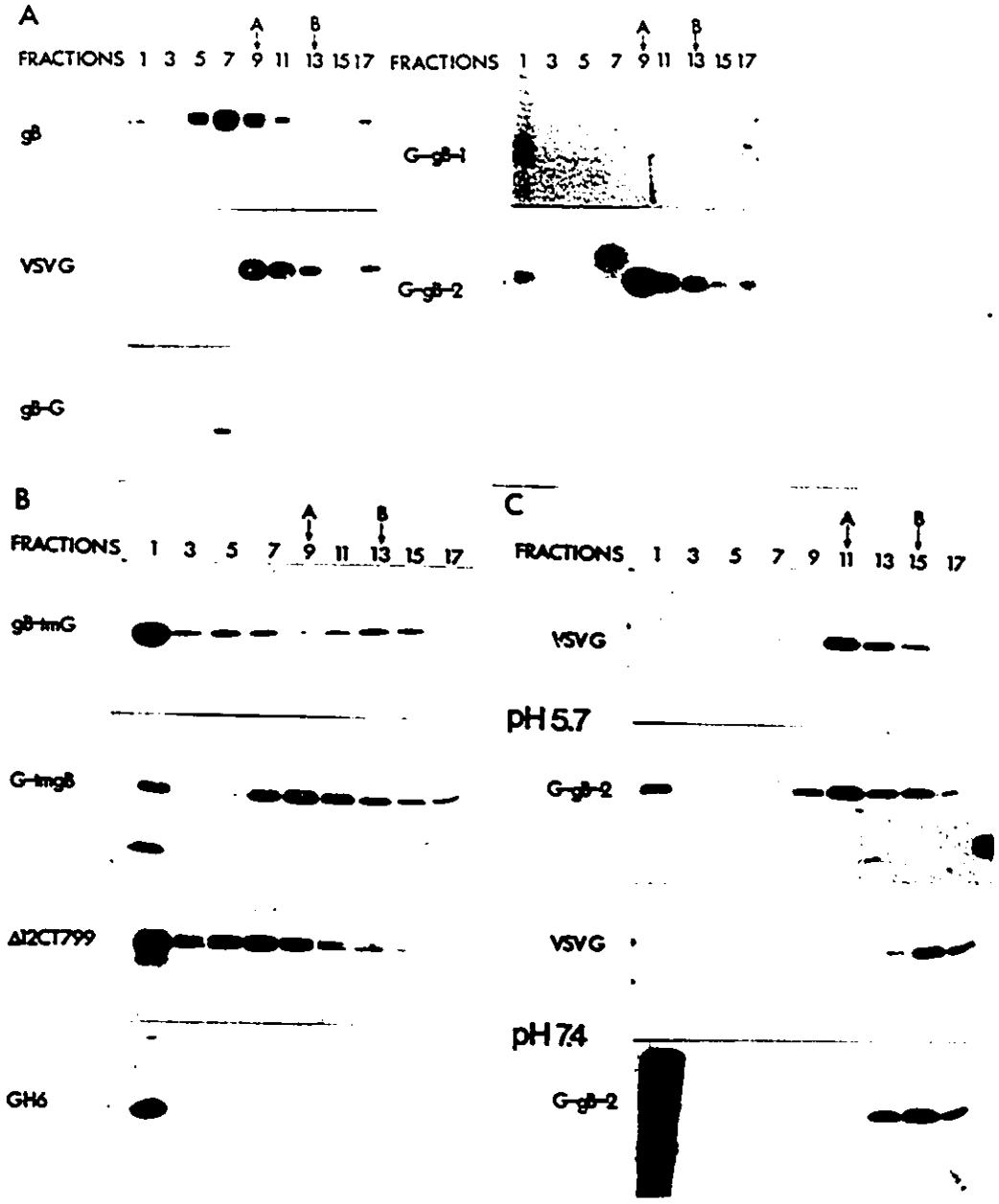

Figure 3.7.1 Sedimentation on sucrose density gradients. COS-1 cells transfected with the different cDNAs were labeled with [^{35}S]-methionine for 1 hr and chased for 1 h 30 min. The cells were lysed and loaded on a continuous 5-20% sucrose gradient and centrifuged for 20 h. Eighteen fractions were collected from the gradients (bottom = 1). The odd fractions were immunoprecipitated and analyzed by SDS-PAGE followed by fluorography. The position of aldolase (A) and BSA (B) at the end of the centrifugation is indicated. A) Sedimentation behaviour of gB, G, gB-G, G-gB-1 and G-gB-2. B) Sedimentation behaviour of gB-tmG, G-tmgB, Δ12CT799 and GH6. C) Effect of an increase of pH on the sedimentation behaviour of G and G-gB-2.

Table 3.7.1 Oligomeric nature estimated by sucrose gradients

Protein	Estimated Mass[a] (kD)	Type of Oligomer
gB	218 (4)[b]	Dimer
G	157 (4)	Trimer
gB-G	214 (2)	Dimer
G-gB-1	≥519 (2)	≥Hexamer
G-gB-2	155 (3)	Dimer
GH6	≥402 (2)	≥Hexamer
G-tmgB	161 (2)	Trimer
gB-tmG	228[c] (3)	Dimer[d]
Δ12CT799	222[c] (3)	Dimer[d]

a: Mass determined by comparison with the position of aldolase at the end of the centrifugation according to the method of Martin and Ames (1961).
b: Number of independent experiments.
c: Position of the major band.
d: Monomers and larger oligomers were also present.

3.8 Distribution by indirect immunofluorescence

Previous studies by immunofluorescence have shown that gB is specifically localized to the NE and on the plasma membrane of transfected COS-1 cells (Ali et al., 1987; Raviprakash et al., 1991). In order to find out which portion of gB is involved in NE localization, the distribution of the chimeric and mutant proteins were investigated by indirect immunofluorescence. COS-1 cells were transfected with the different cDNAs and 24 h following transfection, the distribution of the proteins on the cell surface (non-permeabilized cells) and their internal distribution (Triton permeabilized cells) were studied (Figs 3.8.1 to 3.8.3).

Cells expressing gB and G were strongly labeled on the cell surface and in a compact juxtanuclear region consistent with the Golgi complex (Fig 3.8.1, large arrowheads). The NE was brighter and better defined in cells expressing gB indicating that gB, but not G, was specifically localized to the NE. An ER-like cytoplasmic reticular network was also more apparent in cells expressing gB.

The distribution of the chimera gB-G was very similar to G, because the plasma membrane and structures ressembling to the Golgi complex (Fig. 3.8.1, large arrowhead) were strongly labeled but not the NE. This highly suggests that the EC of gB was not sufficient to specify NE localization in the context of gB-G.

The mutants that remained endo H sensitive such as G-gB-1, G-gB-2, GH6 and gB-tmG, were not transported to the cell surface. G-gB-1 and G-gB-2 were distributed in a reticular network spread in the cytoplasm that most likely corresponds to the ER

(Fig. 3.8.2). The NE was also stained in cells expressing G-gB-1 and G-gB-2 suggesting that the TM and the CT of gB are sufficient to specify NE localization. However, the cells expressing G-gB-2 were usually more strongly labeled than those expressing G-gB-1. A longer stability of G-gB-2 or a stronger affinity of G-gB-2 for the antibody could explain this observation.

Mutant GH6 was distributed in cytoplasmic roundish structures, that were often closely associated with the nucleus, and that were more compact than the network described in cells expressing G-gB-1 and G-gB-2 (Fig. 3.8.2). The NE of cells expressing GH6 was not strongly labeled suggesting that GH6 was not specifically localized in this cellular compartment. This observation suggests that a protein retained in the ER does not necessarily diffuse into the NE.

The NE of cells expressing G-tmgB was strongly labeled as well as an ER-like reticular network spread in the cytoplasm. In cells expressing G-tmgB, a compact juxtanuclear region was also observed (Fig. 3.8.3). This region could correspond to the Golgi complex, but it was generally larger than the juxtanuclear region described in cells expressing gB, G and gB-G. The fact that the NE of cells expressing G-tmgB was strongly labeled suggests that the TM of gB is sufficient to specify NE localization. The chimera G-tmgB was also transported to the plasma membrane (Fig. 3.8.3). However, in contrast with gB and G, G-tmgB was concentrated into small areas of various shapes on the plasma membrane giving rise to a punctuate pattern of distribution.

Mutant Δ12CT799 was also localized on the cell surface, in agreement with the

study using endo H digestion (Fig. 3.8.3). Its internal distribution was very similar to gB except that the NE was not as bright, suggesting that Δ12CT799 was not strongly localized to the NE.

The NE was intensely labeled in cells expressing gB-tmG and its internal pattern of distribution was essentially similar to G-gB-2 (Fig. 3.8.3). This last observation highly suggests that other portions outside the TM of gB are also involved in NE localization.

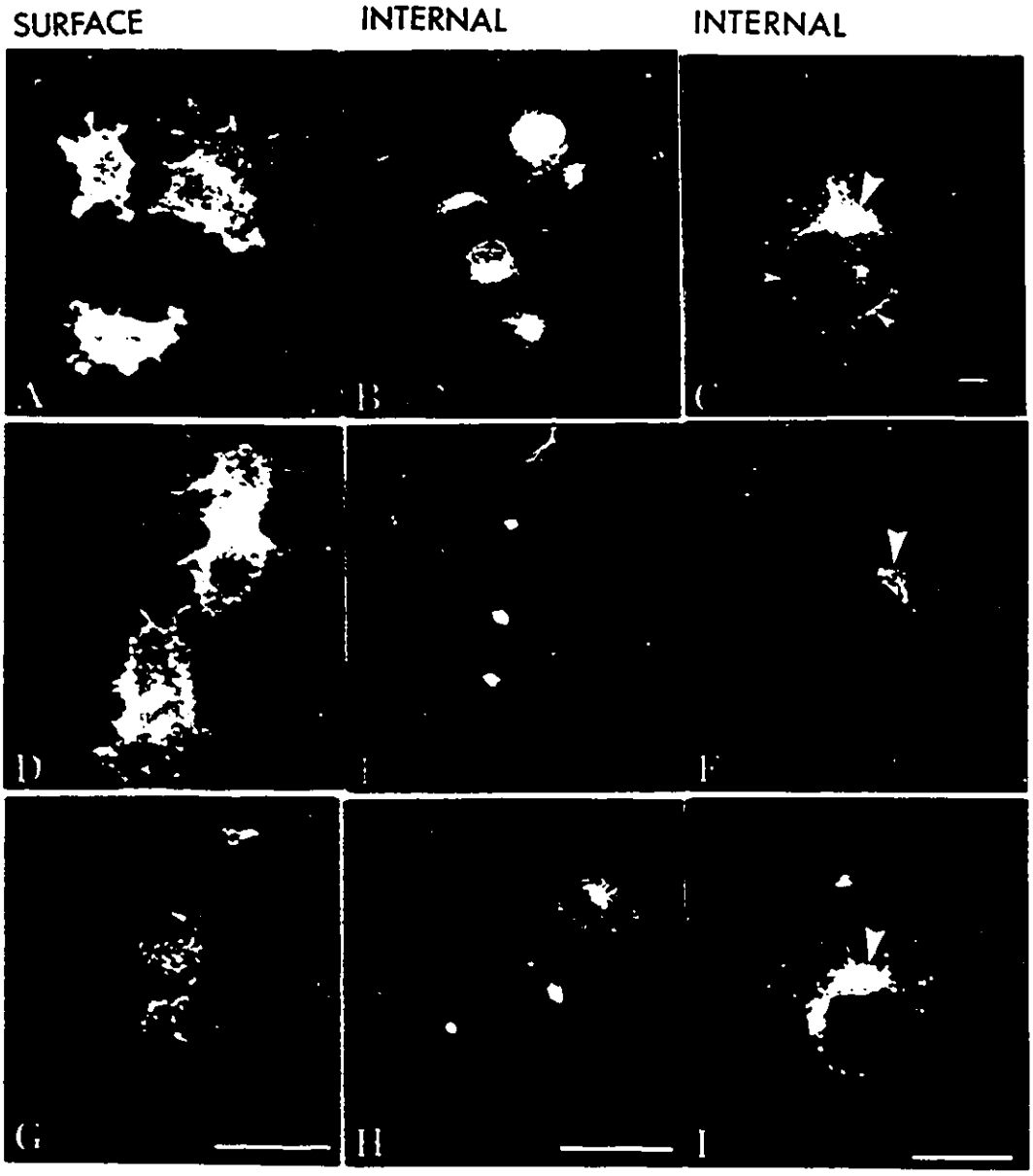

Figure 3.S.1 Immunofluorescence of gB, G and gB-G. COS-1 cells transfected with gB (A,B,C), G (D,E,F) and gB-G (G,H,I) were processed for cell surface and internal immunofluorescence. Small arrowheads: Nuclear envelope; Large arrowheads: Golgi complex; Bars = 60 μm (left and middle columns), or 20 μm (right column).

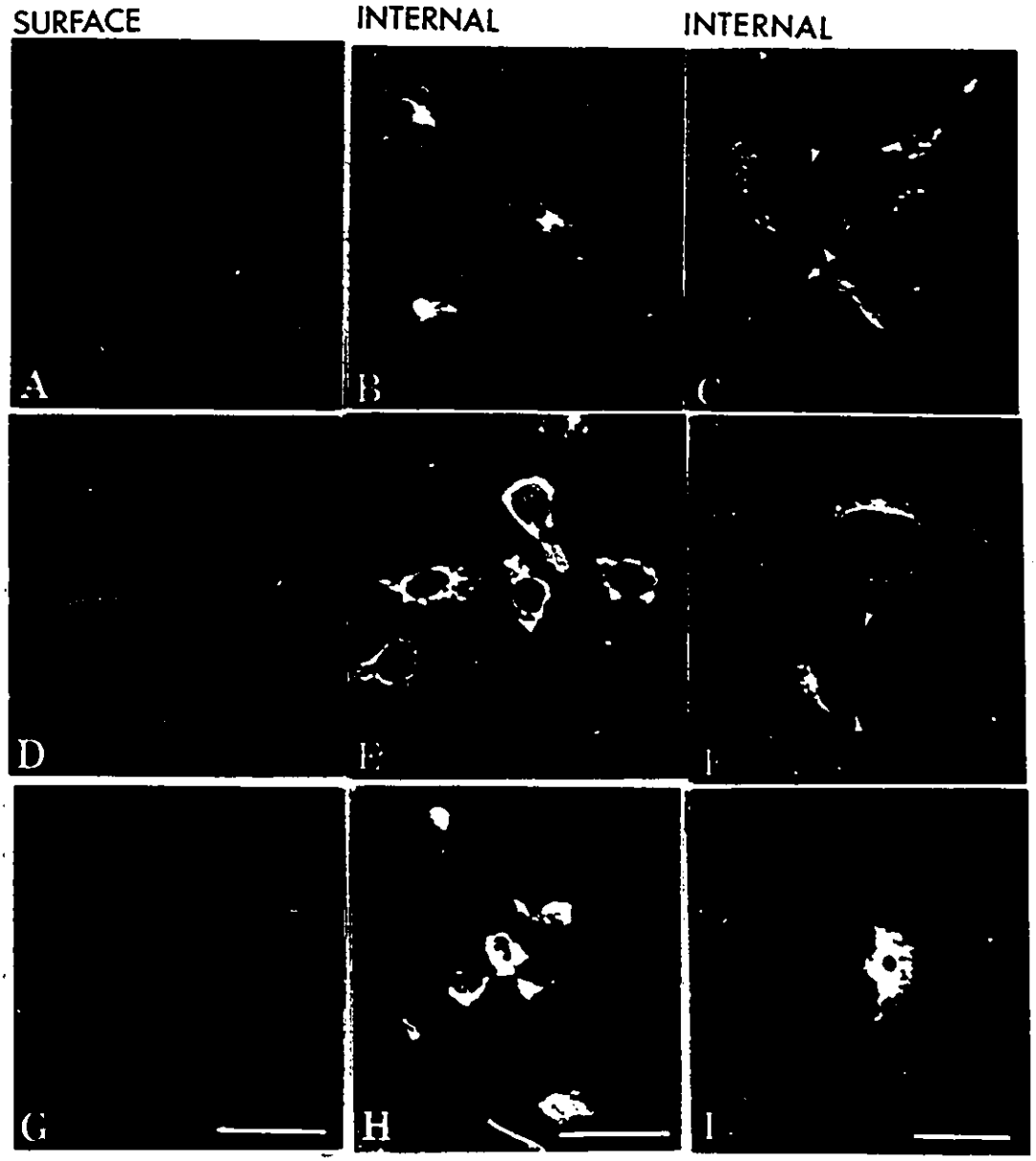

Figure 3.8.2 Immunofluorescence of G-gB-1, G-gB-2 and GH6. COS-1 cells transfected with G-gB-1 (A,B,C), G-gB-2 (D,E,F) and GH6 (G,H,I) were processed for cell surface and internal immunofluorescence. Small arrowheads: Nuclear envelope; Bars = 60 μm (left and middle columns), or 20 μm (right column).

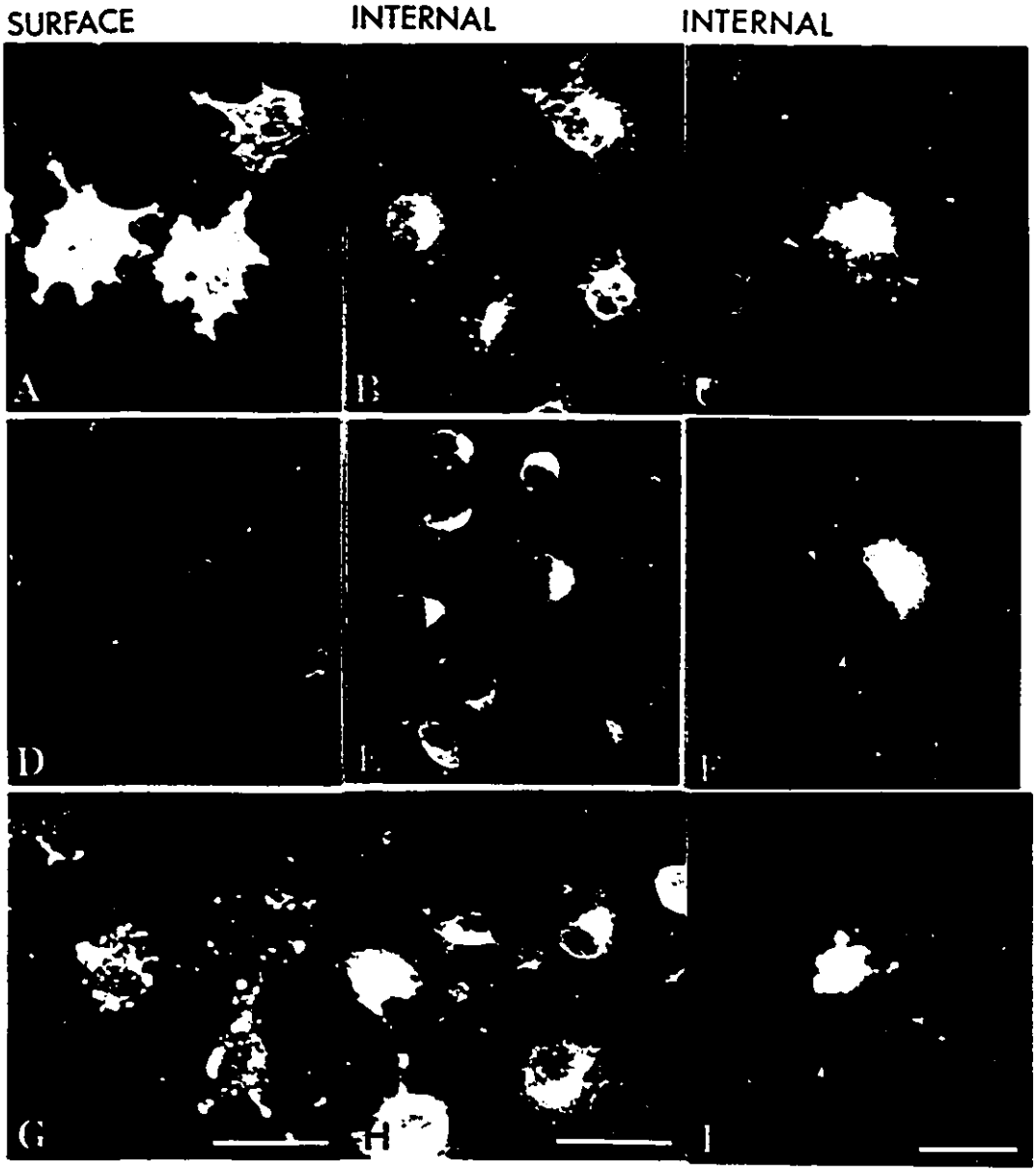

Figure 3.8.3 Immunofluorescence of Δ12CT799, gB-tmG and G-tmgB. COS-1 cells transfected with Δ12CT799 (A,B,C), gB-tmG (D,E,F) and G-tmgB (G,H,I) were processed for cell surface and internal immunofluorescence. Small arrowheads: Nuclear envelope; Bars = 60 μm (left and middle columns), or 20 μm (right column).

3.9 Distribution of gB in infected cells

Before investigating the distribution of the wild types and mutants at the ultrastructural level, the distribution of gB was studied in cells infected with HSV-1. At 14 to 20 h postinfection, COS-1 cells were fixed with glutaraldehyde and embedded at low temperature in Lowicryl K4M. Ultrathin sections were cut and incubated with the affinity purified anti-gB followed by 1 nm gold-conjugated goat anti-rabbit. The gold complex was then visualised by silver enhancement (Danscherg and Nörgaard, 1983). Gold particles of 5 to 10 nm were also used, but the observed signal was much stronger with the 1 nm gold.

The cell morphology is markedly altered during HSV-1 infection. The cells become round and viral particles accumulate on the plasma membrane and in the cytoplasm inside vacuole-like structures. Various modifications of the NE are observed, such as duplication, folding and invagination of the inner membrane inside the nucleus. Also observed is the formation of dense patches in the NE corresponding to thicker, denser and strongly curved portions of the inner membrane (Section 1.4.3).

Results of the immunoelectron microscopy showed that during HSV-1 infection, the virions, the ER and the NE were most intensively labeled, while very few silver particles were found over the nucleoplasm and mitochondria (Figs 3.9.1a and 3.9.2b). No labeling was observed when sections were incubated with the anti-G antibody (Fig 3.9.1c), or when mock infected cells were incubated with affinity purified anti-gB (Table 3.9.1).

Structures corresponding to the ER were easily distinguished (Fig. 3.9.2a), but structures such as stacks of flattened vesicles that represent Golgi complexes were not observed. The cytoplasm was filled with layers of concentric membranes and with vesicles surrounding one or more virions. These membrane structures, which could be derived from the Golgi complex were also labeled (Figs. 3.9.2a and 3.9.2b).

A quantitative analysis of the labeling intensity showed that the NE was more strongly labeled than the plasma membrane, indicating that gB accumulates in this cell compartment (Table 3.9.1). The ER was also strongly labeled and no statistical difference was observed between the labeling intensity of the ER and the NE. The labeling intensity of the plasma membrane is probably overestimated, because in many cases it was difficult to determine if the silver grains were associated with a virus or with the plasma membrane.

The labeling over the NE was evenly distributed and no preferential concentration of silver grains was noticed at the dense patches of the inner membrane where the viruses bud (Figs 3.9.1a and 3.9.2d). It was difficult to distinguish the inner and outer membrane of the NE. In some occasions however, when the perinuclear cisterna enclosed some virions, the two membranes were separated and they were both labeled (Figs 3.9.1b). The duplications and invaginations of the inner membrane of the NE were also strongly and uniformly labeled (Fig. 3.9.2c).

A quantitative analysis of the labeling over the virions in various cell compartments indicated that the virions were labeled when they became associated with

the NE (Table 3.9.2). No significant difference was observed between the labeling intensity of virions in the cytoplasm or on the plasma membrane. This suggests that no concentration or accumulation of gB occurs in the viral envelope during egress to the cell surface.

