IDENTIFICATION AND CHARACTERIZATION OF BOVINE HERPESVIRUS 1 MINOR GLYCOPROTEINS L, H AND M.

A Thesis

Submitted to the Faculty of Graduate Studies and Research

in Partial Fulfillment of the Requirements for

the Degree of Doctor of Philosophy

in the

Department of Veterinary Microbiology

University of Saskatchewan

Saskatoon

by

Sunil Kumar Khattar

FALL, 1997

UNIVERSITY OF SASKATCHEWAN

College of Graduate Studies and Research

SUMMARY OF DISSERTATION