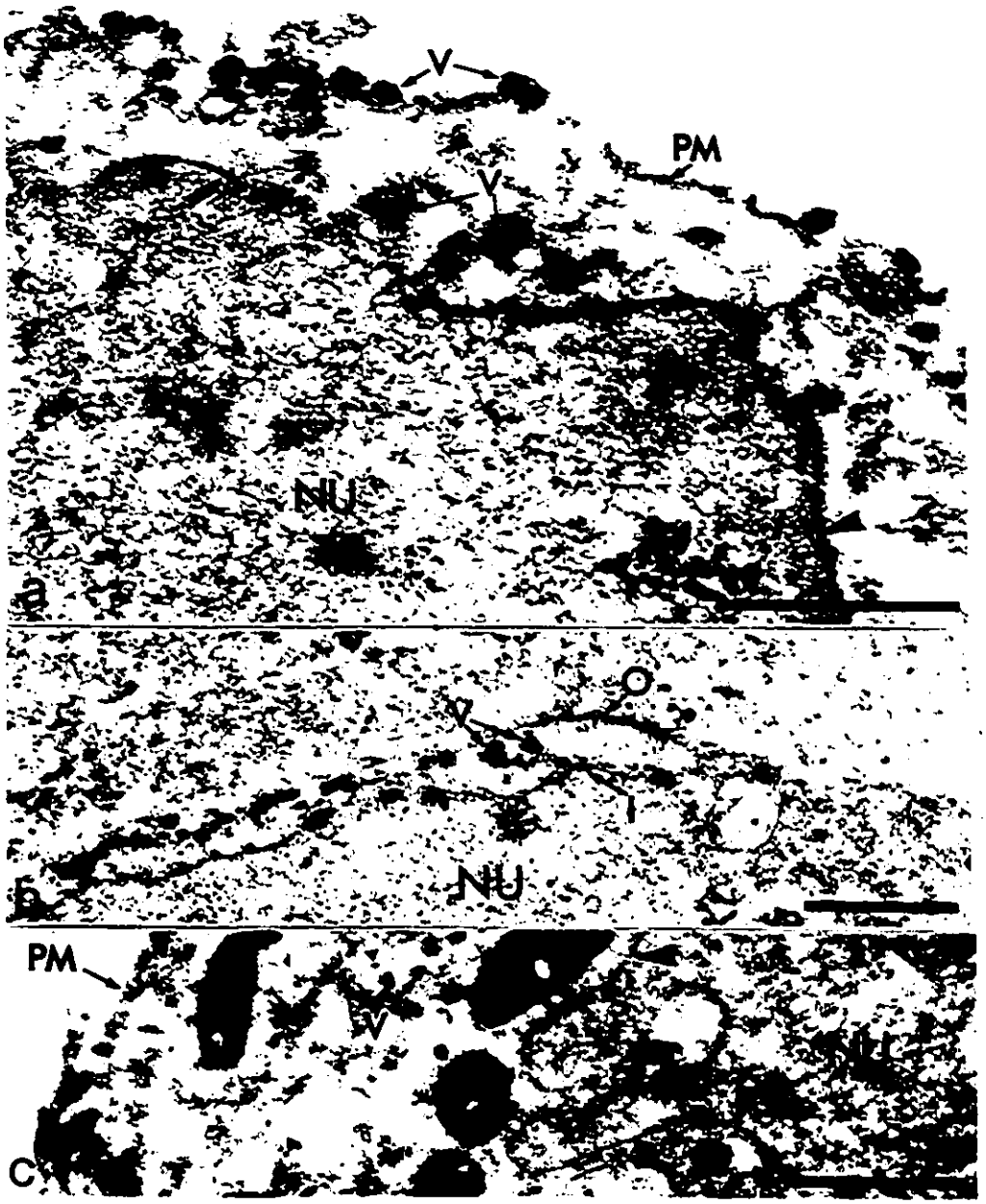

Figure 3.9.1 General distribution of gB in HSV-1 infected cells. a) and b) COS-1 cells infected with HSV-1 were processed for immunoelectron microscopy and labeled with the affinity purified anti-gB. c) similar preparation as a) and b) except the sections were labeled with anti-G. M: mitochondria; NU: nucleus; O and I: outer and inner membrane of the NE; PM: plasma membrane; V: HSV-1 virion; Large arrowheads: dense patches of the NE (a) or NE (c); small arrowheads: nonspecific labeling (c); Bar = 1 μM

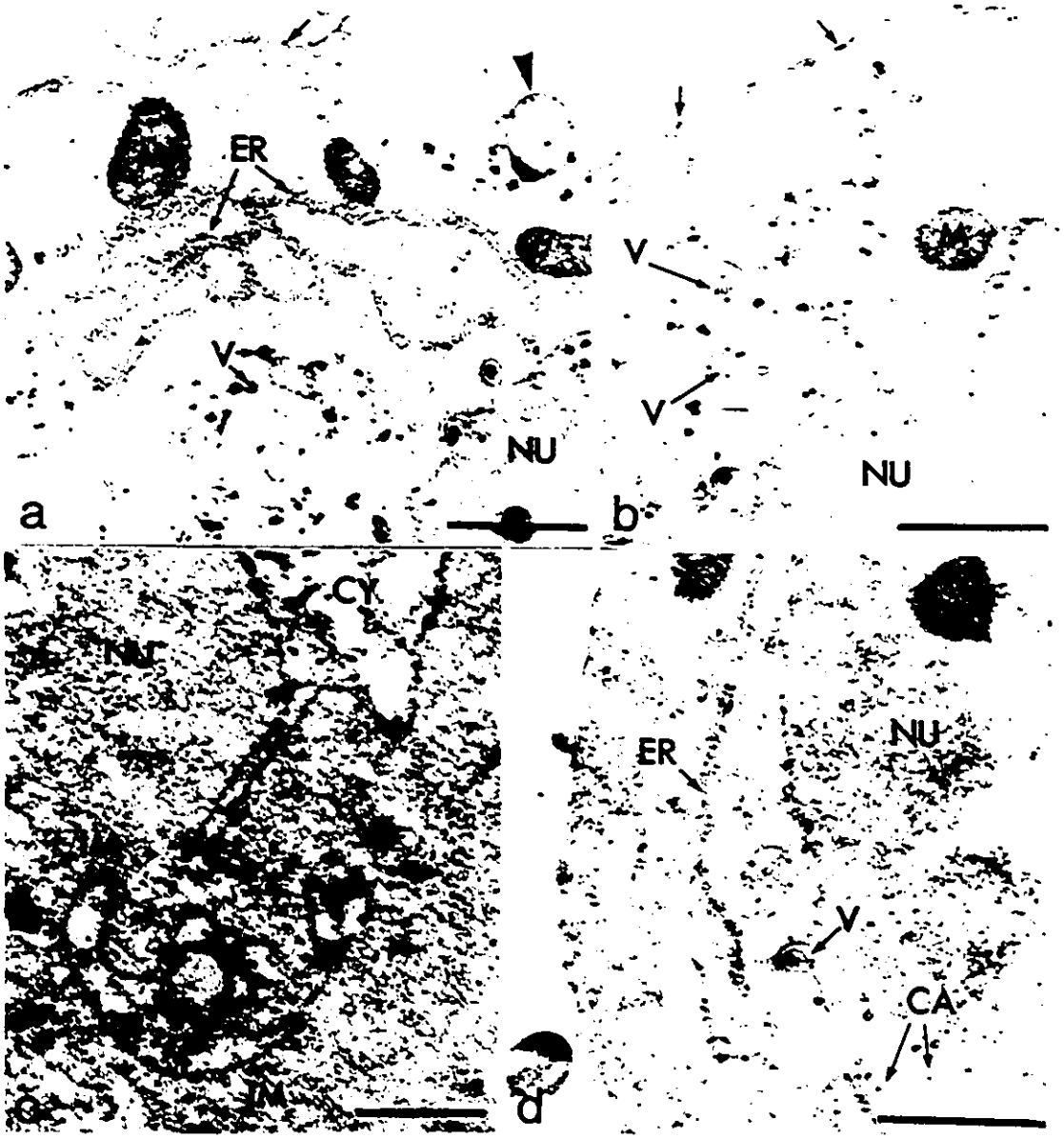

Figure 3.9.2 Distribution of gB in HSV-1 infected cells. COS-1 cells infected with HSV-1 were processed for immunoelectron microscopy and labeled with the affinity purified anti-gB. a) Labeled endoplasmic reticulum (ER) and concentric membrane (arrowheads). b) Cytoplasmic vesicles enclosing many HSV-1 virions (V); c) Intensively labeled invaginated inner membrane of the NE (IM). d) HSV-1 virion (V) in the process of budding at a dense patch. CA: HSV-1 nucleocapsids; CY: cytoplasm; M: mitochondria; NU: nucleus; Arrows: labeling on the plasma membrane (a,b); Bar = 1 μm.

Table 3.9.1 Labeling density of gB in cells infected with HSV-1

Cell type	Grains per μm (Mean $\pm$ SEM) NE[a]	ER	PM	Cells Obs.
Infected	5.7 ± 0.53^{b}	5.0 ± 0.57^{b}	1.1 ± 0.12^{c}	19
Mock infected	0.12 ± 0.044^{d}	0.27 ± 0.09^{d}	0.15 ± 0.03^{d}	10

SEM: Standard error of the mean; NE: Nuclear envelope; ER: Endoplasmic reticulum; PM: plasma membrane

a: The NE is defined as the double membrane system surrounding the cell nucleus, consequently it is composed by the inner and outer membrane.

b,c,d: Means with different letters are significantly different ($P<0.05$)

Table 3.9.2 Labeling density of gB over HSV-1 virions

Cell Compartment Analyzed	Grains per virion Mean $\pm$ SEM	Cells Obs.
Nucleoplasm	0.043 ± 0.067^{a}	15
Nuclear Envelope	1.3 ± 0.49^{b}	16
Cytoplasm	1.9 ± 0.47^{c}	16
Plasma membrane	$1.8 \pm 0.57^{b,c}$	19

SEM: Standard Error of the Mean.

a,b,c: Means with different letters are significantly different ($P<0.05$).

3.10 Immunoelectron microscopic distribution in transfected cells

When the distribution of the proteins was studied by immunofluorescence, it was sometimes difficult, because of the relatively low resolution of the light microscope, to distinguish clearly the NE from other cellular organelles such as the Golgi complex and the ER. To determine more accurately the distribution of the wild types and mutants, COS-1 cells were transfected with the different cDNAs, and 24 h following transfection, the distribution of the proteins were studied by immunoelectron microscopy as described in section 3.9 using the affinity purified anti-gB or anti-G.

3.10.1 Glycoprotein B is localized to the NE

At the ultrastructural level, cells expressing gB were strongly labeled on the NE, the ER, and the plasma membrane, whereas no silver grain was found over mitochondria and over the nucleoplasm (Fig. 3.10.1. and Table 3.10.1). The labeling was homogeneously distributed over these cellular membranes and they were labeled to a similar extent. Vesicles of various sizes were also labeled. The smaller ones most likely correspond to transport vesicles while the larger ones, which were often associated in clusters, probably represent cisternae of the Golgi complex that were markedly altered during the cell processing (Fig. 3.10.1).

No labeling was observed when sections of mock transfected cells were incubated with the affinity purified anti-gB and anti-G antibodies, or when sections of cells expressing gB and G were incubated with the affinity purified anti-G and anti-gB

respectively (Figs 3.10.2 and Table 3.10.1).

In cells expressing G, the plasma membrane as well as small cytoplasmic vesicles and Golgi-like structures were extensively labeled with anti-G, but few silver grains were observed on the ER and on the NE. The distribution of G on the plasma membrane was also homogeneous (Figs 3.10.2a and 3.10.3a and Table 3.10.1). Only 6% of the total number of silver grains were distributed over the NE in cells expressing G in comparison with 26% in cells expressing gB (Table 3.10.2). This observation indicates that gB but not G accumulates in the NE. The ER of cells expressing gB was also strongly labeled. Similarly, the ER of cells expressing the mutants that were localized to the NE was also strongly labeled (see below), suggesting the presence of an ER localization signal as well. Nevertheless we cannot rule out that the presence of these proteins in the ER is a consequence of overexpression (see Section 4.2.2).

The labeling intensity of the NE of cells expressing G was higher than the background, which indicates that a small fraction of G was present on this compartment. Similar observations were made by others who studied the distribution of G in VSV infected cells (Bergman and Singer, 1983) indicating that the NE is involved in protein synthesis.

It was difficult to clearly distinguish the outer and inner membrane of the NE. For this reason no attempt was made to compare the distribution of wild type and mutant proteins on these two membranes.

3.10.2 The carboxy terminus of gB specifies NE localization

The previous studies by immunoelectron microscopy have clearly indicated that gB is localized to the NE. In order to find out which part of gB encodes the NE localization signal, the distribution of the mutants (Fig. 3.1.1) were studied by immunoelectron microscopy. Cells expressing gB-G were strongly labeled on the plasma membrane, whereas few silver grains were observed on the NE and over the ER (Fig. 3.10.3b and Table 3.10.1).

The distribution of gB-G was very similar to the distribution of G and only 7% of the labeling was associated with the NE (Table 3.10.2). This observation and the fact that the NE of cells expressing gB-G was not strongly by immunofluorescence (Section 3.8), indicate that the EC of gB does not specify NE localization in the context of gB-G. The lack of accumulation of gB-G in the NE is not caused by an increase of the transport rate, since gB-G was transported to the Golgi complex at a rate similar to gB (Table 3.5.1).

When cells expressing G-gB-2 were processed for immunoelectron microscopy, the NE as well as the ER were strongly labeled (Fig. 3.10.3c and Table 3.10.1). The plasma membrane and Golgi-like vesicles were not labeled, in agreement with previous studies showing that G-gB-2 remains endo H sensitive and is not expressed on the cell surface. More than 30% of the labeling was associated with the NE (Table 3.10.2) suggesting that the TM and CT of gB are sufficient in the context of G-gB-2 to specify NE localization.

3.10.3 ER residents do not necessarily diffuse to the NE

To investigate if the presence of G-gB-2 in the NE is due to the lack of transport, the distribution of GH6, a mutant of G containing three extra residues in the EC (Section 3.1), was also studied. Previous studies have shown that GH6 is an ER resident protein, because it is not expressed on the cell surface, it remains endo H sensitive and it forms aggregates (Sections 3.6 to 3.8). In cells expressing GH6 and processed for immunoelectron microscopy, most of the labeling was associated into cytoplasmic structures composed of contorted filaments and granules. The granules had the same density, similar sizes and shapes as the ribosomes. Since GH6 is mainly localized in the ER, the contorted filaments could very well correspond to the membranes of the ER. These intensively labeled cytoplasmic structures seem therefore to be formed by the tight packing or agglomeration of individual ER tubules (Fig. 3.10.4a). During the course of this thesis, these structures are referred as "modified ER". The modified ER was not observed in cells expressing the wild type proteins, and no strongly labeled typical ER tubules such as those observed in cells expressing G-gB-2 were observed in cells expressing GH6. Few silver grains were associated with the NE and only 9% of the labeling was associated with this cellular compartment indicating that GH6 did not accumulate in the NE (Tables 3.10.1 and 3.10.2).

The ultrastructural distribution of G-gB-1, a chimera that remains endo H sensitive and aggregates (Section 3.10.6 and 3.10.7), was also studied. In cells expressing G-gB-1, the labeling was also localized over a modified ER network, which

however was more loose than the modified ER described in cells expressing GH6 (Fig. 3.10.4b). The NE of the cells expressing G-gB-1 was relatively more strongly labeled than the NE of cells expressing GH6, because almost 30% of the silver grain was associated with this compartment. The overall labeling was generally very weak, and for this reason the ultrastructural distribution of this protein was not further investigated.

3.10.4 The CT of gB is not essential for NE localization

In order to see if the CT of gB was essential for NE localization, the distribution of mutants CT799 and CT796 were studied by immunoelectron microscopy. These mutants, which were the object of a previous study (Raviprakash et al., 1990), were constructed by introducing a termination codon at amino acid 799 and 796, resulting in truncation proteins lacking the entire CT of gB (CT796) or this domain except for three amino acids (CT799; Fig 3.10.5). It was observed by immunofluorescence and subcellular fractionation, that deletion of the CT of gB did not affect NE localization (Raviprakash et al., 1990). Some properties of these two deletion mutants are summarized in Table 3.10.3.

In cells expressing CT799 and processed for immunoelectron microscopy, the NE and the ER were relatively strongly labeled and 25% of the labeling was associated with the NE, suggesting that deletion of the CT did not affect markedly the NE localization (Fig. 3.10.6a; Tables 3.10.1 and 3.10.2). The plasma membrane was not very strongly labeled in cells expressing CT799. The relatively slow rate of transport of CT799 (Table

3.6.1), could explain this observation. The signal intensity of cells expressing CT799 was generally weaker than for gB. Mutants CT799, CT796, Δ3 and gB-gC (Fig. 3.10.5) were expressed in COS-1 using the vector p91023 (Section 3.2). Cells expressing these proteins were generally more weakly labeled, suggesting expression with p91023 is not as efficient as with pXM.

At the ultrastructural level, mutant CT796 was mainly localized on a modified ER similar to the one described in cells expressing GH6 (Fig. 3.10.4c). The NE was not intensively labeled in cells expressing CT796 and 13% of the protein was associated with the NE (Tables 3.10.1 and 3.10.2). The plasma membrane of cells expressing CT796 was not labeled, in accord with previous studies showing that CT796 was not transported to cell surface (Raviprakash et al., 1990).

3.10.5 The TM of gB specifies NE localization

In order to define further the NE localization signal of gB, the distribution of G-tmgB, in which the TM of G was replaced with the TM of gB (Fig. 3.1.1), was also studied by immunoelectron microscopy. Cells expressing G-tmgB were strongly labeled on the NE and on the ER indicating that the TM of gB was sufficient to specify NE localization in the context of G-tmgB (Fig. 3.10.6c; Tables 3.10.1 and 3.10.2).

Previous studies have shown that G-tmgB acquired endo H resistance relatively rapidly and was transported to the plasma membrane, where the protein was distributed in a punctate pattern (Sections 3.6 to 3.8). Intensively labeled plasma membrane patches

were also observed with the electron microscope (Fig. 3.10.6b). The plasma membrane surrounding these patches of labeling was devoid of silver grains.

Another characteristic of cells expressing G-tmgB was the presence in the cytoplasm of relatively large vacuole-like structures surrounding an intensively labeled core. The endo H resistant form of G-tmgB is degraded (Section 3.6) and it is possible that these strongly labeled structures correspond to lysosomes in the process of degradation of G-tmgB (See discussion, Section 4.8).

3.10.6 Mutants with deletions in the TM of gB

The TM of gB is composed of a 69 amino acids long hydrophobic segment that could theoretically span the membrane three times (Fig. 3.1.1). In order to define more precisely which portion of the TM encodes the NE localization signal, the distribution of mutants lacking one or two segments of the TM were studied by immunoelectron microscopy. The structure of these deletion mutants are schematically presented in Fig. 3.10.5 and some of their biochemical properties, which were the object of other studies (Rasile et al., 1993), are summarized in Table 3.10.3.

Mutants Δ12 and Δ23 were constructed by deletion of segments 1 and 2, and by deletion of segments 2 and 3 respectively, whereas Δ3 was made by deletion of segment 3. As evidenced by carbonate extraction (Rasile et al., 1993), mutant Δ23 was not anchored in the membrane, whereas Δ12 and Δ3 were associated with the membrane. Mutant Δ23 remained endo H sensitive, but was slowly secreted by the cells. Mutant Δ3

became endo H resistant and was expressed on the cell surface, whereas Δ12 remained endo H sensitive and was not transported to the cell surface as demonstrated by immunofluorescence (Table 3.10.3) and cell surface iodination (Rasile et al., 1993).

When cells expressing Δ3 were processed for immunoelectron microscopy, the NE, the ER and the plasma membrane were labeled to a similar extent (Fig. 3.10.7b and Table 3.10.1). Although such cells exhibited a weaker labeling signal, the overall distribution of Δ3 was similar to gB and 20% of the labeling was associated with the NE, which indicates that deletion of segment 3 did not impair NE localization.

The NE was also strongly labeled in cells expressing Δ12 (Fig. 3.10.7a; Tables 3.10.1 and 3.10.2). Well defined ER tubules were strongly labeled and no labeling was observed on the plasma membrane. The overall distribution of Δ12 was identical to G-gB-2 (Fig. 3.10.3c).

Few silver grains were observed on the NE of cells expressing Δ23 suggesting that membrane anchoring is a requirement for NE localization, or that the signal is encoded by segment 2 and 3 (Fig. 3.10.7c; Tables 3.10.1 and 3.10.2). In cells expressing Δ23 most of the labeling was localized over a modified ER network similar to the one described in cells expressing GH6.

The observation that mutant Δ12 was strongly localized to the NE highly suggests that segment 3, although not essential since mutant Δ3 was still localized to this compartment, may be sufficient to specify NE localization. To check this hypothesis further, the distribution of mutant Δ12CT799, which lacks segments 1 and 2 and the CT

of gB except for 3 residues (Fig. 3.1.1), was investigated by immunoelectron microscopy. The plasma membrane was the most intensively labeled compartment in cells expressing Δ12CT799 (Fig. 3.10.8a; Table 3.10.1). The labeling intensity of the NE was higher than cells expressing G, but lower than cells expressing gB. About 15% of the grains of cells expressing Δ12CT799 were associated with the NE. Segment 3 in the context Δ12CT799 was therefore insufficient to promote maximal NE localization, suggesting that the CT is also involved in NE localization.

3.10.7 The CT of gB may also specify NE localization

To study the role of the CT of gB in NE localization, the distribution of gB-tmG, a chimera constructed by replacing the TM of gB with the TM of G (Fig. 3.1.1), was also investigated by electron microscopy. Cells expressing gB-tmG were strongly labeled on the NE, suggesting that the CT of gB contains sufficient information in the context of gB-tmG to specify NE localization (Fig 3.10.8b; Table 3.10.1 and 3.10.2). Well defined ER tubules were also strongly labeled in cells expressing gB-tmG. The plasma membrane was not labeled in agreement with previous studies indicating that gB-tmG did not exit the ER (Sections 3.6 and 3.7). The distribution of gB-tmG was in fact very similar to the distribution of G-gB-2 (Fig. 3.10.3c)

3.10.8 The carboxy terminus of gC specifies NE localization

Glycoprotein gC is also a component of the envelope of HSV-1 (Section 1.4.4).

Glycoprotein gC is a type I membrane protein and it possesses a typical TM of 23 residues and a short CT of 11 residues (Fig. 3.10.5.). Subcellular fractionation and immunofluorescence studies have demonstrated the presence of gC in the NE (Compton and Courtney, 1984; Ghosh-Choudhury et al., 1990). In order to determine if the TM and CT of gC are sufficient to specify NE localization, the distribution of gB-gC was investigated by immunoelectron microscopy using the affinity purified anti-gB.

The chimera gB-gC was made by fusing the EC of gB, except for the last 96 amino acids, to the TM and CT of gC (gB-gC was a gift of Dr. N. Ghosh-Choudhury; Fig. 3.10.5). Immunofluorescence studies have shown that gB-gC was expressed on the cell surface and that it was also localized to the NE of transfected COS-1 cells (Ghosh-Choudhury, 1990). Cells expressing gB-gC and processed for immunoelectron microscopy were labeled on the NE, ER and plasma membrane (Fig. 3.10.7c; Table 3.10.1). The relative distribution of gB-gC was similar to gB, although a smaller proportion of protein was associated with the NE (Table 3.10.2) implying that the TM and CT of gC in the context of gB-gC encode a weak NE localization signal.

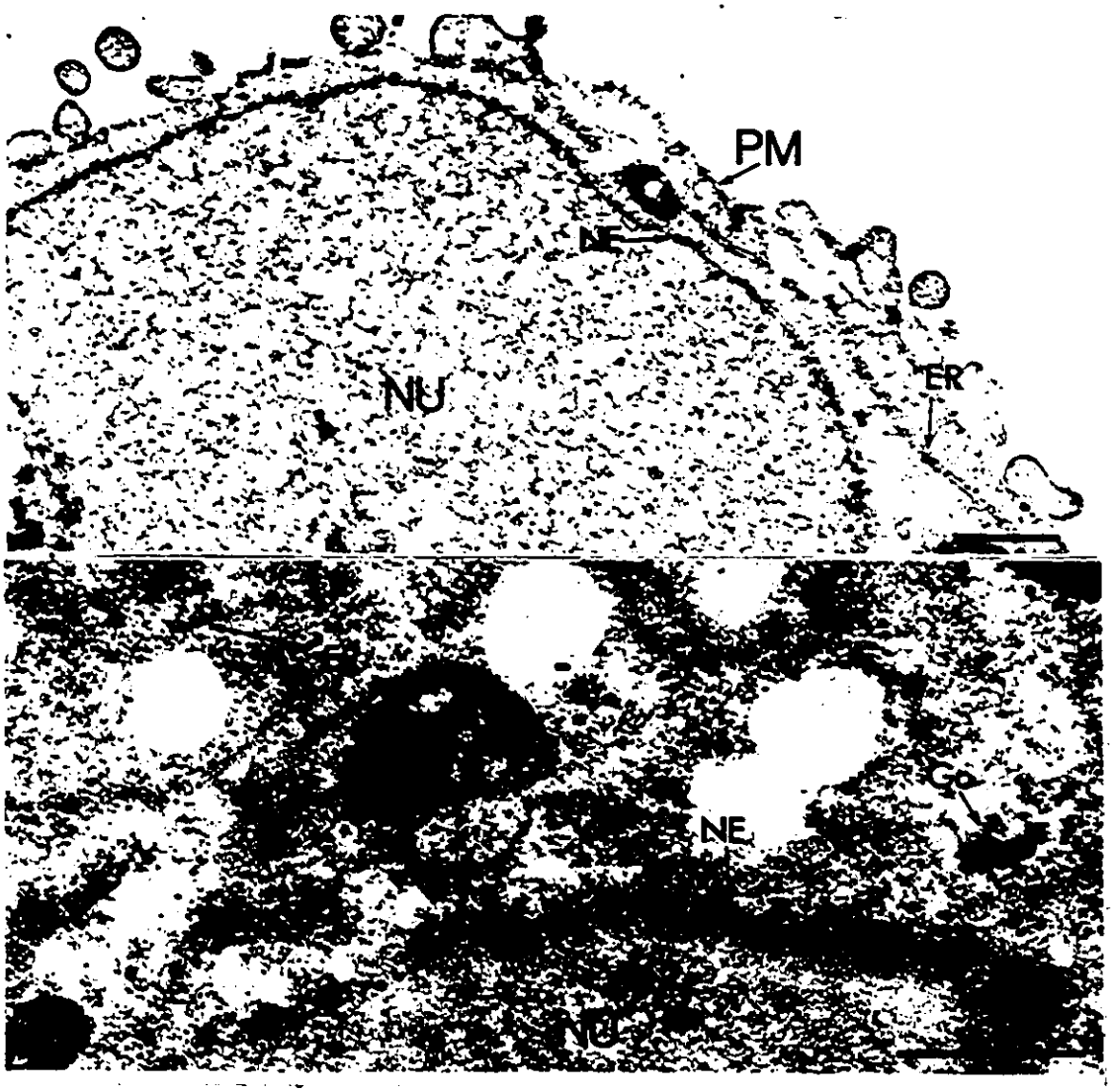

Figure 3.10.1 Distribution of gB in transfected cells. COS-1 cells transfected with gB were processed for immunoelectron microscopy and labeled with the affinity purified anti-gB. ER: endoplasmic reticulum; Go: Golgi-like vesicles; M: mitochondria; NE: Nuclear envelope; NU: nucleus; PM: plasma membrane; Bar = 1 μm.

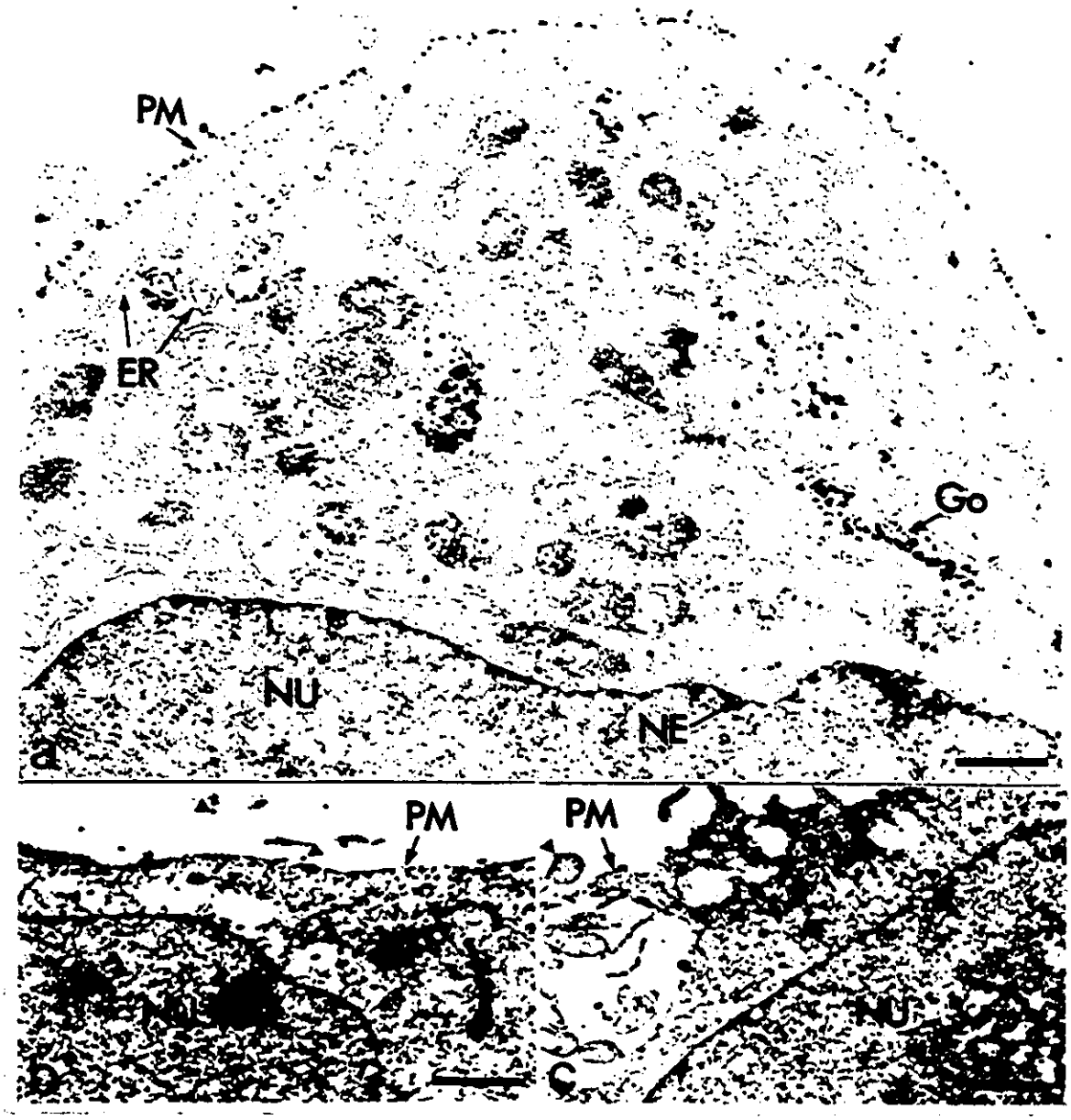

Figure 3.10.2 Distribution of G in transfected cells. COS-1 cells transfected with G (a), gB (c), or mock transfected cells (b), were processed for immunoelectron microscopy and labeled with the affinity purified anti-G (a,c) or the affinity purified anti-gB (b). ER: endoplasmic reticulum; Go: Golgi-like vesicles; NE: Nuclear envelope; NU: nucleus; PM: plasma membrane; Arrowheads nonspecific labeling (b,c); Bar = 1 μm.

Table 3.10.1. Labeling density of wild types and mutants

Protein	Grains per μm (Mean ± SEM) NE	ER	PM	Cells Obs.
gB	6.0 ± 0.67[a]	6.0 ± 0.91[a]	3.8 ± 0.50[a]	33
G	1.4 ± 0.24[a]	2.1 ± 0.35[a]	8.4 ± 0.91[b]	24
gB-G	1.1 ± 0.15[a]	1.9 ± 0.25[a]	6.8 ± 0.89[b]	24
G-gB-1	1.0 ± 0.20[a]	3.2 ± 0.25[b*]	0.15 ± 0.052[c**]	10
G-gB-2	11 ± 1.2[a]	16 ± 2.2[b]	0.18 ± 0.26[c**]	15
GH6	1.2 ± 0.18[a]	11.9 ± 1.1[b*]	0.13 ± 0.039[c**]	24
CT799	3.3 ± 0.30[a]	4.1 ± 0.59[a]	1.4 ± 0.24[b]	19
CT796	1.9 ± 0.18[a]	3.4 ± 0.43[b*]	0.095 ± 0.023[c**]	12
G-tmgB	7.4 ± 0.92[a]	6.9 ± 1.1[a]	2.7 ± 0.72[b]	24
Δ12	5.9 ± 0.93[a]	6.8 ± 1.0[a]	0.15 ± 0.033[b**]	13
Δ3	2.1 ± 0.3[a]	2.7 ± 0.39[a]	2.5 ± 0.49[a]	15
Δ23	0.64 ± 0.17[a]	4.5 ± 0.41[b*]	0.054 ± 0.02[a**]	14
gB-tmG	5.2 ± 0.30[a]	6.5 ± 0.63[a]	0.21 ± 0.096[b**]	13
Δ12CT799	2.6 ± 0.35[a]	4.2 ± 0.75[ab]	5.1 ± 0.55[b]	19
gB-gC	2.4 ± 0.31[a]	3.4 ± 0.74[a]	1.2 ± 0.16[a]	16
Backgr***	0.15 ± 0.053[a]	0.08 ± 0.037[a]	0.13 ± 0.043[a]	11

SEM: Standard error of the mean; NE: nuclear envelope; ER: endoplasmic reticulum; PM: Plasma membrane

a,b,c: For a specific protein, means with different letters are significantly different ($P<0.05$).

*: Agglomerated ER (see text for details).

**: Mean not higher than background ($P<0.05$)

***: Mock transfected cells labeled with anti-gB; similar results were observed with Mock transfected cells labeled with anti-G.

Table 3.10.2 Labeling associated with the NE

Protein	% of grains in NE Mean ± SEM	Cells Obs
gB	25 ± 2.9[b]	14
G	6.3 ± 0.87[a]	11
gB-G	7.1 ± 0.99[a]	15
G-gB-1	28 ± 4.2[b]	8
G-gB-2	33 ± 3.8[b]	11
GH6	9.4 ± 1.8[a]	14
CT799	25 ± 3.2[b]	15
CT796	13 ± 1.8[c]	10
G-tmgB	24 ± 4.7[b]	13
Δ12	38 ± 5.2[b]	8
Δ3	20 ± 3.3[b]	11
Δ23	7.0 ± 1.2[a]	13
Δ12CT799	15 ± 2.1[c]	14
gB-tmG	25 ± 3.7[b]	9
gB-gC	14 ± 1.2[c]	12

NE: Nuclear envelope

a,b,c: Means with different letters are significantly different ($P < 0.05$).

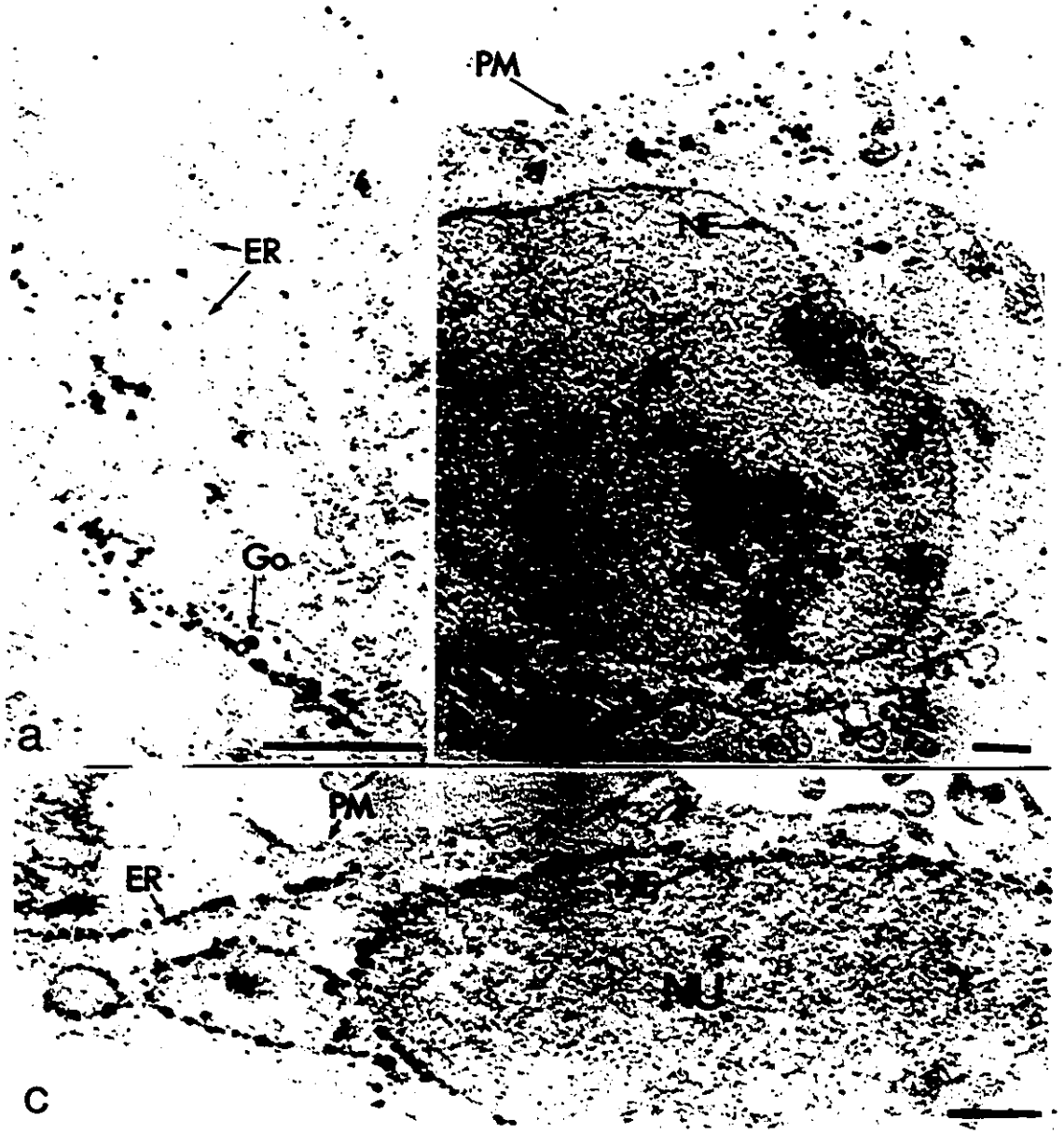

Figure 3.10.3 Distribution of G, gB-G and G-gB-2. COS-1 cells transfected with G (a), gB-G (b) and G-gB-2 (c) were processed for immunoelectron microscopy and labeled with the affinity purified anti-G (a,c) or anti-gB (b). a) Higher magnification of Fig. 3.10.2a showing Golgi-like vesicles (Go); ER: endoplasmic reticulum; NE: nuclear envelope; NU: nucleus; PM: plasma membrane; Bar = 1 μm.

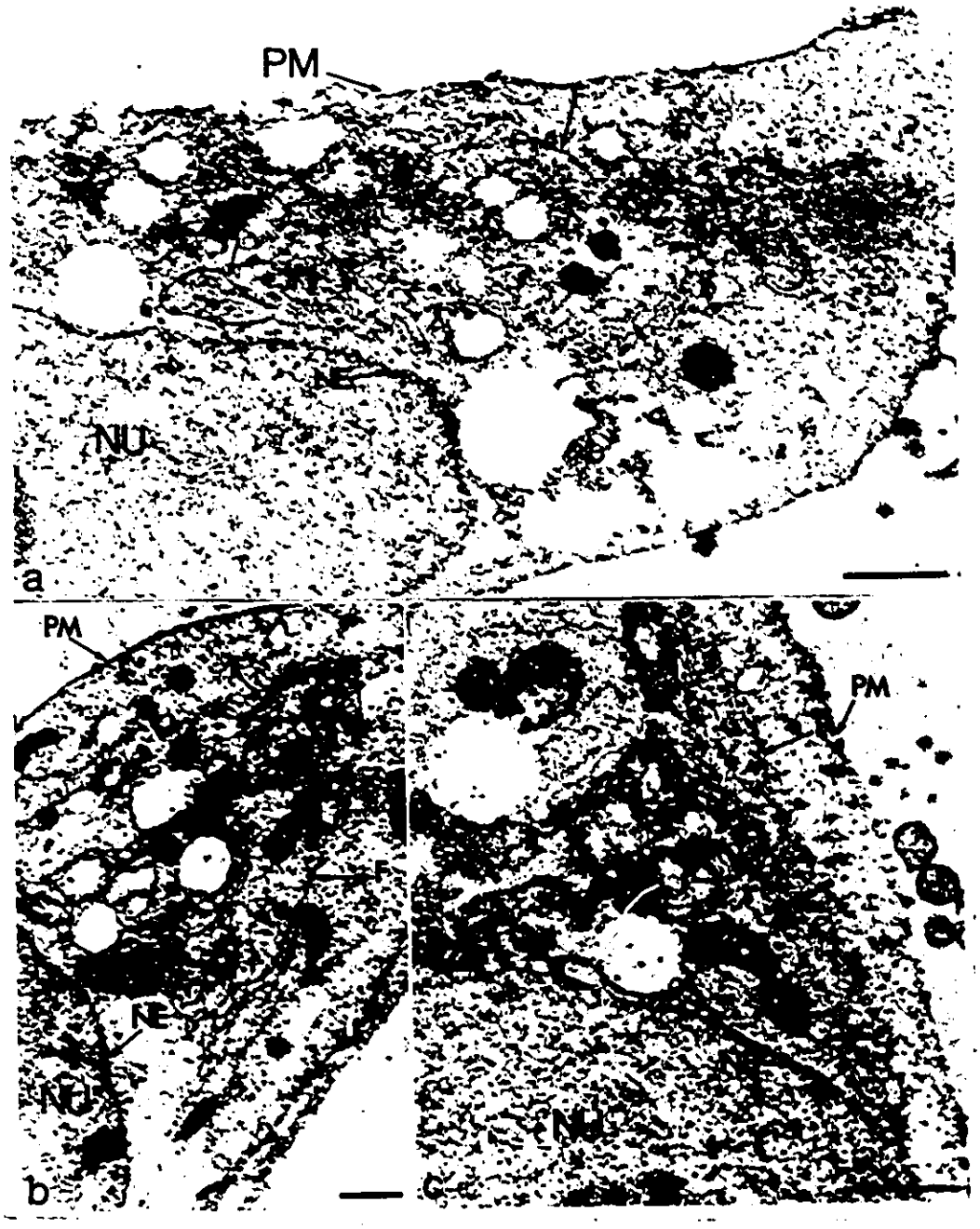

Figure 3.10.4 Distribution of GH6, G-gB-1 and CT796. COS-1 cells transfected with GH6 (a), G-gB-1 (b) and CT796 (c) were processed for immunoelectron microscopy and labeled with the affinity purified anti-G (a,b) or anti-gB (c). F: individual fibrils of a modified endoplasmic reticulum; NE: Nuclear envelope; NU: nucleus; PM: plasma membrane; Bar = 1 μm.

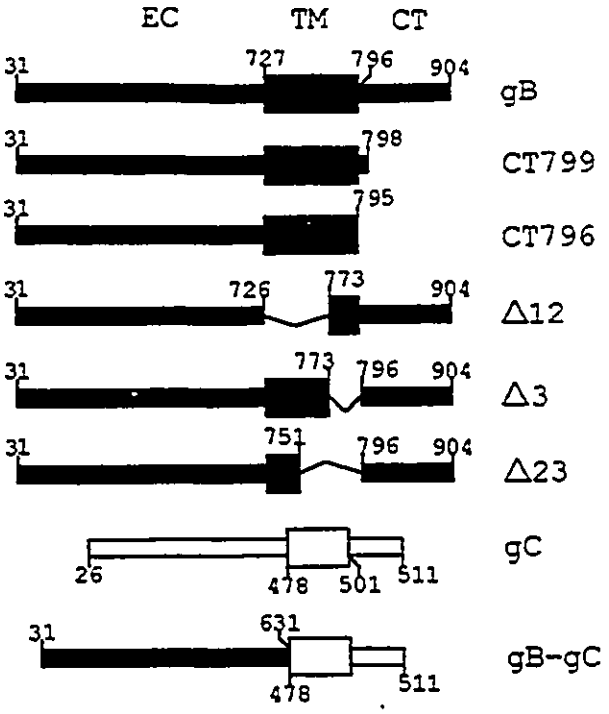

Figure 3.10.5 Structure of some mutants used in this study. The amino acids are numbered from the amino terminus according to Bzik et al. (1986) and Draper et al. (1984). The residues belonging to gB (black) and gC (white) are above and below the bars respectively. For gB and gC, the first residue of each domain (excluding the signal sequence) and the last residue of the protein are indicated. The numbers above and below the mutants correspond to the first and last residues of the segment used to make them. EC: ectodomain, TM transmembrane domain, CT: Cytoplasmic domain.

Table 3.10.3 Localization and rate of transport of other mutants[a]

Protein	Immunofluorescence[b] Surface	NE	Time for 50% Endo H resistance
CT799	++	++++	>3 h
CT796	-	++++	Sensitive
Δ12	-	++++	>3 h
Δ3	++	+++	135 min
Δ23	-	+	Sensitive
gB-gC	++++	++++	Not Done

a: Summary of data presented elsewhere (Raviprakash et al., 1990; Rasile et al., 1993; Ghosh-Choudhury, 1990).
b: The number of + is proportional to the signal intensity; -: No signal observed.
NE: nuclear envelope; Sensitive: completely sensitive to endo H after a chase of 3 h.

Figure 3.10.6 Distribution of CT799 and G-tmgB. COS-1 cells transfected with CT799 (a) and G-tmgB (b,c) were processed for immunoelectron microscopy and labeled with the affinity purified anti-gB (a) or anti-G (b,c). ER: endoplasmic reticulum; NE: nuclear envelope; NU: nucleus; PM: plasma membrane; Arrowheads: strongly labeled areas on the plasma membrane (b), or densely labeled core inside cytoplasmic vesicles (c); Bar = 1 μm.

Figure 3.10.7 Distribution of Δ12, Δ3, Δ23. COS-1 cells transfected with Δ12 (a), Δ3 (b) and Δ23 (c) were processed for immunoelectron microscopy and labeled with the affinity purified anti-gB. ER: endoplasmic reticulum; F: fibrils of an agglomerated ER; NE: nuclear envelope; NU: nucleus; PM: plasma membrane; Bar = 1 μm.

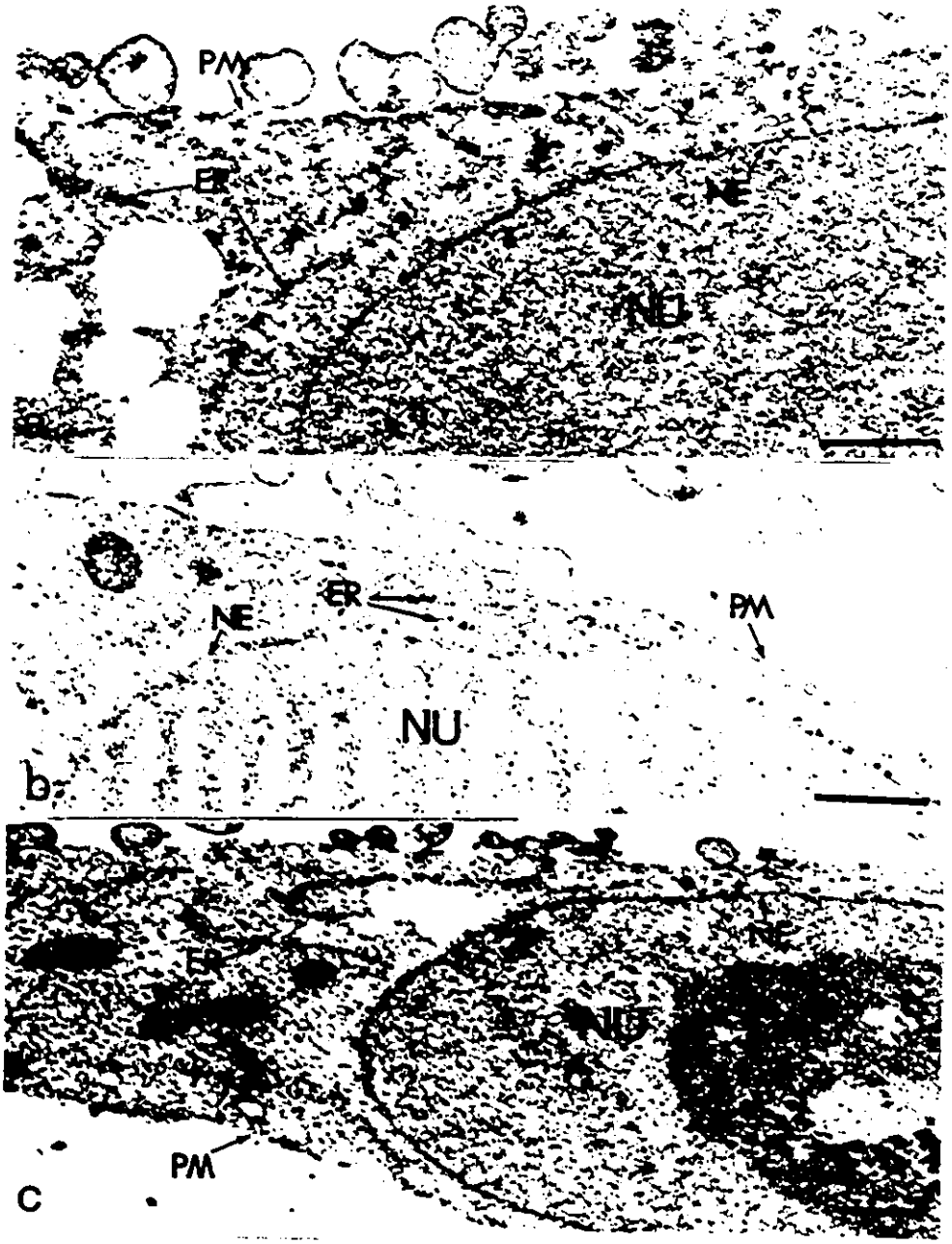

Figure 3.10.8 Distribution of Δ12CT799, gB-tmG and gB-gC. COS-1 cells transfected with Δ12CT799 (a), gB-tmG (b) and gB-gC (c) were processed for immunoelectron microscopy and labeled with the affinity purified anti-gB. ER: endoplasmic reticulum; NE: nuclear envelope; NU: nucleus; PM: plasma membrane; Bar = 1 μm.

3.11 Subcellular fractionation

Subcellular fractionation was used to study the distribution in the NE of gB, G, GH6, gB-G, G-gB-1 and G-gB-2. The method used is essentially the same described by Bos et al. (1988). Briefly, transfected COS-1 cells were labeled with [^{35}S]-methionine and chased for 1.5 h. The cells were swollen by incubation in hypotonic buffer and then treated with 0.5% of Nonidet P40. The nuclei were isolated by low speed centrifugation and washed vigorously with hypotonic buffer containing 0.5% Nonidet P40. The presence of proteins in the postnuclear supernatant and the nuclear fraction was then determined by immunoprecipitation (Fig. 3.11.1).

About 20% of gB was associated with the nuclear fraction, whereas G was completely recovered in the postnuclear supernatant (Fig. 3.11.1 and Table 3.11.1). Mixing COS-1 cells transfected with gB and G together before fractionation did not affect the amount of each protein recovered and the nuclear fraction. The chimera G-gB-2, was not isolated in the nuclear fraction, whereas mutants gB-G, GH6 and G-gB-1 were recovered in relatively large amount with the nuclear fraction (Table 3.11.1). In fact the percentage of gB-G, GH6 and G-gB-1 recovered with the nuclear fraction was higher than for gB.

The proportion of GH6 in the nuclear fraction was quite variable. In some experiments most of the protein was found in the nuclear fraction (Fig. 3.11.1), whereas in some cases a significant fraction was recovered in the postnuclear supernatant. On average more then 50% of GH6 was recovered in the nuclear fraction. It was surprising

to find GH6 in such a large quantity in the nuclear fraction, because G was not recovered in this fraction, and GH6 is not expected to possess a NE localization signal. We have previously seen that GH6 and G-gB-1 form aggregates after their synthesis (Section 3.7). These aggregates were most likely spun down and isolated with the nuclei during the fractionation procedures. Consequently the presence of GH6 and G-gB-1 in the nuclear fraction could be an artifact.

The occurrence of gB-G and the absence of G-gB-2 in the nuclear fraction, are in disagreement with the studies by immunofluorescence and by immunoelectron microscopy which indicate that the NE localization signal of gB is part of the EC. However, we cannot rule out that a certain proportion of gB-G also forms aggregates which were recovered with the nuclei. The reason for the absence of G-gB-2 in the nuclear fraction is not understood, since the immunofluorescence and immunoelectron microscopic studies have clearly indicated the presence of this chimera in the NE. Because of these discrepancies, the fractionation procedure was not utilised to study the distribution of other mutants.

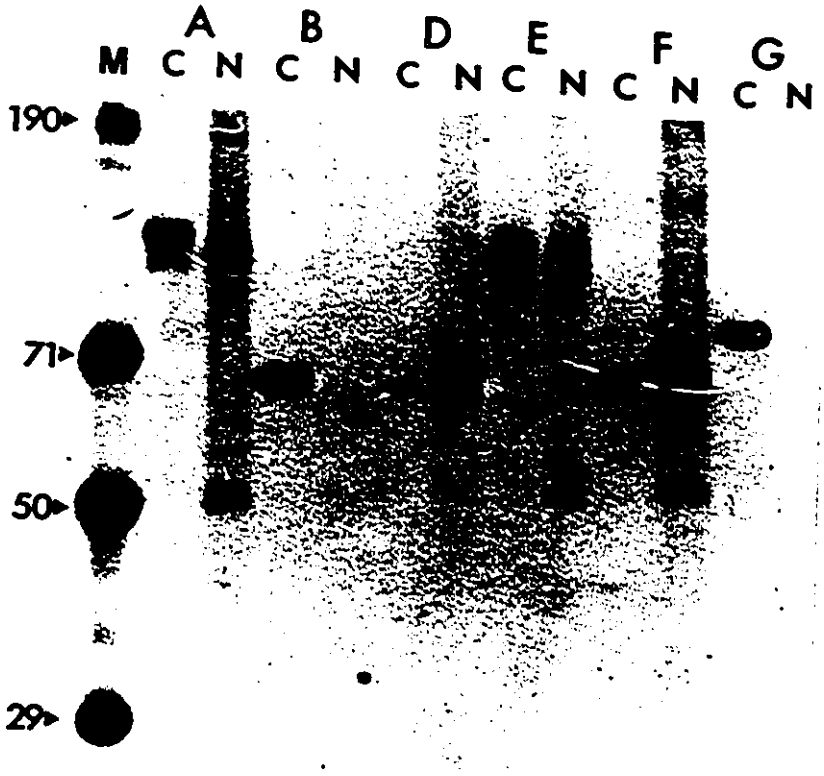

Figure 3.11.1 Subcellular localization by cell fractionation. COS-1 cells transfected with gB (A), G (B), GH6 (D), gB-G (E), G-gB-1 (F) and G-gB-2 (G) were labeled with [^{35}S]-methionine and chased for 1 h 30. The nuclei were then isolated as described in Section 2.46 and the proteins of the postnuclear supernatant (C) and the nuclear fraction (N) were immunoprecipitated and analyzed by SDS-PAGE followed by fluorography. M: labeled VSV (Cocal) was used as mol wt marker (kD).

Table 3.11.1 Subcellular fractionation

Protein	Percentage in Nuclear fraction	Number of Experiments
gB	20	4
G	<1	4
GH6	54	3
gB-G	39	4
G-gB-1	47	4
G-gB-2	<1	2

4.0 DISCUSSION

4.1 Critique of the immunoelectron microscopy

Immunofluorescence and immunoelectron microscopy were used during the course of this thesis to assess the cellular distribution of gB and G and mutants of these proteins. Immunofluorescence is a routine method of investigation utilised by most laboratories interested in the distribution of cellular constituents. In contrast, fewer studies are accomplished with the electron microscope, because immunoelectron microscopy is time consuming, requires expensive equipments and often the help of skilled technicians.

The proteins under investigation were localized using 1 nm colloidal gold instead of the more frequently used 5 or 10 nm colloidal gold, because the signal intensity, as I have myself empirically observed, is known to be higher when the labeling is performed with gold of smaller diameter (Yokata, 1988). Only a handful of studies done with 1 nm gold have been published (See for example: Lah et al., 1990; Shimizu et al., 1992). This can be attributed to the fact that 1 nm gold has become available only recently, and because its employment involves an extra step of silver enhancement, since the 1 nm gold must be enlarged to be visualized.

Several observations suggest that the method of immunoelectron microscopy employed was specific. No labeling was observed when mock transfected or mock infected cells were processed for electron microscopy. Similarly, no labeling was

observed when cells expressing gB were incubated with an anti-G antibody, or when cells expressing G were incubated with an anti-gB antibody. The proteins under investigation were membrane proteins, and the labeling was mainly localized over the cellular membranes. Mitochondria and the nucleoplasm were not labeled. Furthermore, the results obtained by the electron microscopy support the data provided by immunofluorescence and endo H digestion.

One of the main flaws of the present study was the inability to clearly distinguish the labeling associated with the inner and outer membrane of the NE of the transfected cells. I see two reasons for this. Firstly, the inner and outer membranes are separated from each other by an average distance of about 14 nm (Watson, 1958). An antibody possesses an average length of 10 nm (Sarma et al., 1971) and two molecules of antibodies were used for the labeling. The diameter of the grains after silver enhancement was between 5 to 10 nm. Thus, it can be estimated by a crude approximation that the distal face of a silver grain can be located as far as 25 to 30 nm from the antigenic site, a distance larger than the perinuclear cisterna. This problem of resolution could be partly corrected by coupling directly the 1 nm gold to the primary antibody and by reducing the time of silver enhancement in order to generate smaller grains. An other approach would be to mechanically separate the inner and outer membrane before embedding.

Secondly, in my preparation it was difficult to distinguish clearly the outer and inner membranes of the NE, most likely because the lipids of these membranes were extracted during cell processing. The specimens were dehydrated and embedded at low

temperature, a procedure known to improve the overall structural preservation (Roth et al., 1981). However, after a simple fixation with glutaraldehyde, as it was the case in this work, an important fraction of lipids is extracted even when the specimens are processed at low temperature (Weibull et al., 1983). In the future, to obtain better images of the inner and outer membranes, the cells should be processed by cryoultramicrotomy or by cryosubstitution, two technics known to improve the preservation and to limit the extraction of membrane lipids (Weibull et al., 1984; Tokuyasu, 1986).

4.2 Cellular distribution of gB

4.2.1 Distribution of gB in HSV-1 infected cells

I have shown by immunoelectron microscopy that gB is specifically localized in the NE of cells infected with HSV-1. This is in agreement with the observation of Torrisi et al. (1992) who have used a pre-embedding labeling method, namely freeze-fracture label, to demonstrate a specific distribution of gB in the NE. The post-embedding method used in the present study has the advantage over freeze-fracture label, in that an entire section of cell and not just the surface of the fractured specimen, is in contact with the antibody and thus shows a generalized distribution of labeling. This makes the comparison between different compartments easier. Poliquin et al. (1985) have also observed, by electron microscopy using radioautography with [^{3}H]-mannose, that glycoproteins synthesized during HSV-1 infection are localized to the NE.

In my preparations it was difficult to distinguish clearly the inner and the outer

membrane of the NE. However, the alterations of this envelope which occur during infection, such as the reduplication and the invagination of the inner membrane (Roizman and Furlong, 1974; Nii et al., 1968; Darlington and Moss, 1968; Morgan et al., 1959), were strongly labeled, indicating that gB is transported to the inner membrane. On this membrane, the labeling was distributed evenly and no preferential accumulation of gB at specific sites such as the patches where the virus is believed to bud were observed. This is in contrast with similar studies dealing with the glycoproteins of sindbis virus and the glycoprotein G of VSV, where a specific accumulation of viral glycoproteins at the budding sites on the plasma membrane was described (Bergmann et al., 1981; Torrisi and Bonatti, 1985; Pavan et al., 1987). An accumulation of the glycoprotein gp350/220 at the budding sites in the NE has also been described in cells infected with Epstein-Bar virus (Torrisi et al., 1989). However, in the case of the latter protein contradictory results exist, since a specific accumulation of gp350/220 was not observed in the NE of infected cells by cryoultramicrotomy (Gong and Kieff, 1990). Although we can not rule out that the method used in the present study is not sensitive enough to demonstrate such accumulation of proteins, my data indicate that the amount of gB in the NE is high enough so that no further concentration of gB is required at the budding sites.

In accord with this, a comparison of the labeling intensity of gB in the viral envelope and in the NE shows that the density of gB is not much higher on the virus. Hence, the average diameter (d) of HSV-1 virion is about 120 nm (Roizman and Furlong, 1974; Roizman and Sears, 1991) and its circumference is about 0.38 μm (πd).

On the plasma membrane the average number of grains per virus was 1.8, which corresponds to 4.8 grains per μm of viral envelope, a value close to 2.8 grains/μm which was the average density per NE membranes, the envelope being composed of two membranes (Table 3.9.1). The value of 2.8 grains/μm is probably underestimated as we have assumed for its calculation, in accordance with other studies (Bergmann and Singer, 1983; Torrisi et al., 1987), that some membrane proteins are free to diffuse between the inner and outer membranes of the NE. This assumption may not apply for gB, because Torrisi et al. (1992) have observed a slightly higher concentration of gB in the inner membrane.

It was observed that HSV-1 nucleocapsids are labeled with anti-gB only when they become associated with the NE, an indication that gB is incorporated into the virus during envelopment. We cannot rule out that the nucleocapsids are de-enveloped in the cytoplasm and then re-enveloped again by fusion with other cytoplasmic membranes containing gB as recently described for pseudorabis virus (Whealy et al., 1991). However, I think this is very unlikely for HSV-1, because very few de-enveloped nucleocapsids were observed in the cytoplasm. Results presented by Campadelli-Fiume et al. (1991) also suggest that no de-envelopment of HSV-1 particles occurs in the cytoplasm.

A very interesting but poorly documented phenomenon occurring during HSV-1 infection, is the duplication and invagination of the NE (Morgan et al., 1959; Darlington and Moss, 1968; Nii et al., 1968; Roizman and Furlong, 1974). Unfortunately, no study

exits concerning the composition and function of the duplicated and invaginated membrane. A simple calculation indicates that the unmodified NE is not large enough to supply the virions with all the membranes required for envelopment. The average diameter of HSV-1 is 0.12 μm (Roizman and Furlong, 1974; Roizman and Sears, 1991) and its average surface is 0.045 μm^2 ($4\pi r^2$). Under optimal conditions, the yield of HSV-1 varies between 10 000 virions to no more than 100 000 virions per infected cells (Roizman and Furlong, 1974). Therefore at least 450 μm^2 of membrane are required for envelopment (0.045 μm^2 x 10 000). The average diameter of a cell nucleus is about 6 μm (Alberts et al., 1989) and thus, only 113 μm^2 of membrane ($4\pi r^2$) can be supplied by the nucleus. Clearly, the duplication and infolding of the NE must occur to supplement the virus with enough membrane for envelopment. In accordance with this is the observation made by Ben-Porat and Kaplan (1972) that the transfer of lipids from the cytoplasm to the NE occurs during infection with HSV-1.

4.2.2 Distribution of gB in transfected cells

The results presented in this thesis corroborate the previous studies by cell fractionation and by immunofluorescence indicating a specific localization of gB in the NE of transfected cells (Ali et al., 1987; Raviprakash et al., 1990). However, the present work is the first to have employed the high resolution of the electron microscope to demonstrate such specific localization of gB. The previous immunofluorescence studies were useful to demonstrate the presence of gB in the NE, but they suffered from the lack

of quantification and from the relatively low resolution of the light microscope, which did not allow a clear distinction of the NE from other cellular constituents such as the ER and Golgi complex.

It is clear from the data of the present work and from previous studies, that gB is associated with the compartments of the secretory pathway namely the NE, ER, Golgi complex and plasma membrane. Similarly, we have observed that G was also associated with all of these compartments, except in the NE, where G was present in very low amount in accord with other studies (Bergmann et al., 1981; Bergeron et al., 1982; Bergman and Singer, 1983). These observations were expected since these two membrane glycoproteins possess typical signal sequences that target them to the secretory pathway (Section 1.2). It should be noted however, that the relative cellular distribution of gB and G were different. The density of G on the NE was about six times lower than the density on the plasma membrane. In contrast, the density of gB on these two compartments was quite similar, indicating the presence of an NE localization signal in gB.

As measured by digestion with endo H, gB is transported more slowly than G to the Golgi complex. The slower transport rate of gB has nothing to do with its stronger association with the NE, because the chimera gB-G, which is transported to the Golgi at a rate similar to gB, showed very little localization to the NE.

A surprising and intriguing observation is the fact that gB was also strongly associated with the ER. This observation was made in transfected and in HSV-1 infected cells. The mutants that were strongly associated with the NE were also strongly

associated with the ER as determined by immunofluorescence and immunoelectron microscopy. Two possibilities can account for this. Firstly, gB contains a NE as well as an ER localization signal. Secondly, the ER distribution is an artifact generated by over expression of the proteins. In accordance with the last hypothesis, an intense ER distribution has been described when some NE and Golgi complex membrane proteins were over expressed (Munro, 1991; Smith and Blobel, 1993). With the current data it is impossible to distinguish between these two possibilities. This point could be clarified if the ER/NE ratio of gB is measured as a function of the time following transfection. If the presence of gB in the ER is due to overexpression, the ratio should increase with time. Similarly, the same ER/NE ratio should be determined as a function of the expression level by using weak and strong promoters.

Few things are known concerning the reasons that gB is transported to the plasma membrane. In the case of G, the reason is obvious since envelopment of VSV occurs at this site. As discussed in the introduction (Section 1.6.4), evidence exists indicating that gB is involved in membrane fusion (Cai et al., 1988a; Butcher et al., 1990; Gage et al., 1993). The presence of gB on the cell surface of the infected cells most likely facilitates the spreading of the infection by promoting fusion with the adjacent cells (See Navarro et al., 1992 for a discussion of the topic).

Glycoprotein B and other HSV-1 glycoproteins are transported faster in transfected cells than in infected cells (Johnson and Smiley, 1985; Campadelli-Fiume et al., 1988). These glycoproteins are also transported faster in early infection than in late

infection (Campadelli-Fiume et al., 1988; Sommer and Courtney, 1991). These two observations would account for the relatively higher expression of gB on the plasma membrane of the transfected cells, assuming that the amount of protein transported to this site is proportional to the transport rate. As discussed in the introduction (Section 1.5.2), the reduction in the transport rate could be due to a shutoff of the host macromolecular synthesis or it could be due to the incorporation of the glycoproteins into the viral envelope.

As discussed above (Section 4.1), it was not possible, even with the high resolution provided by the electron microscope, to distinguish the inner and outer membranes of the NE. Consequently it is not certain if gB and the mutants were localized on the inner membrane of the NE in transfected cells. Studies exist however indicating that membrane proteins can freely reach the inner membrane of the NE by lateral diffusion at the level of the nuclear pore complexes (Bergmann and Singer, 1983; Torrisi and Bonatti, 1985; Torrisi et al., 1987).

4.3 The NE localization determinants of gB

A summary of the transport and localization to the NE of the proteins investigated in this thesis is presented in Table 4.3.1. It is clear from this table that a segment of 75 amino acids starting at residue 721 and ending at residue 795 of gB is sufficient to specify NE, because all the mutants containing this segment (G-gB-2, CT799, G-tmgB, CT796, G-gB-1) were localized to the NE. The fact that mutants CT796 and G-gB-1

Table 4.3.1 Summary of the distribution and transport

Protein	Accumulation In Nuclear Envelope	Transport To Cell Surface
gB	YES	YES
G	NO	YES
gB-G	NO	YES
G-gB-1	±	NO
G-gB-2	YES	NO
GH6	NO	NO
CT799	YES	SLOW[a]
CT796	±	NO
G-tmgB	YES	YES
Δ12	YES	NO
Δ3	YES	YES
Δ23	NO	NO
gB-tmG	YES	NO
Δ12CT799	±	YES
gB-gC	±	YES

±: Weak accumulation in the nuclear envelope
a: Not efficiently transport to the cell surface

were not targeted to the NE very efficiently could be due to some folding problems. This segment of 75 residues encompasses completely the 69 amino acid long TM of gB, suggesting the latter domain encodes the NE localization signal. During the past few years other membrane proteins have also been shown to contain specific targeting determinants within their TM. These membrane proteins include residents of the Golgi complex (Swift and Machamer, 1991; Munro, 1991; Tang et al., 1992; Section 1.2.11), a constituent of the ER (Poruchynsky and Atkinson, 1988; Stirzaker and Both, 1989; Section 1.2.10.2) and two NE proteins (Wozniak and Blobel, 1992; Smith and Blobel, 1993; Section 1.3.2).

Other portions of gB may also be sufficient to specify NE localization, since mutant gB-tmG, which lacks amino acids 722 to 795, was also strongly localized to the NE. This suggests that the CT of gB, or the CT in conjunction with the EC of gB contain NE localization determinant. The EC of gB is not sufficient by itself to promote NE localization, because the chimera gB-G did not accumulate in the NE.

Evidence exists indicating that other membrane proteins possess targeting determinants spanning more than one domain. It has been shown that the TM and the stem region of sialyltransferase (ST) are both sufficient to promote Golgi retention (Munro, 1991; Colley et al., 1992). Similarly, the TM and the CT of gp210, a transmembrane protein of the nuclear pore complex, are both sufficient to promote NE localization when fused separately to a reporter protein (Wozniak and Blobel, 1992). The apparent contradiction suggesting that the NE determinant of the lamin B receptor is

either located in the nucleoplasmic tail (Soullam and Worman, 1993) or within the first TM (Smith and Blobel, 1993) could be reconciled by assuming that these two domains are both sufficient to specify NE when placed in the right context. These observations suggest that the targeting determinants of many membrane proteins is either redundant, or is an elusive one, lacking the properties of other targeting signals, such as the signal mediating accumulation in the nucleus (Silver, 1991) or the ER retention signal of soluble proteins (Pelham, 1989; Pelham, 1990; Section 1.2.10.2), which are built around short peptides made of characteristic residues.

A major criticism of this work concerns the distribution of gB-tmG and Δ12 in the NE which could be nonspecific. Mutant GH6 was used to demonstrate that a protein lacking a NE localization determinant and deficient in transport does not accumulate in the NE. However, GH6 forms aggregates and it is presently not known if proteins that remain in the ER but that do not form aggregates, such as mutant gB-tmG and Δ12, accumulate nonspecifically in the NE, most likely by diffusion from the ER.

In favour of the existence of a NE localization determinant in the CT of gB is the observation that mutants Δ12 and Δ3 are both targeted to the NE, and that deletion of the CT of Δ12 reduces the NE localization efficiency, since Δ12CT799 is not strongly localized in the NE. However, the result of the latter deletion is complicated by the fact that Δ12 does not exit the ER, whereas Δ12CT799 is transported quite efficiently. Furthermore, the weaker NE localization of Δ12CT799, indicates that segment 3 is not sufficient in the context of Δ12CT799 to promote full localization in the NE. If a signal

exists in the CT of gB, it is functional only when the protein is anchored in the membrane, because mutant Δ23, which is completely translocated in the lumen of the ER (Rasile et al., 1993), is not localized to the NE. I presume that the most direct way to demonstrate the presence of a NE localization determinant in the CT of gB, is to study the distribution of a chimera made by fusing the EC and TM of G to the CT of gB.

Glycoproteins gD and gC are also known to be specifically localized to the NE (Compton and Courtney, 1984a; Ghosh-Choudhury et al., 1990; Torrisi et al., 1992). In the case of gC, the result of immunoelectron microscopy, in agreement with previous studies by immunofluorescence (Ghosh-Choudhury, 1990), indicates that the TM and CT of this glycoprotein encodes some NE localization information, because mutant gB-gC was localized, although not very efficiently, to this compartment.

The amino acid sequence of the polypeptide specifying NE localization of gB (residues 721 to 795) was compared with the homologous region of the gB glycoprotein of 6 other herpes viruses (Fig. 4.3.1). The most conservative region corresponds to a peptide of 19 residues starting at G^{758} and ending at G^{776}, which spans 2/3 of segment 2 and the beginning of segment 3 of the TM of gB (See also Figs 1.5.1 and 3.1.1). If this peptide is involved in NE localization, it would explain why mutant Δ3 (assuming no signal in the CT of gB) and Δ12CT799 are both targeted to the NE, since they both posses a portion of this peptide. Three amino acids (G^{766}, F^{770}, P^{774}) are totally conserved among the 7 herpesviruses. The residues around the P^{774} are also well conserved. Furthermore, polar and charged residues are positioned at the same place. In analogy

```
                            1         *              2    *
       721        *       *         *  *           *   * * *
HSV-1:   TVIH---ADANAAMFAGLGAFF-EGMGDLGRAVGKVVMGIVGGVVSAVSGVSS
CMV:     KYVEDKVVDPLPPYLKGLDDLM-SGLGAAGKAVGVAIGAVGGAVASVVEGVAT
EBV:     KDLDNAVSNGRNQFVDGLGELM-DSLGSVGQSITNLVSTVGGLFSSLVSGFIS
VZV:     KVVQ---YDSGTAIMQGMAQFF-QGLGTAGQAVGHVVLGATGALLSTVHGFTT
MDV:     KVIE---VDTNYAGMNGLAELF-NGMGQVGQAIGKVVVGAAGAIVSTISGVSA
ILTV:    TVIR---GDRGDAIFRAIADFFGNTLGEVGKALGTVVMTAAAAVISTVSGIAS
HVS:     KDFDNSQRNNRDRIIQDFSEIL-ADLGSIGKVIVRVASGAFSLFGGIVTGILN

         *   *         3
         *  ****     *   *        795
HSV-1:   FMSNPFGALAVGLLVLAGLAAAFFAF
CMV:     FLKNPFGAFTIILVAIAVVIITYLIY
EBV:     FFKNPFGGMLILVLVAGVVILVISLT
VZV:     FLSNPFGALAVGLLVLAGLVAAFFAY
MDV:     FMSIPLFLSAIGLIIIAGLVAAFLAY
ILTV:    FLSNPFAALGIGIAVVVSIILGLLAF
HVS:     FIKNPLGGMFTFLLIGAVIILVILLV
```

Figure 4.3.1 Amino acid homology between the TM of gB homologues. The amino acid sequences of the TM region of the gB glycoprotein of seven different herpesviruses were aligned. The positions of T^{721} and F^{795} of the gB of HSV-1 (Bzik et al., 1986) are indicated. Segments 1, 2 and 3 of the TM of gB are underlined. The charged and polar residues are bold. Two *: residues conserved among the 7 proteins; One *: residues present more then four times. HSV-1: Herpes Simplex virus type 1 (Bzik et al., 1986); CMV: Cytomegalovirus (Cranage et al., 1986); EBV: Epstein-Barr virus (Gong et al., 1987); VZV: Varicell-Zoster virus (Davison and Scott, 1986); MDV: Marek's disease virus (Ross et al., 1989); ILTV: Infectious laryngotracheitis virus (Griffin, 1991); HVS: Herpesvirus saimiri (Albrecht and Fleckenstein, 1990).

with some Golgi membrane proteins, where conserved polar residues within the TM have been shown to be directly involved in targeting (Swift and Machamer, 1991; Machamer, 1991; Aoki et al., 1992), the aligned polar residues could be essential components of the NE localization signal of gB.

According to the model of Pellet et al. (1985) (See Figs 1.5.1 and 3.1.1), P^{774} is part of a β-turn that links segments 2 and 3. It is therefore plausible that P^{774} and the residues around it, are conserved because they are part of a secondary structure essential for the proper folding of gB. Similarly, we can not rule out that the relatively conserved region is involved in other essential functions of gB such as the fusogenic activity for example.

The amino acid sequence of the TM of gB was also compared with the amino acid sequence of the TM of four transmembrane proteins known to be specifically localized to the NE. These proteins are glycoprotein C and D of HSV-1 (Compton and Courtney, 1984a; Torrisi et al., 1992), the pore complex glycoprotein gp210 (Wozniak and Blobel, 1992) and the lamin B receptor (Smith and Blobel, 1993). As can be seen in Fig. 4.3.2, no region of high homology exists between the TM of these proteins. The position of polar residues is not conserved either. It should be noted however, that among the three segments making up the TM of gB, segment 3 is the one sharing the highest similarity with the other 4 TM. Among the most interesting points is the presence of a leucine and a glycine conserved between gB, gC and gD and an alanine between gp210, LBR and gB (stars Fig. 4.3.2).

```
                  1                                   2
gB:      ANAAMFAGLGAFFEGMGDLGRAVGKVVMGIVGGVVSAVSGVSSFMSNP
gC:      m-----vGIG-----------------------------------------
gD:                                      MGLIaGaVggs---------l
gP210:   vmfftFfaL-----------------------------------------
LBR:
            *   *  3 *
gB:      FGALAV-GLLVLAGLAAAFFAF
gC:      iGvLAa-GVLVVTaIv---Yvv
gD:      laALvIcGIVywm
gP210:   ----------LAGtAvTiiAY
LBR:     FGTfmL-mffLpAtVlylvlmc
```

Figure 4.3.2 Homology of gB with NE membrane proteins. The amino acid sequence of the TM of four transmembrane proteins localized to the NE were compared with the TM of gB. The sequence were aligned according to the method of Myers and Miller (1988) using the softwar PCGENE. Residues identical to gB are capitalized and bold. Residues similar to gB are capitalized. Segments 1, 2 and 3 of the TM of gB are indicated. *: position of conserved residues between three proteins. gB: glycoprotein B of HSV-1 (Bzik et al., 1986); gC: glycoprotein C of HSV-1 (Frink et al., 1983); gD: glycoprotein D of HSV-1 (Watson et al., 1982). gp210: Nuclear pore membrane glycoprotein of 210 kD (Wozniak et al., 1989). LBR: lamin B receptor (Worman et al., 1990).

4.4 Models for NE localization of gB

It is presently not understood how gB is targeted to the inner membrane of the NE. Three models can be conceived to explain this specific targeting. In model A, the fraction synthesized on the outer membrane of the NE is retained in this compartment and is transported by lateral diffusion to the inner membrane at the level of a nuclear pore complex. In favour of this model evidence exists indicating that some membrane proteins are free to diffuse between the outer and inner membrane of the NE (Bergmann and Singer, 1983; Torrisi and Bonatti, 1985; Torrisi et al., 1987).

In model B, the proteins synthesized on the ER diffuse to the outer membrane and then to the inner membrane of the NE at the level of a nuclear pore complex. This model is based on morphological evidence indicating that the outer membrane of the NE is directly connected with the ER membranes (Fawcett, 1981; Frank et al., 1981), and on the observation that diffusion from the ER to the NE can occur (Powell and Burke, 1990). Model B does not necessarily exclude model A. Glycoprotein B could very well be targeted to the NE by retention in the NE and by diffusion from the ER. A similar model has been proposed to explain the NE localization of the lamin B receptor, and gp210 (Wozniak and Blobel, 1992; Smith and Blobel, 1993; Soullam and Worman, 1993).

Although lateral diffusion in the membrane is the most attractive model, we cannot rule out that gB is targeted to the NE by means of transport vesicles originating from the ER (Model C). The existence of such vesicles has not yet been demonstrated.

If targeting of gB to the NE involves transport vesicles, these vesicles are not derived from the Golgi complex, because the fraction of gB associated with the NE has not been processed by Golgi enzymes (Compton and Courtney, 1984b; Poliquin et al., 1985; Torrisi et al., 1992).

It is not known if the transport between the ER and the outer and inner membranes of the NE is passive or if it involves specific receptors shuffling back and forth between these membranes. Retention in the NE and/or transport from the ER to the NE, must involve some residues located between amino acids 721 to 795 (Section 4.3). This segment could bind to some uncharacterized receptors or it could promote the aggregation of gB in the NE. Specific aggregation has been proposed as a mechanism used by some Golgi proteins to escape the bulk flow and be specifically retained in the Golgi complex (Machamer, 1991; Munro, 1991).

As discussed in Section 4.3, the CT of gB could be equally involved in NE localization. It was proposed by Torrisi et al. (1992) that gB is targeted to the inner membrane by means of its CT. The CT of gB contains two short segments of basic residues (HKAKKK^{866} and RKRR^{875}) that could form the core of a determinant analogue to the consensus signal PKKKRKV which specifies nuclear uptake (Silver, 1991). Proteins bearing such determinants are recognized and become associated with the nuclear pore complexes before being actively translocated inside the nucleus by an obscure mechanism involving hydrolysis of ATP. In the case of gB, a similar mechanism would occur, but the end result is the transport to the inner membrane, because gB is

tethered in the membrane by its TM. This attractive model, could account for the NE localization of proteins containing the CT of gB (gB, G-gB-2, gB-tmG, Δ12, and Δ3), but not for those lacking this domain (G-tmgB, CT799, CT796, Δ12CT799).

In contrast to other proteins localized to the NE namely, the lamin B receptor and gp210, gB is also transported to the plasma membrane. This suggests that a subpopulation of gB is able to escape retention in the NE/ER system and be incorporated into the transport vesicles, followed by transport to the plasma membrane.

4.5 Transport to the plasma membrane

It is generally recognized that transport to the plasma membrane occurs by default and that no specific signals are needed to exit the ER (Pfeffer and Rothman, 1987; Pelham, 1989; Section 1.2.8). Nevertheless, to be transported efficiently a protein must generally fold properly and form correct oligomers, because misfolded proteins are retained in the ER by a system of quality control (Pelham, 1989; Hurtley and Helenius, 1989; Section 1.2.10.1). In agreement with the model of bulk flow, the proteins that were transported efficiently to the cell surface (gB, G, gB-G, Δ12CT799, G-tmgB) appeared to fold properly because they form correct oligomers, whereas two proteins that did not exit the ER formed aggregates (G-gB-1, GH6). It is more difficult to understand why G-gB-2 and gB-tmG were not transported efficiently to the plasma membrane, since they were able to oligomerize to a certain extent. In the case of G-gB-2, misfolding of the CT could be responsible for the ER retention, because G-gB-2 was poorly recognized

by a polyclonal antibody raised against the carboxy terminus of gB (Section 3.5). Consequently, not only correct folding of the EC is essential for transport, but also correct folding of the CT, suggesting the existence of a system of quality control in the cytoplasm. Similarly, a subtle folding defect that could be detected only by specific conformational antibodies, could be responsible for the retention of gB-tmG in the ER.

Deletion of the CT of gB abolishes transport or reduces the rate of transport to the cell surface, which has lead to the hypothesis that this domain carries essential information for transport (Raviprakash et al., 1990). We have seen that mutant Δ12CT799, which lacks the CT and segments 1 and 2 of the potential TM of gB, was transported rapidly to the cell surface, thus ruling out the previous hypothesis (compare Tables 3.10.3 and 3.5.1). The reason why deletion of segments 1 and 2 allows rapid transport of Δ12CT799 is not understood, but we can speculate that deletion of these two relatively hydrophobic segments, allows Δ12CT799 to fold and to oligomerize more efficiently.

It was proposed that the CT of G accelerates transport to the cell surface by facilitating incorporation into transport vesicles (Doms et al., 1988; Guan et al., 1988; Rose and Doms, 1988). Although the rate of transport of G-tmgB was difficult to measure, because of the degradation of the endo H resistant form, my results suggest that G-tmgB is transported as rapidly as G. This is in agreement with the involvement of the CT of G in rapid transport. It should be noted that G-tmgB contains the EC of G, and we cannot rule out that the rapid transport of G-tmgB is caused by efficient folding of

the EC, a process known to occur relatively rapidly with the wild type protein (Doms et al., 1987). In the context of gB-G, the CT of G does not promote efficient transport, since gB-G was transported more slowly than G. The chimera gB-G forms dimers and it is plausible that the CT of G promotes efficient transport solely when associated with a trimeric structure.

4.6 Oligomerization

The oligomeric nature of the proteins made in this work was estimated by sedimentation on sucrose density gradients, using aldolase and BSA as internal markers according to the method of Martin and Ames (1961). With this method, I have observed in agreement with previous studies (Sarmianto and Spear, 1979; Ali et al., 1990; Doms et al., 1987; See Sections 1.5.1 and 1.6.3), that gB and G oligomerize as dimers and trimers respectively. The fact that gB-G forms dimers shows that the EC of gB contains sufficient information for dimerization as was previously demonstrated (Ali, 1990; Qadri et al., 1991; Navarro et al., 1993). It was also observed that mutants gB-tmG and Δ12CT799 oligomerize into dimers, although a subpopulation remained monomeric or was associated into larger oligomeric structures. It is presently not known if these larger oligomers are constituted by a single species or are associated with molecular chaperons such as BIP.

I have assigned the oligomeric nature of G-gB-2 to correspond to a dimer (mol wt: 160 kD), because this chimera cosedimented with aldolase (mol wt: 161 kD) and

with G (mol wt: 169 kD). The oligomeric nature of G-tmgB was assigned to be a trimer (210 kD), because it sedimented slightly faster than aldolase and G. According to the mol wt of the monomers of G-tmgB (70 kD) and G-gB-2 (80 kD) estimated by PAGE-SDS (Section 3.5), If G-gB-2 was a trimer (240 kD) it would have sedimented faster than aldolase and G. On the other hand a G-tmgB dimer (140 kD) would have sedimented slightly slower than these two markers. To further confirm the true oligomeric nature of G-tmgB and G-gB-2, experiments could be done using crosslinking agents such as dimethylsuberimidate which was used efficiently to demonstrate the trimeric nature of G (Doms et al., 1987).

It is well recognized that the EC of G contains sufficient information for proper oligomerization, since deletion of the TM and CT does not abolish trimerization of G (Doms et al., 1988; Crise et al., 1989; Schmidt et al., 1992). According to the previous observation, the fact that G-tmgB forms trimers, while G-gB-2 oligomerizes into dimers highly suggests that the CT of gB is too bulky to be organized into a trimeric structure. When the pH was raised from 5.7 to 7.4, the G-gB-2 oligomers behaved like the G trimers and dissociated into monomers (Section 3.7). In contrast, the gB dimers are stable at neutral pH (Sarmiento and Spear, 1979; Ali, 1990). This suggests that the G-gB-2 and G monomers are maintained into an oligomeric structure using similar intermolecular interactions, which involve most likely charged residues that are sensitive to fluctuations of pH.

4.7 The modified ER

Mutants GH6 and G-gB-1 were localized in cytoplasmic structures composed of filaments and granules that were not observed in cells expressing gB and G. These structures were termed modified ER, because they seem to originate from a tight packing or agglomeration of ER tubules (Section 3.10.3). I believe these structures correspond to the ER, because GH6 and G-gB-1 remain endo H sensitive indicating that they are not transported beyond the cis-Golgi. Furthermore, GH6 and G-gB-1 aggregate, and it is well recognized that protein aggregates do not leave the ER (Section 1.2.10.1).

Chimeric proteins and incomplete oligomers have been observed to accumulate in a post-ER compartment distinct from the cis-Golgi network (Rizzolo et al., 1985; Hobman et al., 1992). This post-ER compartment differs from the modified ER, because it possesses a different morphology (it was described as being made of short tubules and narrow membrane bounded cisternae, some being in continuity with the ER), and because a temperature sensitive mutant of G, which aggregates at the restrictive temperature, was not specifically concentrated into this post-ER compartment (Hobman et al., 1992). I am not aware of any other reports indicating that protein aggregates are distributed in a structure analogue to the modified ER. It is not known if the modified ER corresponds to the entire ER or if it represents only a subdomain of this organelle. Double labeling studies using antibodies against ER markers should be undertaken to clarify this point.

The modified ER is not an artifact of transfection, because similar structures were not observed in cells expressing mutants G-gB-2 and gB-tmG, which were not transported

to the cell surface. The formation of the modified ER could be induced by the synthesis of large amounts of aggregates. It is plausible that the modified ER is involved in the degradation and recycling of aggregates, since mutant GH6 was degraded. The modified ER could also correspond to a normal structure, that is greatly amplified by the high expression of GH6 and G-gB-1. Clearly, more studies are required to understand the function of this newly described structure.

During the course of this study, I have observed that cells expressing mutants CT796 and Δ23 were also showing strongly labeled cytoplasmic structures similar to the modified ER (Figs 3.10.4c and 3.10.7c). Although Δ23 remains in a monomeric state (Rasile et al., 1993), whereas mutant CT796 oligomerizes into dimers (Dr. K. Ghosh, personal communication), we can speculate that a subpopulation of Δ23 and CT796 also forms aggregates that are localized into a modified ER.

4.8 Transport and processing of G-tmgB

Of the mutants investigated in this thesis, G-tmgB possesses unique characteristics. Firstly, the endo H resistant form of G-tmgB disappears with time, suggesting the protein is either degraded or secreted by the cell. Preliminary immunoprecipitation experiments have been unable to demonstrate the presence of G-tmgB in the extracellular medium, suggesting the protein is degraded intracellularly. Secondly, G-tmgB is localized on the plasma membrane, but on this compartment the

protein is concentrated into patches, that could very well correspond to coated pits. In agreement with this, endocytosis of chimeric proteins by means of coated pits has been previously described (Roth et al., 1986). Thirdly, G-tmgB is concentrated into densely stained vacuole-like structures near the nucleus that could correspond to lysosomes. From these observations, a model for the transport and processing of G-tmgB can be proposed: following synthesis, a subpopulation of G-tmgB is transported to the cell surface by default. On this compartment, the protein is concentrated into coated pits, followed by endocytosis and delivery to lysosomes where the protein is degraded. This model would account for the punctuate distribution of G-tmgB on the plasma membrane and would explain why the endo H resistant form of G-tmgB is degraded. A similar scheme was proposed by Rizzolo et al. (1985) to explain the rapid degradation of one of their chimeric proteins. In favour of this model, lysosomal membrane proteins have been described to transiently accumulate on the plasma membrane, before being delivered to the lysosomes (Mathews et al., 1992).

To test this model, double labeling studies with lysosomal markers should be undertaken to see if the cytoplasmic vacuole-like structures correspond effectively to lysosomes. The fate of the plasma membrane associated G-tmgB should be investigated by labeling living cells with tagged antibodies specific for G-tmgB, or by cell surface iodination. Furthermore, treatments with lysosotropic agents, such as chloroquine, should abolish the degradation of G-tmgB.

The reason for the degradation of the endo H resistant form of G-tmgB remains

obscure. It is plausible that G-tmgB possesses a particular conformation that is recognized by a post-Golgi system of quality control. Scavenger receptors have been described on the plasma membrane. These receptors recognize and become associated with modified or abnormal proteins floating in the extracellular medium. The binding is then followed by endocytosis and transport to the lysosomes, where the abnormal protein is degraded (Schnitzer and Bravo, 1993). It is possible to envision the existence of similar cell surface receptors, whose function is to recognize and destroy aberrantly folded plasma membrane proteins.

4.9 Subcellular fractionation

The NE distribution of gB, G and the mutants was also investigated by subcellular fractionation. Although similar conclusions were reached concerning the distribution of gB and G by subcellular fractionation and by microscopy, conflicting results were obtained with the mutants. Mutants GH6, G-gB-1 and gB-G were found by subcellular fractionation to be strongly localized to the NE, whereas the same proteins, except for G-gB-1, were not strongly localized to the NE as determined by light and electron microscopy. On the other hand, no G-gB-2 was isolated in the nuclear fraction whereas this chimera was strongly localized to the NE by light and electron microscopy.

Protein aggregates can be isolated from cell lysate by low speed centrifugation (Dubois et al., 1991). This could easily explain the presence of GH6 in the nuclear fraction, since this protein aggregates. The same argument could be used to explain the

presence of G-gB-1 in the nuclear fraction, since this mutant also forms aggregates. Although the chimera gB-G oligomerizes into dimers (Section 3.7), we cannot rule out that a subpopulation forms aggregates that were likewise recovered in the nuclear fraction. It is equally conceivable that the EC of gB interacts with some insoluble components that are recovered with the nuclei (see below).

The reason that G-gB-2 was not isolated in the nuclear fraction is intriguing. The fractionation procedure of this thesis involves the use of 0.5% of a nonionic detergent, namely Nonidet P40 (Section 2.46). Similar concentrations of nonionic detergent have been employed to solubilise the membrane of the NE and most integral membrane proteins associated with this compartment (Dwyer and Blobel, 1986; Senior and Gerace, 1988). For example, when isolated NE were treated with a low salt buffer containing 1% Triton X100, most major integral membrane proteins were solubilised except for the pore complex proteins, the lamina, and some proteins associated with the lamina (Senior and Gerace, 1988). We can thus speculate that G-gB-2 was not recovered in the nuclear fraction because it was solubilized during the fractionation procedure.

According to the preceding theory, gB should have been completely solubilized and no gB should have been found with the nuclear fraction. However, 20% was recovered in the nuclear fraction. It is conceivable that the EC of gB interacts with some cellular components that are associated with the ER or with the NE and which, because of their size, are recovered by centrifugation with the nuclei. It is unlikely that the EC of gB interacts directly with the lamina, since this proteinaceous network is contiguous

with the nucleoplasmic side of the inner membrane, whereas the EC of gB faces the perinuclear cisterna. The presence of gB and gB-G in the nuclear fraction could also be due to a propensity of the EC of gB to form aggregates.

5.0 CONCLUSION

We have seen in this thesis that gB is specifically localized to the NE of cells infected with HSV-1 and of cells transfected with the gB gene. Glycoprotein B and all the mutants that were strongly localized in the NE were also strongly localized in the ER, suggesting the presence of an ER retention signal as well. However, concerning the latter point, it remains to be demonstrated that the presence of protein in the ER was not an artifact generated by high expression of gB and the mutants. We have also seen that a peptide of 75 residues, which spans the TM of gB, is sufficient to promote NE localization. However, some observations suggest that other domains, most likely the CT of gB, also encode some NE localization determinants. The transport and distribution of a chimera constructed by fusing the EC and TM of G with the CT of gB should be investigated to clarify this point.

Experiments should be undertaken to define further the minimal sequence located within the 75 amino acid peptide that is responsible for NE localization of gB. One approach would be to study the distribution of chimeras in which the TM of G is replaced by one of the three segments making up the TM of gB. Is the signal universal i.e do other herpes virus glycoproteins contain within their TM determinants specifying NE localization? Preliminary results suggest the affirmative, since the TM and CT of gC were sufficient to target the EC of gB to the NE. More experiments are required to

demonstrate conclusively the presence of a NE localization determinant within the TM of gC. Other herpes virus glycoproteins should also be investigated.

The main flaw of this study was the inability to distinguish the inner and outer membrane of the NE in transfected cells. It is therefore possible that some proteins were localized to the outer membrane but not to the inner membrane of the NE. I believe this problem could be overcome if the cells are processed by cryosubstitution or cryoultramicrotomy, two methods known to improve the ultrastructural preservation (Weibull et al., 1984; Tokuyasu, 1986). Another approach would be to mechanically separate the inner and outer membrane before cell processing. This could be done by first isolating salt-washed NE as described by Dwyer and Blobel (1976), followed by strong vortexing or ultrasound treatments to break the NE.

The mechanism used by gB to achieve its NE localization remains obscure. For example, it is not known if this process is passive i.e occurring solely by diffusion, or if it involves the action of specific receptors or requires input of energy. Treatments with components that perturb protein transport such as brefeldin A or aluminium fluoride, could give some clues about the mechanism. Similarly, cells could be treated with inhibitor of translation in order to see if gB can be chased out of the NE.

Two novel and intriguing observations were made during the course of this work. One concerns the modified ER structure termed the modified ER into which protein aggregates seem to accumulate. Its function is unknown but it could be involved in the managing and destruction of protein aggregates. I believe we could learn a great deal

about this fundamental biological process by improving the microscopic data and by biochemical characterization of the constituents of the modified ER. One way to improve the microscopic data would be to enhance the cellular preservation by processing the specimens using cryotechnics such as cryosubstitution (Weibull et al., 1984). Better fixatives, such as osmium tetroxide, could also be used to improve the ultrastructure of the membranes (Hayot, 1981). Secondly, the processing and transport of G-tmgB highly suggests the existence of a system of quality control on the cell surface. Crosslinking studies could be undertaken in order to characterize potential receptors which recognize and direct G-tmgB for degradation.

6.0 APPENDICES

MKCLLYLAFLFIGVNCKFTIVFPHNQKGNWKNVPSNYHYCPSSSDLNWHN 50

DLIGTAIQVKMPKSHKAIQADGWMCHASKWVTTCDFRWYGPKYITQSIRS 100

FTPSVEQCKESIEQTKQGTWLNPGFPPQSCGYATVTDAEAVIVQVTPHHV 150

LVDEYTGEWVDSQFINGKCSNYICPTVHNSTTWHSDYKVKGLCDSNLISM 200

DITFFSEDGELSSLGKEGTGFRSNYFAYETGGKACKMQYCKHWGVRLPSG 250

VWFEMADKDLFAAARFPECPEGSSISAPSQTSVDVSLIQDVERILDYSLC 300

QETWSKIRAGLPISPVDLSYLAPKNPGTGPAFTIINGTLKYFETRYIRVD 350

IAAPILSRMVGMISGTTTERELWDDWAPYEDVEIGPNGVLRTSSGYKFPL 400

YMIGHGMLDSDLHLSSKAQVFEHPHIQDAASQLPDDESLFFGDTGLSKNP 450

IELVEGWFSSWKSSIASFFFIIGLIIGLFLVLRVGIHLCIKLKHTKKRQI 500

YTDIEMNRLGK

Figure 6.1 Amino acid sequence of glycoprotein G. The sequence corresponds to G of VSV, serotype Indiana (Rose and Gallione, 1981). The signal sequence and the transmembrane domain are bold.

MHQGAPSWGRRWFVVWALLGLTLGVLVASAAPSSPGTPGVAAATQAANGG 50

PATPAPPPLGAAPTGDPKPKKNKKPKNPTPPRPAGDNATVAAGHATLREH 100

LRDIKAENTDANFYVCPPPTGATVVQFEQPRRCPTRPEGQNYTQGIAVVF 150

KENIAPYKFKATMYYKDVTVSQVWFGHRYSQFMGIFEDRAPVPFEEVIDK 200

INAKGVCRSTAKYVRNNLETTAFHRDDHETDMELKPANAATRTSRGWHTT 250

DLKYNPSRVEAFHRYGTTVNCIVEEVDARSVYPYDEFVLATGDFVYMSPF 300

YGYREGSHTEHTTYAADRFKQVDGFYARDLTTKARATAPTTRNLLTTPKF 350

TVAWDWVPKRPSVCTMTKWQEVDEMLRSEYGGSFRFSSDAISTTFTTNLT 400

EYPLSRVDLGDCIGKDARDAMDRIFARRYNATHIKVGQPQYYQANGGFLI 450

AYQPLLSNTLAELYVREHLREQSRKPPNPTPPPPGASANASVERIKTTSS 500

IEFARLQFTYNHIQRHVNDMLGRVAIAWCELQNHELTLWNEARKLNPNAI 550

ASVTVGRRVSARMLGDVMAVSTCVPVAADNVIVQNSMRISSRPGACYSRP 600

LVSFRYEDQGPLVEGQLGENNELRLTRDAIEPCTVGHRRYFTFGGGYVYF 650

EEYAYSHQLSRADITTVSTFIDLNITMLEDHEFVPLEVYTRHEIKDSGLL 700

DYTEVQRRNQLHDLRFADIDTVIHA**DANAAMFAGLGAFFEGMGDLGRAVG** 750

KVVMGIVGGVVSAVSGVSSFMSNPFGALAVGLLVLAGLAAAFFAFRYVMR 800

LQSNPMKALYPLTTKELKNPTNPDASGEGEEGGDFDEAKLAEAREMIRYM 850

ALVSAMERTEHKAKKKGTSALLSAKVTDMVMRKRRNTNYTQVPNKDGDAD 900

EDDL

Figure 6.2. Amino acid sequence of glycoprotein B. The sequence corresponds to gB of HSV-1, strain KOS (Bzik et al., 1986). The signal sequence and the carboxy terminal hydrophobic region are bold.

7.0 BIBLIOGRAPHY

Aebi, U., Cohn, J., Buhle, L., and Gerace, L. (1986). The nuclear lamina is a meshwork of intermediated filaments. Nature 323, 560-564.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. (1989). Molecular biology of the cell, Sec. Ed. Garland Publishing, Inc., New York and London.

Albrecht, J.C., and Fleckenstein, B. (1990). Structural organization of the conserved gene block of herpesvirus Saimiri coding for DNA polymerase, glycoprotein B, and major DNA binding protein. Virology 174, 533-542.

Ali, M.A. (1990). Oligomerization of herpes simplex virus glycoprotein B occurs in the endoplasmic reticulum and a 102 amino acid cytosolic domain is dispensable for dimer assembly. Virology 178, 588-592.

Ali, M.A., Butcher, M., and Ghosh, H.P. (1987). Expression and nuclear envelope localization of biologically active fusion glycoprotein gB of herpes simplex virus in mammalian cells using cloned DNA. Proc. Nat. Acad. Sci. U.S.A. 84, 5675-5679.

Andersson, H., Bakker, E., and von Heijne, G. (1992). Different positively charged amino acids have similar effects on the topology of a polytopic transmembrane protein in Escherichia coli. J. Biol Chem. 267, 1491-1495.

Andrews, D.W., and Rachubinski, R.A. (1990). Secretion and organelle biogenesis in: Tip growth in plant and fungal cells. Heath, I.B. Ed., Academic Press, pp 317-343.

Andrews, D.W., Walter, P., and Ottensmeyer, F.P. (1985). Structure of the signal recognition particle by electron microscopy. Proc. Natl. Acad. Sci. U.S.A. 82, 785-789.

Andrews, D.W., Young, J.C., Mirels, L.F., and Czarnota, G.J. (1992). The role of the N region in signal sequence and signal-anchor function. J. Biol. Chem. 267, 7761-7769.

Aoki, D., Lee, N., Yamaguchi, N., Dubois, C., and Fukuda, M.N. (1992). Golgi retention of a trans-golgi membrane protein, galactosyltransferase, requires cysteine and histidine residues within the membrane-anchoring domain: Proc. Natl. Acad. Sci. U.S.A. 89, 4319-4323.

Bailer, S.M., Eppenberger, H.M., Griffiths, G., and Nigg, E.A. (1991). Characterization of 54-kD protein of the inner nuclear membrane: evidence for cell cycle-dependent interaction with the nuclear lamina. J. Cell Biol. 114, 389-400.

Baines, J.D., and Roizman, B. (1993). The U_L10 gene of herpes simplex virus 1 encodes a novel viral glycoprotein, gM, which is present in the virion and in the plasma membrane of infected cells. J. Virol. 67, 1441-1452.

Baucke, R.B., and Spear, P.G. (1979). Membrane proteins specified by herpes simplex viruses V. Identification of an Fc-binding glycoproteins. J. Virol. 32, 779-789.

Bendayan, M., Nanci, A., and Kan, F.W.K. (1987). Effect of tissue processing on colloidal gold cytochemistry. J. Histochem. Cytochem. 35, 983-996.

Ben-Porat, T., and Kaplan, A.S. (1971). Phospholipid metabolism of herpesvirus-infected and uninfected rabbit kidney cells. Virology. 45, 252-264.

Ben-Porat, T., and Kaplan, A.S. (1972). Studies on the biogenesis of herpesvirus envelope. Nature 235, 165-166.

Bergeron, J.J.M, Kotwal, G.J., Levine, G., Bilan, P., Rachubinski, R., Hamilton, M., Shore, G.C., and Ghosh, H.P. (1982). Intracellular transport of the transmembrane glycoprotein G of vesicular stomatitis virus through the golgi apparatus as visualized by electron microscope radioautography. J. Cell. Biol. 94, 36-41.

Bergmann, J.E., Tokuyasu, K.T., and Singer, S.J. (1981). Passage of an integral membrane protein, the vesicular stomatitis virus glycoprotein, through the golgi apparatus en route to the plasma membrane. Proc. Nat. Acad. Sci. U.S.A. 78, 1746-1750.

Bergmann, J.E., and Singer, S.J. (1983). Immunoelectron microscopic studies of the intracellular transport of the membrane glycoprotein (G) of vesicular stomatitis virus in infected chinese hamster ovary cells. J. Cell Biol. 97, 1777-1787.

Bernstein, H.D., Poritz, M.A., Strub, K., Hoben, P.J., Brenner, S., and Walter, P. (1989). Model for signal sequence recognition from amino-acid sequence of 54k subunit of signal recognition particle. Nature 340, 482-486.

Birnboim, H.C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7, 1513-1523.

Bole, D.G., Dowin, R., Doriaux, M., and Jamieson J.D. (1989). Immunocytochemical localization of BiP to the rough endoplasmic reticulum: evidence for protein sorting by selective retention. J. Histochem. Cytochem. 37, 1817-1823.

Bonatti, S., Migliaccio, G., and Simons, K. (1989). Palmitylation of viral membrane glycoproteins takes place after exit from the ER. J. Biol. Chem. 264, 12590-12595.

Bonifacino, J.S., Cosson, P., Shah, N., and Klausner, R.D. (1991). Role of potentially charged transmembrane residues in targeting proteins for retention and degradation within the endoplasmic reticulum. EMBO J. 10, 2783-2793.

Bonner, W.M., and Laskey, R.A. (1974). A film detection method for tritium-labelled proteins and nucleic polyacrylamide gels. Eur. J. Biochem. 46, 83-88.

Bos, T.J., Bohmam, D., Tsuchie, H., Tjian, R., and Vogt, P.K. (1988). V-Jun encodes a nuclear protein with enhancer binding properties of AP-1. Cell 52, 705-712.

Boyd, D., and Beckwith, J. (1990). The role of charged amino acids in the localization of secreted and membrane proteins. Cell 62, 1031-1033.

Braakman, I., Helenius, J., and Helenius, A. (1992). Role of ATP and disulphide bonds during protein folding in the endoplasmic reticulum. Nature 356, 260-262.

Bradford, M. (1976). A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Burke, J., Pettitt, J.M., Schachter, H., Sarkar, M., and Gleeson, P.A. (1992). The transmembrane and flanking sequences of β1,2-N-acetylglucosaminyltransferase I specify medial-golgi localization. J. Biol Chem. 267, 24433-24440.

Butcher, M., Raviprakash, K., and Ghosh, H.P. (1990). Acid pH-induced fusion of cells by herpes simplex virus glycoproteins gB and gD. J. Biol. Chem. 265, 5862-5868.

Bzik, D.J., Debroy, C., Fox, B.A., Pederson, N.E., and Person, S. (1986). The nucleotide sequence of the gB glycoprotein gene of HSV-2 and comparison with the corresponding gene of HSV-1. Virology 155, 322-333.

Bzik, D.J., Fox, B.A., DeLuca, N.A., and Person, S. (1984). Nucleotide sequence specifying the glycoprotein gene, gB, of herpes simplex virus type 1. Virology 133, 301-314.

Cai, W., Gu, B., and Person, S. (1988a). Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. J. Virol. 62, 2596-2604.

Cai, W., Person, S., Debroy, C., and Gu, B. (1988b). Functional regions and structural features of the gB glycoprotein of Herpes Simplex Virus Type 1. J. Mol. Biol. 201, 575-588.

Campadelli-Fiume, G., and Serafini-Cessi, F. (1985). Processing of the oligosaccharide chains of herpes simplex virus type 1 glycoproteins. In B. Roizman ed., The herpes virus, vol 3, pp 357-382, Plenum Press, New York and London.

Campadelli-Fiume, G., Farabegoli, F., Di Gaeta, S., and Roizman, B. (1991). Origin of unenveloped capsids in the cytoplasm of cells infected with herpes simplex virus 1. J. Virol. 65, 1589-1595.

Campadelli-Fiume, G., Lombardo, M.T., Foa-Tomasi, L., Avitabile, E., and Serafini-Cessi, F. (1988). Individual herpes simplex virus 1 glycoproteins display characteristic rates of maturation from precursor to mature form both in infected cells and in cells that constitutively express the glycoproteins. Virus Res. 10, 29-40.

Capone, J., Toneguzzo, F., and Ghosh, H.P. (1982). Synthesis and assembly of membrane glycoproteins. J. Biol. Chem. 257, 16-19.

Capone, J., Leblanc, P., Gerber, H.E., and Ghosh, H.P. (1983). Localization of membrane proteins by the use of a photoreactive fatty acid incorporated in vivo into vesicular stomatitis virus. J. Biol. Chem. 256, 1395-1398.

Castle, A.M., Stahl, L.E., and Castle, J.K. (1992). A 13-amino acid N-terminal domain of a basic proline rich protein is necessary for storage in secretory granules and facilitates exit from the endoplasmic reticulum. J. Biol. Chem. 267, 13093-13100.

Chamberlain, J.P. (1979). Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. Ana. Biochem. 98, 132-135.

Chanat, E., and Huttner, W.B. (1991). Milieu-induced, selective aggregation of regulated secretory proteins in the trans-golgi network. J. Cell Biol. 115, 1505-1519.

Chege, N.W., and Pfeffer, S.R. (1990). Compartmentation of the golgi complex: brefeldin-A distinguishes trans-golgi cisternae from the trans-golgi network. J. Cell Biol. 111, 893-899.

Chung, K.-N., Walter, P., Aponte, G.W., and Moore, H.-P.H. (1989). Molecular sorting in the secretory pathway. Science 243, 192-197.

Claesson-Welsh, L. and Spear, P.G. (1986). Oligomerization of herpes simplex virus glycoprotein B. J. Virol. 60, 803-806.

Claesson-Welsh, L., and Spear, P.G. (1987). Amino-terminal sequence, synthesis, and membrane insertion of glycoprotein B of herpes simplex virus type 1. J. Virol. 61, 1-7.

Clarke, S. (1992). Protein isoprenylation and methylation at carboxyl-terminal cysteine residues. Ann. Rev. Biochem. 61, 355-386.

Cluett, E.B., Wood, S.A., Banta, M., and Brown, W.J. (1993). Tubulation of golgi membranes in vivo and in vitro in the absence of brefeldin A. J. Cell Biol. 120, 15-24.

Cohen, S.N., and Chang, A.C.Y., and Hsu, L. (1972). Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of Escherichia coli by R-factor DNA. Proc. Natl. Acad. Sci. U.S.A. 69, 2110-2114.

Compton, T., and Courtney, R.J. (1984a). Virus-specific glycoproteins associated with the nuclear fraction of herpes simplex virus type 1-infected cells. J. Virol. 49, 594-597.

Compton, T., and Courtney, R.J. (1984b). Evidence for post-translational glycosylation of a nonglycosylated precursor protein of herpes simplex virus type 1. J. Virology 52, 630-637.

Colley, K.J., Lee, E.U., and Paulson, J.C. (1992). The signal anchor and stem regions of the β-galactoside α2,6-sialyltransferase may each act to localize the enzyme to the golgi apparatus. J. Biol. Chem. 267, 7784-7793.

Cooper, M.S., Cornell-Bell, A.H., Chernjavsky, A., Dani, J.W., and Smith, S.J. (1990). Tubulovesicular processes emerge from trans-golgi cisternae, extend along microtubules, and interlink adjacent trans-golgi elements into a reticulum. Cell 61, 135-145.

Cranage, N.P., Kouzarides, T., Bankier A.T., Satchwell, S., Weston K., Tomlinson P., Barrell, B., Hart H., Bell S.E., Minson, A.E., and Smith, G.L. (1986). Identification of the human cytomegalovirus glycoprotein B gene and induction of neutralizing antibodies via its expression in recombinant vaccinia virus. EMBO J. 5, 3057-3063.

Crise, B., Ruusala, A., Zagouras, P., Shaw, A., and Rose, J.K. (1989). Oligomerization

of glycolipid-anchored and soluble forms of the vesicular stomatitis virus glycoprotein. J. Virol. 63, 5328-5333.

Dahllöf, B., Wallin, M., and Kvist, S., (1991). The endoplasmic reticulum retention signal of the E3/19K protein of adenovirus-2 is microtubule binding. J. Biol. Chem. 266, 1804-1808.

Danscherg, G., and Nörgaard, R. (1983). Light microscopic visualization of colloidal gold on resin-embedded tissue. J. Histochem. Cytochem. 31, 1394-1398.

Darlington, R.W., and Moss, L.H. (1968). Herpesvirus envelopment. J. Virol. 2, 28-55.

Davis, L.I., and Blobel, G. (1986). Identification and characterization of a nuclear pore complex protein Cell 45, 699-709.

Davison, A.J. and Scott J.E. (1986). The complete DNA sequence of varicella-zoster virus. J. Gen. Virol. 67, 1759-1816.

Dawidowicz, E.A. (1987). Dynamics of membrane lipid metabolism and turnover. Ann. Rev. Biochem. 56, 43-61.

de Boer, A.D., and Weisbeek, P.J. (1991). Chloroplast protein topogenesis: import, sorting and assembly. Biochem. Biophys. Acta. 1071, 221-253.

Desai, P.J., Schaffer, P.A., and Minson, A.C. (1988). Excretion of non-infectious virus particles lacking glycoprotein H by a temperature-sensitive mutant of herpes simplex virus type 1: Evidence that gH is essential for virion infectivity. J. Gen. Virol. 69, 1147-1156.

De Silva, A., Braakman, I., and Helenius, A. (1993). Posttranslational folding of vesicular stomatitis virus G protein in the ER: involvement of noncovalent and covalent complexes. J. Cell Biol. 120, 647-655.

Dessev, G.N. (1992). Nuclear envelope structure. Curr. Opi. Cell Biol. 4, 430-435.

Devolt, M.R., Graham, F.L., Hanke, T., and Johnson, D.C. (1989). Protection of mice against lethal challenge with herpes simplex virus by vaccination with an adenovirus vector expressing HSV glycoprotein B. Virology 169, 244-247.

Dingwall, C., and Laskey, L. (1992). The nuclear membrane. Science 258, 942-947.

Doms, R.W., Keller, D.S., Helenius, A. and Balch, W.E. (1987). Role for adenosine triphosphate in regulating the assembly and transport of vesicular stomatitis virus G protein trimers. J Cell Biol. 105, 1957-1969.

Doms, R.W., Ruusala, A., Machamer, C., Helenius, J., Helenius, A., and Rose, J.K. (1988). Differential effects of mutations in three domains on folding, quaternary structure, and intracellular transport of vesicular stomatitis virus G protein. J. Cell Biol. 107, 89-99.

Donaldson, J.G., Finazzi, D., and Klausner, R.D. (1992). Brefeldin A inhibits golgi membrane-catalysed exchange of guanine nucleotide onto ARF protein. Nature 360, 350-352.

Donaldson, J.G., Lippincott-Schwartz, J., Bloom, G.S., Kreis, T.E., and Klausner, R.D. (1990). Dissociation of a 110-kD peripheral membrane protein from the golgi apparatus is an early event in brefeldin A action. J. Cell Biol. 111, 2295-2306.

Draper, K.G., Costa, R.H., Lee, G.T.-Y., Spear, P.G., and Wagner, E.K. (1984). Molecular basis of the glycoprotein-C-negative phenotype of herpes simplex virus type 1 macroplaque strain. J. Virol. 51, 578-585.

Dubois, M.F., Hovanessian, A.G., and Bensaude, O. (1991). Heat-shock-induced denaturation of proteins. J. Biol. Chem. 266, 9707-9711.

Duncan, J.R., and Kornfeld, S. (1988). Intracellular movement of two mannose 6-phosphate receptors: return to the golgi apparatus. J. Cell Biol. 106, 617-628.

Dwyer, N., and Blobel, G. (1976). A modified procedure for the isolation of a pore complex-lamina fraction from rat liver nuclei. J. Cell Biol. 70, 581-591.

Eberle, R., and Courtney, R.J. (1980a). Preparation and characterization of specific antisera to individual glycoprotein antigens comprising the major glycoprotein region of herpes simplex virus type 1. J. Virol. 35, 902-917.

Eberle, R., and Courtney, R.J. (1980b). gA and gB glycoproteins of herpes simplex virus type 1: two forms of a single polypeptide. J. Virol. 36, 665-675.

Emerson, S.U. (1985). Rhabdoviruses. In B.N. Field et al., eds, Virology, pp 119-132, Raven Press, New York.

Etchison, J.R., Roberstson, J.S., and Summers, D.F. (1977). Partial structural analysis

of the oligosaccharide moieties of the vesicular stomatitis virus glycoprotein by sequential chemical and enzymatic degradation. Virology 78, 375-392.

Farnsworth, C.C., Wolda, S.L., Gelb, M.H., and Glomset, J.A. (1989). Human lamin B contains a farnesylated cysteine residue. J. Biol. Chem. 264, 20422-20429.

Farquhar, M.G., and Palade, G.E. (1981). The golgi apparatus (complex)-(1954-1981)-from artifact to center stage. J. Cell Biol. 91, 77s-103s.

Fawcett, D.W. (1981). The Cell. Sec. Ed., W.B. Saunders company, Philadelphia.

Foisner, R., and Gerace, L. (1993). Integral membrane proteins of the nuclear envelope interact with the lamins and chromosomes, and binding is modulated by mitotic phosphorylation. Cell, 1267-1269.

Franke, W.W, Scheer, U., Krohne, G., and Jarasch, E.D. (1981). The nuclear envelope and the architecture of the nuclear periphery. J. Cell Biol. 91, 39s-50s.

Frink, R.J., Eisenberg, R., Cohen, G., and Wagner, E.K. (1983). Detailed analysis of the portion of the herpes simplex virus type 1 genome encoding glycoprotein C. J. Virol. 45, 634-647.

Frost, E., and Williams, J. (1978). Mapping temperature-sensitive and host-range mutations of adenovirus type 5 by marker rescue. Virology 91, 39-50.

Fujiki, Y., Hubbard, A.L., Fowler, S., and Lazarow, P.B. (1982). Isolation of intracellular membranes by means of sodium carbonate treatment: Application to endoplasmic reticulum. J. Cell Biol. 93, 97-102.

Fujiwara, T., Oda, K., Yokota, S., Takatsuki, A., and Ikehara, Y. (1988). Brefeldin A causes disassembly of the golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. J. Biol. Chem. 263, 18545-18552.

Fukuda, M. (1991). Lysosomal membrane glycoproteins. Structure, biosynthesis, and intracellular trafficking. J. Biol. Chem. 266, 21327-21330.

Gage, P.J., Levine, M., and Glorioso, J.C. (1993) Syncytium-inducing mutations localize to two discrete regions within the cytoplasmic domain of herpes simplex virus type I glycoprotein G. J. Virol. 64, 2191-2201.

Gerace, L., and Burke, B. (1988). Functional organization of the nuclear envelope. Ann.

Rev. Cell Biol. 4, 336-374.

Gerdes, H.-H., Rosa, P., Phillips, E., Baeuerle, P.A., Franke, R., Argos, P., and Huttner, W.B. (1989). The primary structure of human secretogranin II, a widespread tyrosin-sulfated secretory granule protein that exhibits low pH- and calcium-induced aggregation. J. Biol. Chem. 246, 12009-12015.

Gething, M.-J., and Sambrook, J. (1992). Protein folding in the cell. Nature 355, 33-38.

Gething, M.-J., McCammon, K., and Sambrook, J. (1986). Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. Cell 46, 939-950.

Ghosh, H.P. (1980). Synthesis and maturation of glycoproteins of enveloped animal viruses. Rev. Inf. Dis. 2, 26-39.

Ghosh-Choudhury, N. (1990). Biosynthesis, processing and intracellular transport of herpes simplex virus glycoproteins. Ph.D thesis, McMaster University, Hamilton, Canada.

Ghosh-Choudhury, N., Butcher, M., and Ghosh, H.P. (1990). Expression from cloned DNA of biologically active glycoprotein C of herpes simplex virus type 1 in mammalian cells. J. Gen. Virol. 71, 689-699.

Ghosh-Choudhury, N., Graham, A., and Ghosh, H.P. (1987). Herpes simplex virus type 2 glycoprotein biogenesis: effect of monensin on glycoprotein maturation, intracellular transport and virus infectivity. J. Gen. Virol. 68, 1939-1949.

Gibson, R., Schlesinger, S., and Kornfeld, S. (1979). The nonglycosylated glycoprotein of vesicular stomatitis virus is temperature-sensitive and undergoes intracellular aggregation at elevated temperatures. J. Biol. Chem. 254, 3600-3607.

Gilbert, R. (1988). Etude ultrastructurale et cytochimique du noyau de cellules végétales préparées a l'aide de méthodes cryogéniques. Mémoire de maîtrise, Université laval, Québec, Canada.

Glick, B.S., Beasley, E.M., and Schatz, G. (1992). Protein sorting in mitochondria. Trends Biochem. Sci. 17, 453-459.

Glomset, J.A., Gelb, M.H., and Farnsworth, C.C. (1990). Prenyl proteins in eukaryotic cells: a new type of membrane anchor. Trends Biochem. Sci. 15, 139-142.

Gluzman, Y. (1981). SV40-transformed simian cells support the replication of early SV40 mutants. Cell 23, 175-182.

Goldfarb, D.S. (1992). Are the cytosolic components of the nuclear, ER, and mitochondrial import apparatus functionally related? Cell 70, 185-188.

Goldman, A.E, Moir, R.D., Montag-Lowy M., Stewart, M., and Goldman, R.D. (1992). Pathway of incorporation of microinjected lamin A into the nuclear envelope. J. Cell Biol. 119, 725-735.

Gompels, H., and Minson, A. (1986). The properties and sequence of glycoprotein H of herpes simplex virus type I. Virology 153, 230-247.

Gong, M., and Kieff, E. (1990). Intracellular trafficking of two major Epstein-Barr virus glycoproteins, gp350/220 and gp110. J. Virol. 64, 1507-1516.

Gong, M.T., Ooka, T., Matsuo, T., and Kieff, E. (1987). Epstein-Barr virus glycoprotein homologous to herpes simplex virus gB. J. Virol. 61, 499-508.

Graeve, L., Garreis-Wabnitz, C., Zauke, M., Breindl, M., and Kruppa, J. (1986). The soluble glycoprotein of vesicular stomatitis virus is formed during or shortly after the translation process. J. Virol. 57, 968-975.

Graham, F.L., and Van Der EB, A.J. (1973). A new technique for the assay of infectivity of human adenovirus sDNA. Virology 52, 456-467.

Graham, W. (1990). Salvage receptors: two of a kind? Cell 62, 1-2.

Greber, U.F., Senior, A., and Gerace, L. (1990). A major glycoprotein of the nuclear pore complex is a membrane-spanning polypeptide with a large lumenal domain and a small cytoplasmic tail. EMBO J. 9, 1495-1502.

Griffin, A.M. (1991). The nucleotide sequence of the glycoprotein gB gene of infectious laryngotracheitis virus: analysis and evolutionary relationship to the homologous gene from other herpesviruses. J. Gen. Virol. 72, 393-398.

Grigera, P.R., Mathieu, M.E., and Wagner, R.R. (1991). Effect of glycosylation on the conformational epitopes of the glycprotein of vesicular stomatitis virus (New Jersey serotype). Virology 180, 1-9.

Guan, J.-L., Ruusala, A., Cao, H., and Rose, J.K. (1988). Effects of altered cytoplasmic

domains on transport of the vesicular stomatitis virus glycoprotein are transferable to other proteins. Mol. Cell. Biol. 8, 2869-2874.

Haeuple, M.-T., Flint, N., Gough, N.M., and Dobberstein, B. (1989). A tripartite structure of the signals that determine protein insertion into the endoplasmic reticulum membrane. J. Cell Biol. 108, 1227-1236.

Hallberg, E., Wozniak, R.W., and Blobel, G. (1993). An integral membrane protein of the pore membrane domain of the nuclear envelope contains a nucleoporin-like region. J. Cell Biol. 122, 513-521.

Hanahan, D. (1985). Techniques for transformation of E. coli. In D.M Glover ed., DNA cloning a practical approach, vol I, pp 109-135, IRL Press, Oxford, Washington DC.

Harding, C.V., Collins, D.S., Slot, J.W., Geuze, H.J., and Unanue, E.R. (1991). Liposome-eccapsulated antigens are processed in lysosomes, recycled, and presented to T cells. Cell 64, 393-401.

Harlow, E.D., and Lane, D. (1988). Antibodies, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Hawkes, R. (1982). Identification of concanavalin A-binding proteins after sodium dodecyl sulfate-gel electrophoresis and protein blotting. Anal. Biochem. 123, 143-146.

Hayot, M.A. (1981). Principles and techniques of electron microscopy. Sec. Edi. University Park Press, Baltimore

Heine, J.W., Spear, P.G., and Roizman, B. (1972). Proteins specified by herpes simplex virus. VI. Viral proteins in the plasma membrane. J. Virol. 9, 431-439.

Helms, J.B., and Rothman, J.E. (1992). Inhibition by brefeldin A of a golgi membrane enzyme that catalyses exchange of guanine nucleotide bound to ARF. Nature 360, 352-354.

High, S., and Dobberstein, B. (1991). The signal sequence interacts with the methionine-rich domain of the 54-kD protein of signal recognition particle. J. Cell Biol. 113, 229-233.

Highlander, S.L., Goins, W.F., Person, S., Holland, T.C., Levine, M., and Glorioso, J.C. (1991). Oligomer formation of the gB glycoprotein of herpes simplex virus type 1. J. Virol. 65, 4275-4283.

Hinshaw, J.E., Carragher, B.O., and Milligan, R.A. (1992). Architecture and design of the nuclear pore complex. Cell 69, 1133-1141.

Hobman, T.C., Woodward, L., and Farquhar, M.G. (1992). The rubella virus E1 glycoprotein is arrested in a novel post-ER, pre-golgi compartment. J. Cell Biol. 118, 795-811.

Hochstenbach, F., David, V., Watkins, S., and Brenner, M.B. (1992). Endoplasmic reticulum resident protein of 90 kilodaltons associates with the T- and B-cell antigen receptors and major histocompatibility complex antigens during their assembly. Proc. Natl. Acad. Sci. U.S.A. 89, 4734-4738.

Holmes, D.S., and Quigley, M. (1981). A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. 114, 193-197.

Holtz, D., Tanaka, R.A., Hartwig, J., and McKeon, F. (1989). The CaaX motif of lamin A functions in conjunction with the nuclear localization signal to target assembly to the nuclear envelope. Cell 59, 969-977.

Hong, W., and Doyle, D. (1990). Molecular dissection of the NH_2-terminal signal/anchor sequence of rat dipeptidyl peptidase IV. J. Cell Biol. 111, 323-328.

Huang, C.C., and Aminoff, D. (1972). Enzymes that destroy blood group specificity. V. The oligosaccharidase of Clostridium perfringens. J. Biol. Chem. 247, 6737-6742.

Huovila, A.-P.J., Eder, A.M., and Fuller, S.D. (1992). Hepatitis B surface antigen assembles in a post-ER, pre-golgi compartment. J. Cell Biol. 118, 1305-1320.

Hurt, E.C. (1990). Targeting of a cytosolic protein to the nuclear periphery. J. Cell Biol. 111, 2829-2837.

Hurtley, S.M., and Helenius, A. (1989). Protein oligomerization in the endoplasmic reticulum. Ann. Rev. Cell Biol. 5, 277-307.

Hurtley, S.M., Bole, D. G., Hoover-Litty, H., Helenius, A., and Copeland, C.S. (1989). Interactions of misfolded influenza virus hemagglutinin with binding protein (BiP). J. Cell Biol. 108, 2117-2126.

Hutchinson, L., Browne, H., Wargent, V., Davis-Poynter, N., Primorac, S., Goldsmith, K., Minson, A.C., and Johnson, D.C. (1992a). A novel herpes simplex virus glycoprotein, gL, forms a complex with glycoprotein H (gH) and affects normal folding

and surface expression of gH. J. Virol. 66, 2240-2250.

Hutchinson, L., Goldsmith, K., Snoddy, D., Ghosh, H., Graham, F.L., and Johnson, D.C. (1992b). Identification and characterization of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion. J. Virol. 66, 5603-5609.

Irving, R.A., and Ghosh, H.P. (1982). Shedding of vesicular stomatitis virus soluble glycoprotein by removal of carboxy-terminal peptide. J. Virol. 42, 322-325.

Irving, R.A., Toneguzzo, F., Rhee, S.H., Hofmann, T., Ghosh, H.P. (1979). Synthesis and assembly of membrane glycoproteins: presence of leader peptide in nonglycosylated precursor of membrane glycoprotein of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. 76, 570-574.

Ish-Horowicz, D., and Burke, J.F. (1981). Rapid and efficient cosmid cloning. Nucl. Acids Res. 9, 2989-2998.

Jackson, M.R., Nilsson, T., and Peterson, P.A. (1990). Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. EMBO J. 9, 3153-3162.

Jarnik, M., and Aebi, U. (1991). Toward a more complete 3-D structure of the nuclear pore complex. J. Struc. Biol. 107, 291-308.

Jennings, M.A. (1989). Topography of membrane proteins. Ann. Rev. Biochem. 58, 999-1027.

Johnson, D.C., and Smiley, J.R. (1985). Intracellular transport of herpes simplex virus gD occurs more rapidly in uninfected cells than in infected cells. J. Virol. 54, 682-689.

Johnson, D.C., and Spear, P.G. (1982). Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. J. Virol. 43, 1102-1112.

Johnson, D.C., and Spear, P.G. (1983). O-linked oligosaccharides are acquired by herpes simplex virus glycoproteins in the golgi apparatus. Cell 32, 987-997.

Johnson, D.C., Frame, M.C., Ligas, M.W., Cross, A.M., and Stow, N.D. (1988a). Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. J. Virol. 62, 1347-1354.

Johnson, D.C., Ghosh-Choudhury, G., Smiley, J.R., Fallis, L., and Graham, F.L. (1988b). Abundant expression of herpes simplex virus glycoprotein gB using an adenovirus vector. Virology 164, 1-14.

Johnson, K.F., and Kornfeld, S. (1992). The cytoplasmic tail of the mannose 6-phosphate/insulin-like growth factor-II receptor has two signals for lysosomal enzymes sorting in the golgi. J. Cell Biol. 119, 249-257.

Kabcenell, A.K., and Atkinson, P.A. (1985). Processing of the rough endoplasmic reticulum membrane glycoproteins of rotavirus SA11. J. Cell Biol. 101, 1270-1280.

Kaiser, C.A., and Botstein, D. (1990). Efficiency and diversity of protein localization by random signal sequences. Mol. Cell. Biol. 8, 1915-1922.

Kaiser, C.A., Preuss, D., Grisafi, P., and Botstein, D. (1987). Many random sequences functionally replace the secretion signal sequence of yeast invertase. Science 235, 321-317.

Kang, C.Y., and Prevec, L. (1970). Proteins of vesicular stomatitis virus. II. Immunological comparaisons of viral antigens. J. Virol. 6, 20-27.

Karrenbauer, A., Jeckel, D., Just, W., Birk, R., Schmidt, R.R., Rothmam, J.E., and Wieland, F.T. (1990). The rate of bulk flow from the golgi to the plasma membrane. Cell 63, 259-267.

Kaufman, R.J. (1985). Identification of the components necessary for adenovirus translational control and their utilization in cDNA expression vectors. Proc. Natl. Acad. Sci. U.S.A. 82, 689-693.

Kaufman, R.J. (1990). Vectors used for the expression in mammalian cells. Meth. Enz. 185, 487-511.

Kaufman, R.J., and Murtha, P. (1987). Translational control mediated by eucaryotic initiation factor-2 is restricted to specific mRNAs in transfected cells. Mol. Cell Biol. 7, 1568-1571.

Kelley, J.M., Emerson, S.U., and Wagner, R.R. (1972). The glycoprotein of vesicular stomatitis virus is the antigen that gives rise to and reacts with neutralizing antibody. J. Virol. 10, 1231-1235.

Kim, P.S., Bole, D., and Arvan, P. (1992). Transient aggregation of nascent

thyroglobulin in the endoplasmic reticulum: relationship to the molecular chaperone, BiP. J. Cell Biol. 118, 541-549.

Klausner, R.D., and Sitia, R. (1990). Protein degradation in the endoplasmic reticulum. Cell 62, 611-614.

Kitten, G.T., and Nigg, E.A. (1991). The CaaX motif is required for isoprenylation, carboxyl methylation, and nuclear membrane association of lamin B_2. J. Cell Biol. 113, 13-23.

Klausner, R.D., Donaldson, J.G., and Lippincott-Schwartz, J. (1992). Brefeldin A: insights into the control of membrane traffic and organelle structure. J. Cell Biol. 116, 1071-1080.

Knipe, D., Baltimore, D., and Lodish, H. (1977). Separate pathways of maturation of the major structural proteins of vesicular stomatitis virus glycoprotein. J. Virol. 21, 1128-1139.

Knittler, M.R., and Haas, I.G. (1992). Interaction of BiP with newly synthesized immuoglobulin light chain molecules: cycles of sequential binding and release. EMBO J. 11, 1573-1581.

Koga, J., Chatterjee, S., and Whitley, R.J. (1986). Studies on herpes simplex virus type 1 glycoproteins using monoclonal antibodies. Virology 151, 385-389.

Kornfeld, R., and Kornfeld, S. (1985). Assembly of asparagine-linked oligosaccharides. Ann. Rev. Biochem. 54, 631-664.

Kotwal, G.J, and Ghosh, H.P. (1984). Role of fatty acid acylation of membrane glycoproteins. J. Biol. Chem. 259, 4699-4701.

Kreis, T.E, and Lodish, H.F. (1986). Oligomerization is essential for transport of vesicular stomatitis viral glycoprotein to the cell surface. Cell 46, 929-937.

Krohne, G., Waizenegger, I., and Höger, T.H. (1989). The conserved carboxy-terminal cysteine of nuclear lamins is essential for lamin association with the nuclear envelope. J. Cell Biol. 109, 2003-2011.

Kundu, A., Jabbar, M.A., and Natak, D. (1991). Cell surface transport, oligomerization, and endocytosis of chimeric type II glycoproteins: role of cytoplasmic and anchor domains. Mol. Cell. Biol. 11, 2675-2685.

Kunkel, T.A., Roberts, J.D., and Zakour, R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. Meth. Enz. 154, 367-382.

Kuroiwa, T., Sakaguchi, M., Mihara, K., and Omura, T. (1991). Systematic analysis of stop-transfer sequence for microsomal membrane. J. Biol. Chem. 266, 9251-9255.

Kyte, J., and Doolittle, R.F. (1982). A simple method for displaying the hydrophathic character of a protein. J. Mol. Biol. 157, 105-120.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lah, J.J., Hayes, D.M., Burry, R.W. (1990). A neutral pH silver development method for the visualization of 1-nanometer gold particles in pre-embedding electron microscopy immunocytochemistry. J. Histochem. Cytochem. 38, 503-508.

Lanini, L., Bachs, O., and Carafoli, E. (1992). The calcium pump of the liver nuclear membrane is identical to that of endoplasmic reticulum. J. Biol. Chem. 267, 11548-11552.

Laskey, R.A., and Mills, A.D. (1975). Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. 56, 335-341.

Lazarovits, J., Shia, S.-P., Ktistakis, N., Lee, M.-S., Bird, C., and Roth, M.G. (1990). The effects of foreign transmembrane domains on the biosynthesis of the influenza virus hemagglutinin. J. Biol. Chem. 264, 4760-4767.

Lee, C., and Chen, L.B. (1988). Behavior of endoplasmic reticulum in living cells. Cell 54, 37-36.

Letourneur, F., and Klausner, R.D. (1992). A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains. Cell 69, 1143-1157.

Lewis, M.J., and Pelham, H.R.B. (1992). Ligand-induced redistribution of a human KDEL receptor from the golgi complex to the endoplasmic reticulum. Cell 68, 353-364.

Lingappa, V.R. (1991). More than just a channel: provovative new features of protein traffic across the ER membrane. Cell 65, 527-530.

Lipp, J., and Dobberstein, B. (1986). The membrane spanning segment of invariant

chain (Iγ) contains a potentially cleavable signal sequence. Cell. 46, 1103-1112.

Lippincott-Schwartz, J., Donaldson, J.G., Schweizer, A., Berger, E.G., Hauri, H.-P., Yuan, L.C., and Klausner, R.D. (1990). Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. Cell 60, 821-836.

Lippincott-Schwartz, J., Yuan, L.C., Bonifacino, J.S., and Klausner, R.D. (1989). Rapid redistribution of golgi proteins into the ER in the cells treated with brefeldin A: evidence for membrane cycling form the golgi to ER. Cell 56, 801-813.

Lippincott-Schwartz, J., Yuan, L., Tipper, C., Amherdt, M., Orci, L., and Klausner, R.D. (1991). Brefeldin A's effects on endosomes, lysosomes, and the TGN suggest a general mechanism for regulating organelle structure and membrane traffic. Cell 67, 601-616.

Little, S.L., and Huang, A.S. (1978). Shedding of the glycoprotein from vesicular stomatitis virus-infected cells. J. Virol. 27, 330-339.

Locker, J.K., Rose, J.K., Horzinek, M.C., and Rottier, P.J.M. (1992). Membrane assembly of the triple-spanning coronavirus M protein. J. Biol. Chem. 267, 21911-21918.

Lodish, H.F. (1988). Transport of secretory and membrane glycoproteins from the rough endoplasmic reticulum to the golgi. J. Biol. Chem. 263, 2107-2110.

Logan, J., and Shenk, T. (1984). Adenovirus tripartite leader sequence enhances translation of mRNAs late after infection. Proc. Natl. Acad. Sci. U.S.A. 81, 3655-3659.

Longnecker, R., Chatterjee, S., Whitley, R.J., and Roizman, B. (1987). Identification of a herpes simplex virus 1 glycoprotein gene within a gene cluster dispensable for growth in cell culture. Proc. Nat. Acad. Sci. U.S.A. 84, 4303-4307.

Lopez-Iglesias, C., and Puvion-Dutilleul, F. (1988). Visualization of glycoproteins after tunicamycin and monensin treatment of herpes simplex virus infected cells. J. Ultrastruc. Mol. Struc. Res. 191, 75-91.

Lotti, L.V., Torrisi, M.-R., Pascale, M.C., and Bonatti, S. (1992). Immunocytochemical analysis of the transfer of vesicular stomatitis virus G glycoprotein from the intermediate compartment to the golgi complex. J. Cell Biol. 118, 43-50.

Machamer, C.E. (1991). Golgi retention signals: do membranes hold the key. Trends Cell Biol. 1, 141-144.

Machamer, C.E., and Rose, J.K. (1987). A specific transmembrnae domain of a coronavirus E1 glycoprotein is required for its retention in the Golgi region. J. Cell Biol. 105, 1205-1214.

Machamer, C.E., Doms, R.W., Bole, D.G., Helenius, A., and Rose, J.K. (1990a). Heavy chain binding protein recognized incompletely disulfide-bonded forms of vesicular stomatitis virus G protein. J. Biol. Chem. 265, 6879-6883.

Machamer, C.E., Mentone, S.A., Rose, J.K., and Farquhar, M.G. (1990b). The E1 glycoprotein of an avian coronavirus is targeted to the cis Golgi complex. Proc. Natl. Acad. Sci. USA. 87, 6944-6948.

Mack, D., and Kruppa, J. (1988). Fatty acid acylation at the single cysteine residue in the cytoplasmic domain of the glycoprotein of vesicular-stomatitis virus. Biochem. J. 256, 1021-1027.

Mallabiabarrena, A., Fresno, M., and Alarcon, B. (1992). An endoplasmic reticulum retention signal in the CD3e chain of the T-cell receptor. Nature 357, 593-596.

Manservigi, R., Spear, P.G., and Bucham, A. (1977). Cell fusion induced by herpes simplex virus is promoted and suppressed by different viral glycoporteins. Proc. Nat. Acad. Sci. U.S.A. 74, 3913-3917.

Marquardt, T., and Helenius, A. (1992). Misfolding and aggregation of newly synthesized proteins in the endoplasmic reticulum. J. Cell Biol. 117, 505-513.

Martin, R.G., and Ames, B.N. (1961). A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. 236, 1372-1379.

Mathews, P.M., Martinie, J.B., and Fambrough, D.M. (1992). The pathway and targeting signal for delivery of the integral membrane glycoprotein LEP100 to lysosomes. J. Cell Biol. 118, 1027-1040.

McDermott, M.R., Graham, F.L., Hanke, T., and Johnson, D.C. (1989). Protection of mice against lethal challenge with herpes simplex virus by vaccination with an adenovirus vector expressing HSV glycoprotein B. Virology 169, 244-247.

McGeoch, D.J., Dalrymple, M.A., Davison, A.J., Dolan, A., Frame, M.C., McNab,

D., Perry, L.J., Scott, J.E., and Taylor, P. (1988). The complete DNA sequence of the long unique region in the genome of herpes simplex virus type I. J. Gen. Virol. 69, 1531-1574.

McGovern, K., Ehrmann, M., and Beckwith, J. (1991). Decoding signals for membrane protein assembly using alkaline phosphatase fusions. EMBO J. 10, 2773-2782.

Mellman, I., and Simons, K. (1992). The golgi complex: in vitro veritas? Cell 68, 829-840.

Melnick, J., Aviel, S., and Argon, Y. (1992). The endoplasmic reticulum stress protein GRP94, in addition to BiP, associates with unassembled immunoglobulin chains. J. Biol. Chem. 267, 21303-21306.

Miller, S.G., Carnell, L., and Moore, H.-P. (1992). Post-golgi membrane traffic: brefeldin A inhibits export form distal golgi compartments to the cell surface but not recycling. J. Cell Biol. 118, 267-283.

Morgan, C., Rose, H.M., Holden, M., and Jones, E.P. (1959). Electron microscopic observation on the development of herpes simplex virus. J. Exp. Med. 110, 643-656.

Munro, S. (1991) Sequences within and adjacent to the transmembrane segment of α-2,6-sialytransferase specify Golgi retention. EMBO J. 12, 3577-3588.

Munro, S. and Pelham, H.R.B. (1987). A C-terminal signal prevent secretion of luminal ER proteins. Cell 48, 899-907.

Myers, E.W., and Miller, W. (1988). Optimal alignments in linear space. Comput. Appli. Biosci. 4, 11-7.

Navarro, D., Paz, P., and Pereira, L. (1992). Domains of herpes simplex virus 1 glycoprotein B that function in virus penetration, cell-to-cell spread, and cell fusion. Virology 186, 99-112.

Navarro, D., Qadri, I., and Pereira, L. (1991). A mutation in the ectodomain of herpes simplex virus 1 glycoprotein B causes defective processing and retention in the endoplasmic reticulum. Virology, 184, 253-264.

Navarro, D., Qadri, I., and Pereira, L. (1993). Transport and secretion of truncated derivatives of herpes simplex virus 1 glycoprotein B. Virology 192, 234-245.

Nigg, E.A. (1992). Assembly-disassembly of the nuclear lamina. Curr. Opi. Cell Biol. 4, 105-109.

Nii, S., Morgan, C., and Rose, H. M. (1968). Electron microscopy of Herpes simplex virus II. sequence of development. J. Virol. 2, 517-536.

Nilsson, T., Jackson, M., and Peterson, P.A. (1989). Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. Cell 58, 707-718.

Nilsson, T., Lucocq, J.M., Mackay, D., and Warren, G. (1991). The membrane spanning domain of the β-1,4-galactosyltransferase specifies trans golgi localization. EMBO J. 10, 3567-3575.

Oloffson, S., Blomberg, J., and Lycke, E. (1981). O-glycosidic carbohydrate-peptide linkages of herpes simplex virus glycoproteins. Arch. Virol. 70, 321-329.

Orci, L., Tagaya, M., Amherdt, M., Perrelet, A., Donaldson, J.G., Lippincott-Schwartz, J., Klausner, R.D., and Rothman, J.E. (1991). Brefeldin A, a drug that blocks secretion, prevents the assembly of non-clatrin-coated buds on golgi cisternae. Cell 64, 1183-1195.

Pachl, C., Burke, R.L., Stuve, L.L., Sanchez-Pescador, L., Van Nest, G., Masiarz, F., and Dina, D. (1987). Expression of cell-associated and secreted forms of herpes simplex virus type 1 glycoprotein gB in mammalian cells. J. Virol. 61, 315-325.

Padan, R., Nainudel-Epszteyn, S., Goitein, R., Fainsod, A., and Gruenbaum Y. (1990). Isolation and characterization of the Drosophila nuclear envelope otefin cDNA. J. Biol. Chem. 265, 7808-7813.

Para, M.F., Baucke, R.B., and Spear, P.G. (1982). Glycoprotein gE of herpes simplex virus type 1: effects of anti-gE on virion infectivity and on virus-induced Fc-binding receptors. J. Virol. 41, 129-136.

Parks, G.D., and Lamb, R.A. (1991). Topology of eukaryotic type II membrane proteins: importance of N-terminal positively charged residues flanking the hydrophobic domain. Cell 64, 777-787.

Pavan, A., Lotti, L.V., Torrisi, M.R., Migliaccio, G., and Bonatti, S. (1987). Regional distribution of Sindbis virus glycoproteins on the plasma membrane of infected baby hamster kidney cells. Exp. Cell Res. 168, 53-62.

Pearse, B.M.F., and Robinson, M.S. (1990). Clathrin, adaptors, and sorting. Ann. Rev. Cell Biol. 6, 151-171.

Pelham, H.R.B. (1987). Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. EMBO J. 7, 913-818.

Pelham, H.R.B. (1989). Control of protein exit from the endoplasmic reticulum. Ann. Rev. Cell Biol. 5, 1-23.

Pelham, H.R.B. (1990). The retention signal for luminal ER proteins. Trends Biochem. Sci. 15, 483-486.

Pelham, H.R.B. (1991). Multiple targets for brefeldin A. Cell 67, 449-451.

Pellet, P.E., Kousoules, K.G., Pereira, L., and Roizman, B. (1985). Anatomy of the herpes simplex virus 1 strain f glycoprotein B gene: primary sequence and predicted protein structure of the wild-type and of monoclonal antibody-resistant mutants. J. Virol. 53, 243-253.

Perez-Vilas, J., Hidalgo, J., and Velasco, A. (1991). Presence of terminal N-acetylgalactosamine residues in subregions of the endoplasmic reticulum is influenced by cell differentiation in culture. J. Biol. Chem. 266, 23967-23976.

Persson, R., and Pettersson, R.F. (1991). Formation and intracellular transport of a heterodimeric viral spike protein complex. J. Cell Biol. 112, 257-266.

Pfanner, N., Rassow, J., van der Klei, I.J., and Neupert W. (1992). A dynamic model of the mitochondrial protein import machinery. Cell 68, 999-1002.

Pfeffer, S.R., and Rothman, J.E. (1987). Biosynthetic protein transport and sorting by the endoplasmic reticulum and golgi. Ann. Rev. Biochem. 56, 829-852.

Pitta, A.M., Rose, J.K., and Machamer, C.E. (1989). A single-amino-acid substitution eliminates the stringent carbohydrate requirement for intracellular transport of a viral glycoprotein. J. Virol. 63, 3801-3809.

Poliquin, L., Levine, G., and Shore, G.C. (1985). Involvement of the golgi apparatus and a restructured nuclear envelope during biogenesis and transport of herpes simplex virus glycoproteins. J. Histochem. Cytochem. 33, 875-883.

Poruchynsky, M.S., and Atkinson, P.H. (1988). Primary sequence domains required for

the retention of rotavirus VP7 in the endoplasmic reticulum. J. Cell Biol. 107, 1697-1706.

Powell, L., and Burke, B. (1990). Internuclear exchange of an inner nuclear membrane protein (p55) in heterokaryons: in vivo evidence for the interaction of p55 with the nuclear lamina. J. Cell Biol. 111, 2225-2234.

Pryer, N.K., Wuestehube, L.J., and Schekman, R. (1992). Vesicle-mediated protein sorting. Ann. Rev. Biochem. 61, 471-516.

Puddington, L., Lively, M.O., and Lyles, D.S. (1985). Role of the nuclear envelope in synthesis, processing, and transport of membrane glycoproteins. J. Biol. Chem. 260, 5641-5647.

Qadri, I., Gimeno, C., Navarro, D., and Pereira, L. (1991). Mutations in conformation-dependent domains of herpes simplex virus 1 glycoprotein B affect the antigenic properties, dimerization, and transport of the molecule. Virology 180, 135-152.

Radsak, K., Brücher, K.H., Britt, W., Shiou, H., Schneider, D., and Kollert, A. (1990). Nuclear compartmentation of glycoprotein B of human cytomegalovirus. Virology 177, 515-522.

Radu, A., Blobel, G., and Wozniak, R. (1993). Nup155 is a novel nuclear pore complex protein that contains neither repetitive sequence motif nor reacts with WGA. J. Cell Biol. 121, 1-9.

Rambourg, A., and Clermont, Y. (1990). Three-dimensional electron microscopy: structure of the golgi apparatus. Eur. J. Cell Biol. 51, 189-200.

Rapoport, T.A. (1992). Transport of proteins across the endoplasmic reticulum membrane. Science 258, 931-935.

Rasile, L., Ghosh, K., Raviprakash, K., and Ghosh, H.P. (1993). Effects of deletions in carboxy-terminal hydrophobic region of herpes simplex virus glycoprotein gB on intracellular transport and membrane anchoring. J. Virol. 67, 4856-4866.

Raviprakash, K., Rasile, L., Ghosh, K., and Ghosh, H.P. (1990). Shortened cytoplasmic domain affects intracellular transport but not nuclear localization of a viral glycoprotein. J. Biol Chem. 265, 1777-1782.

Reading, C.L., Penhoet, E.E, and Ballou, C.E. (1978). Carbohydrate structure of

vesicular stomatitis virus glycoprotein. J. Biol. Chem. 253, 5600-5612.

Reaves, B., and Banting, G. (1992). Perturbation of the morphology of the trans-golgi network following brefeldin A treatment: redistribution of a TGN-specific integral membrane protein, TGN38. J. Cell Biol. 116, 85-94.

Reichelt, R., Holzenburg, A., Buhle, E.L., Jarnick, M., Engel, A., and Aebi, U. (1990). Correlation between structure and mass distribution of the nuclear pore complex and of distinct pore complex components. J. Cell Biol. 110, 883-894.

Richman, D.D., Buckmaster, A., Bell, S., Hodgman, C., and Minson, A.C. (1986). Identification of a new glycoprotein of herpes simplex virus type I and genetic mapping of the gene that codes for it. J. Virol. 57, 647-655.

Rizzolo, L.J., Finidori, J., Gonzalez, A., Arpin, M., Ivanov, I.E., Adesnik, M., and Sabatini, D.D. (1985). Biosynthesis and intracellular sorting of growth hormone-viral envelope glycoprotein hybrids. J. Cell Biol. 101, 1351-1362.

Roizman, B. (1991). Herpesviridae: a brief introduction. In B.N. Fields, D.M. Knipe et al. (eds), Fundamemtal Virology, 2nd ed., pp 841-846. Raven Press, New York.

Roizman, B., and Furlong, D. (1974). The replication of herpesviruses, In H. Fraenkel-Conrat and R.R. Wagner eds, Comprehensive Virology, Vol. 3., pp. 229-403, Plenum Press, New York.

Roizman, B., and Sears, A.E. (1991). Herpes simplex viruses and their replication. In B.N. Fields, D.M. Knipe et al. eds, Fundamental Virology, 2nd ed., pp. 849-895, Raven Pres, New York.

Roop C., Hutchinson L.. and Johnson D.C. (1993). A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells, and its particles lack glycoprotein H. J. Virol. 67, 2285-2297.

Rose, J.K., and Bergmann, J.E. (1982). Expression from cloned cDNA of cell-surface secreted forms of the glycoprotein of vesicular stomatitis virus in eucaryotic cells. Cell 30, 753-762.

Rose, J.K., and Bergmann, J.E. (1983). Altered cytoplasmic domains affect intracellular transport of the vesicular stomatitis virus glycoprotein. Cell 34, 513-534.

Rose, J.K., and Doms, R.W. (1988). Regulation of protein export from the endoplasmic

reticulum. Ann. Rev. Cell Biol. 4, 257-288.

Rose, J.K., and Gallione, C.J. (1981). Nucleotides sequences of the mRNA'encoding the vesicular stomatitis virus G and M proteins determined from cDNA clones containing the complete coding regions. J. Virol. 39, 519-528.

Rose, J.K., Adams, G.A., and Gallione, C.J. (1984). The presence of cysteine in the cytoplasmic domain of the vesicular stomatitis virus glycoprotein is required for palmitate addition. Proc. Natl. Acad. Sci. U.S.A. 81, 2050-2054.

Ross, L.J.N., Sanderson M., Scott S.D., Binns, M.M., Doel, T. and Milne, B. (1989). Nucleotide sequence and characterization of the Marek's disease virus homologue of glycoprotein B of herpes simplex virus. J. Gen. Virol. 70, 1789-1804.

Roth, J. (1987). Subcellular organization of glycosylation in mammalian cells. Biochem. Biophy. Act. 906, 405-436.

Roth, J., Bendayan, M., Carlemalm, E., Villiger, W., and Garavito, M. (1981). Enhancement of structural preservation and immunocytochemical staining in low temperature embedded pancreatic tissue. J. Histochem. Cytochem. 29, 663-671.

Roth, M.G., Doyle, C., Sambrook, J., and Gething, M.-J. (1986). Heterologous transmembrane and cytoplasmic domains direct functional chimeric influenza virus hemagglutinins into the endocytic pathway. J. Cell Biol. 102, 1271-1283.

Rothman, J.E, and Orci, L. (1992). Molecular dissection of the secretory pathway. Nature 355, 409-415.

Russo, R.N., Shaper, N.L., Taatjes, D.J., and Shaper, J.H. (1992). β1,4-Galactosyltransferase: A short NH_2-terminal fragment that includes the cytoplasmic and transmembrane domain is sufficient for golgi retention. J. Biol. Chem. 267, 9241-9247.

Sakaguchi, M., Tomiyoshi, R., Kuroiwa, T., Mihara, K., and Omura, T. (1992). Functions of signal and signal-anchor sequences are determined by the balance between the hydrophobic segment and the N-terminal charge. Proc. Natl. Acad. Sci. U.S.A. 89, 16-19.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning. A Laboratory Manual (2nd edition). Cold Spring Harbor Laboratory Press, Cold Spring Habor, NY.

Sanders, S.L., and Schekman, R. (1992). Polypeptide translocation across the

endoplasmic reticulum membrane. J. Biol. Chem. 267, 13791-13794.

Sanger, F., Niklen, S., and Coulson, A.R. (1977). DNA sequencing with the chain-terminating inhibitors. Proc. Nat. Acad. Sci. U.S.A. 74, 5463-5467.

Saraste, J., and Kuismanen, E. (1984). Pre- and post-golgi vacuoles operate in the transport of semliki forest virus membrane glycoproteins to the cell surface. Cell 38, 535-549.

Saraste, A., and Svensson, K. (1991). Distribution of the intermediate elements operating in ER to Golgi transport. J. Cell Sci. 100, 415-430.

Sarma, V.R., Davies, D.R., Labaw, L.W., Silverton, E.W., and Terri, W.D. (1971). Crystal structure of an immunoglobulin molecule by X-ray diffraction and electron microscopy. Cold Spr. Harb. Symp. Quant. Biol. XXXVI, 413-419.

Sarmiento, M., and Spear, P.G. (1979). Membrane proteins specified by herpes simplex viruses IV. Conformation of the virion glycoprotein designated VP7 (B_2). J. Virol. 29, 1159-1167.

Sarmiento, M., Haffey, M., and Spear, P.G. (1979). Membrane proteins specified by herpes simplex viruses III. Role of glycoprotein VP7(B2) in virion infectivity. J. Virol. 29, 1149-1158.

Sato, T., Sakaguchi, M., Mihara, K., and Omura, T. (1990). The amino-terminal structures that determine topological orientation of cytochrome p-450 in microsomal membrane. EMBO J. 9, 2391-2397.

Schmidt, C., Grunberg, J., and Kruppa, J. (1992). Formation of heterotrimers between the membrane-integrated and the soluble glycoproteins of vesicular stomatitis virus leads to their intracellular cotransport. J. Virol. 66, 2792-2797.

Schmidt, M.F., and Schlesinger, M.J. (1979). Fatty acid binding of vesicular stomatitis virus glycoprotein: a new type of post-translational modification of the viral glycoprotein. Cell 17, 813-819.

Schneider, M., Graham, F.L. and Prevec, L. (1989). Expression of the glycoprotein of vesicular stomatitis virus by infectious adenovirus vectors. J. Gen. Virol. 70, 417-427.

Schneider, R.J., Weinberger, C., and Shenk, T. (1984). Adenovirus VAI RNA facilitates the initiation of translation in virus-infected cells. Cell 37, 291-298.

Schnitzer, J.E., and Bravo, J. (1993). High affinity binding, endocytosis, and degradation of conformationally modified albumins. J. Cell Biol. 268, 7562-7570.

Schweizer, A., Matter, K., Ketcham, C.M., and Hauri, H.-P. (1991). The isolated ER-golgi intermediated compartment exhibits properties that are different from the ER and cis-golgi. J. Cell Biol. 113, 45-54.

Senior, A., and Gerace, L. (1988). Integral membrane proteins specific to the inner muclear membrane and associated with the nuclear lamina. J. Cell Biol. 107, 2029-2036.

Shimizu, H., Ishida-Yamamoto, A., and Eady, R.A.J. (1992). The use of silver-enhanced 1-nm gold probes for light and electron microscopic localization of intra- and extracellular antigens in skin. J. Histochem. Cytochem. 40, 883-888.

Shin, J., Dunbrack, R.L., Lee, S., and Strominger, J.K. (1991). Signals for retention of transmembrane proteins in the endoplasmic reticulum studied with CD4 truncation mutants. Proc. Natl. Acad. Sci. U.S.A. 88, 1918-1922.

Silver, P.A. (1991). How proteins enter the nucleus. Cell 64, 489-497.

Simon, S.M., and Blobel, G. (1991). A protein-conducting channel in the endoplasmic reticulum. Cell 65, 371-380.

Simons, K., and Griffiths, G. (1986). The trans golgi network: sorting at the exit site of the golgi complex. Science 234, 438-443.

Simos, G., and Georgatos, S.D. (1992). The inner nuclear membrane protein p58 associates in vivo with a p58 kinase and the nuclear lamins. EMBO J. 11, 4027-4036.

Singer, S.J. (1990). The structure and insertion of integral proteins in membranes. Ann. Rev. Cell Biol. 6, 247-296.

Singh, L., Doms, R.W., Wagner, K.R., and Helenius, A. (1990). Intracellular transport of soluble and membrane-bound glycoproteins: folding, assembly and secretion of anchor-free influenza hemagglutinin. EMBO J. 9, 631-639.

Smith, S., and Blobel, G. (1993). The first membrane spanning region of the lamin B receptor is sufficient for sorting to the inner nuclear membrane. J. Cell Biol. 120, 631-637.

Sommer, M., and Courtney, R.J. (1991). Differential rates of processing and transport

of herpes simplex virus type 1 glycoproteins gB and gC. J. Virol. 65, 520-525.

Song, J., Lee, C., Lin, C.-H., and Chen, L.B. (1991). Electron microscopic studies of the endoplasmic reticulum in whole-mount cultured cells fixed with potassium permanganate. J. Structural. Biol. 107, 106-115.

Soullam, B., and Worman, H.J. (1993). The amino-terminal domain of the lamin B receptor is a nuclear envelope targeting signal. J. Cell Biol. 120, 1093-1100.

Spear, P.G. (1976). Membrane proteins specified by herpes simplex viruses. I. Identification of four glycoprotein precursors and their products in type 1-infected cells. J. Virol. 17, 991-1008.

Spear, P.G. (1984). Glycoproteins specified by herpes simplex viruses. In B. Roizman ed., The herpesviruses, Vol. 3, pp 315-356, Plenum Press, New York.

Spear, P.G., and Roizman, B. (1968). The proteins specified by herpes simplex virus I. Time of synthesis, transfer into nuclei, and properties of proteins made in productively infected cells. Virology 36, 545-553.

Spear, P.G., and Roizman, B. (1972). Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. J. Virol. 9, 143-159.

Spear, P.G., and Roizman, B. (1981). Herpes simplex viruses. In Molecular biology of tumor viruses. Sec edi., part 2. J. Tooze ed., pp 616-746e. Cold Spring Harbor Laboratory, Cold Spring Harbor.

Stannard, L.M., Fuller, A.O., Spear, P.G. (1987). Herpes simplex virus glycoproteins associated with different morphological entities progecting from the virion envelope. J. Gen. Virol. 68, 714-725.

Stirzaker, S.C., and Both, G.W. (1989). The signal peptide of the rotavirus glycoprotein VP7 is essential for its retention in the ER as an integral membrane protein. Cell 56, 741-747.

Stirzaker, S.C., Poncet, D., and Both, G.W. (1990). Sequences in rotavirus glycoprotein VP7 that mediate delayed translocation and retention of the protein in the endoplasmic reticulum. J. Cell Biol. 111, 1343-1350.

Stuve, L.L., Brown-Shimer, S., Pachl, C., Najarian, R., Dina, D., and Burke, R.L. (1987). Structure and expression of the herpes simplex virus type 2 glycoprotein gB

gene. J. Virol. 61, 326-335.

Sukegawa, J., and Blobel, G. (1993). A nuclear pore complex protein that contains zinc finger motifs, binds DNA and faces the nulcleoplasm. Cell 72, 29-38.

Swift, A.N., and Machamer, C.E. (1991). A golgi retention signal in a membrane-spanning domain of coronavirus E1 protein. J. Cell Biol. 115, 19-30.

Tang, B.L., Wong, S.H., Low, S.H., and Hong, W. (1992a). The transmembrane domain of N-glucosaminyltransferase 1 contains a golgi retention signal. 267, 10122-10126.

Tang, B.L., Wong, S.H., Low, S.H., and Hong, W. (1992b). Retention of a type II surface membrane protein in the endoplasmic reticulum by the Lys-Asp-Glu-Leu sequence. J. Biol. Chem. 267, 7072-7076.

Tang, B.L., Wong, S.H., Qi, X.L., Low, S.H., and Hong, W. (1993). Molecular cloning, characterization, subcellular localization and dynamics of p23, the mammalian KDEL receptor. J. Cell Biol. 120, 325-338.

Tarentino, A.L., Plummer, T.H., JR., and Maley, F. (1974). The release of intact oligosaccharides from specific glycoproteins by endo-β-N-acetylglucosaminidase H. J. Biol. Chem. 249, 818-824.

Teasdale, R.D., D'Agostaro, G., and Gleeson, P.A. (1992). The signal for golgi retention of bovine β1,4-galactosyltransferase is in the transmembrane domain. J. Biol. Chem. 267, 4084-4096.

Tokuyasu, K.T. (1986). Application of cryoultramicrotomy to immunocytochemistry. J. Microsc. 143, 139-149.

Toneguzzo, F. (1977). Synthesis in vitro of vesicular stomatitis virus messenger RNA and proteins. Ph.D thesis, McMaster University, Hamilton, Canada.

Tonegusso, F., and Ghosh, H.P. (1977). Synthesis and glycosilation in vitro of glycoprotein of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. 74, 1516-1520.

Tooze, S.A., Tooze, J., and Warren, G. (1988). Site of addition of N-acetylgalactosamine to E1 glycoprotein of a mouse hepatitis virus-A59. J. Cell Biol. 106, 1475-1487.

Torrisi, M.R., and Bonatti, S. (1985). Immunocytochemical study of the partition and distribution of Sindbis virus glycoproteins in freeze-fractured membranes of infected baby hamster kidney cells. J. Cell Biol. 101, 1300-1306.

Torrisi, M.R., Cirone, M., Pavan, A., Zompetta, C., Barile, G., Frati, L., and Faggioni, A. (1989). Localization of Epstein-Barr virus envelope glycorproteins on the inner nuclear membrane of virus-producing cells. J. Virol. 63, 828-832.

Torrisi, M.R., Di Lazzaro, C., Pavan, A., Pereira, L., and Campadelli-Fiume, G. (1992). Herpes simplex virus envelopment and maturation studied by fracture label. J. Virol. 66, 554-561.

Torrisi, M.R., Lotti, L.V., Pavan, A., Migliaccio, G., and Bonatti, S. (1987). Free diffusion to and from the inner nuclear membrane of newly synthesized plasma membrane glycoproteins. J. Cell Biol. 104, 733-737.

Tumilowicz, J.J., and Powell, B.B. (1990). Role of intranuclear sacs, intrasaccate tubes, and cytoplasmic dense bodies in the structural ccmpletion of cytomegalovirus. Intervirol. 31, 14-22.

von Heijne, G. (1983). Patterns of amino acids near signal sequence cleavage. Eur. J. Biochem. 133, 17-21.

von Heigne, G. (1985). Signal sequence: the limits of variation. J. Mol. Biol. 184, 99-105.

von Heijne, G. (1988). Transcending the impenetrable: how proteins come to terms with membranes. Bioch. Biophy. Act. 947, 307-333.

von Heijne, G. (1989). Control of topology and mode of assembly of a polytopic membrane protein by positively charged residues. Nature 341, 456-458.

von Heijne, G. and Gavel, Y. (1988). Topogenic signal in integral membrane proteins. Eur. J. Biochem. 174, 671-678.

Walter, P., and Lingappa, V.R. (1986). Mechanism of protein translocation across the endoplasmic reticulum membrane. Ann. Rev. Cell. Biol. 2, 499-516.

Watson, M.L. (1958). Further observations on the nuclear envelope of the animal cell. J. Biophys. Biochem. Cytol. 6, 147-155.

Watson, R.J., Weis, J.H., Salstron, J.S., Enquist, L.W. (1982). Herpes simplex virus type-1 glycoprotein D gene: nucleotide sequence and expression in Escherichia coli. Science 218, 381-384.

Weibull, C., Villiger, W., and Carlemalm, E. (1984). Extraction of lipids during freeze-sustitution of Acholeplasma laidlawii-cells for electron microscopy. J. Microsc. 134, 213-216.

Weibull, C., Villiger, W., and Carlemalm, E. (1983). Extraction of membrane lipids during fixation dehydration and embedding of Acholeplasma laidlawii-cells for electron microscopy. J. Microsc. 129, 201-207.

Weibull, C., Villiger, W., and Carlemalm, E. (1984). Extraction of lipids during freeze-sustitution of Acholeplasma laidlawii-cells for electron microscopy. J. Microsc. 134, 213-216.

Weinstein, J., Lee, E.U., McEntee, K., Lai, P.-H., and Paulson, J.C. (1987). Primary structure of β-galactoside α 2,6-sialyltrasnferase. J. Biol Chem. 262, 17735-17743.

Wenske, E.A., Bratton, M.W., and Courtney, R.J. (1982). Endo-β-N-Acetylglucosaminidase H sensitivity of precursors to herpes simplex virus type 1 glycoproteins gB and gC. J. Virol. 44, 241-248.

Wente, S.R., Rout, M.P., and Blobel, G. (1992). A new family of yeast nuclear pore complex proteins. J. Cell Biol. 119, 705-723.

Whealy, M.E., Card, J.P., Meade, R.P., Robbins, A.K., and Enquist, L.W. (1991). Effect of brefeldin A on alphaherpesvirus membrane protein glycosylation and virus egress. J. Virol. 65, 1066-1081.

Whitt, M.A., and Rose, J.K. (1991). Fatty acid acylation is not required for membrane fusion activity or glycoprotein assembly into VSV virions. Virology 185, 875-878.

Wieland, F.T., Gleason, M.L., Serafini, T.A., and Rothman, J.E., (1987). The rate of bulk flow from the endoplasmic reticulum to the cell surface. Cell 50, 289-300.

Wong, G.G., Witek, J.S., Temple, P.A., Wilkens, K.M., Leary, A.C., Luxemberg, D.P., Jones, S.S., Brown, E.L., Kay, R.M., Orr, E.C., Shoemaker, C., Golde, D.W., Kaufman, R.J., Hewick, R.M., Wong, E.A., and Clark, S.C. (1985). Human GM-CSF: molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. Science 228, 810-815.

Wong, S.H., Low, S.H., and Hong, W. (1992). The 17-residue transmembrane domain of β-galactoside α2,6-sialyltransferase is sufficient for golgi retention. J. Cell Biol. 117, 245-258.

Wood, S.A., Park, J.E., and Brown, W.J. (1991). Brefeldin A causes a microtubule-mediated fusion of the trans-golgi network and early endosomes. Cell 67, 591-600.

Woolson, R.F. (1987). Statistical methods for the analysis of biomedical data. John Wiley & Sons, New York.

Worman, H.J., Evans, C.D., and Blobel, G. (1990). The lamin B receptor of the nuclear envelope inner membrane: a polytopic protein with eight potential trasnmembrane domains. J. Cell Biol. 111, 1535-1542.

Worman, H.J., Yuan, J., Blobel, G., and Georgatos, S. (1988). A lamin B receptor in the nuclear envelope. Proc. Natl. Acad. Sci. U.S.A. 85, 8531-8534.

Wozniak, R.W., and Blobel, G. (1992). The single transmembrane segment of gp210 is sufficient for sorting to the pore membrane domain of the nuclear envelope. J. Cell Biol. 119, 1441-1449.

Wozniak, R.W., Bartnik, E. and Blobel, G. (1989). Primary structure analysis of an integral membrane glycoprotein of the nuclear pore. J. Cell Biol. 108, 2083-2092.

Yamane, K., Akiyama, Y., Ito, K., and Mizushima, S. (1990). A positively charged region is a determinant of the orientation of cytoplasmic membrane proteins in Escherichia coli. J. Biol. Chem. 265, 21166-21171.

Yang, Y.-C., Ciarletta, A.B., Temple, P.A., Chung, M.P., Kovacic, S., Witek-Giannoti, J.S., Leary, A.C., Kriz, R., Donahue, R.E., Wong, G.G., and Clark, S.C. (1986). Human Il-3 (multi-CSF): Identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. Cell 47, 3-10.

Yokata, S. (1988). Effect of particle size on labeling density for catalase in protein A-gold immunocytochemistry. J. Histochem. Cytochem. 36, 107-109.

Young, W.W., Jr., Lutz, M.S., Blackburn, W.A. (1992). Endogenous glycosphingolipids move to the cell surface at a rate consistent with bulk flow estimates. J. Biol. Chem. 267, 12011-12015.

Zhang, H., Scholl, R., Browse, J., and Somerville, C. (1988). Double stranded DNA

sequencing as a choice for DNA sequencing. Nucl. Acids Res. 16, 1220.

Zilberstein, A., Snider, M.D., and Lodish, H.F. (1981). Synthesis and assembly of the vesicular stomatitis virus glycoprotein. Cold Spr. Harb. Symp. Quant. Biol. XLVI, 785-795.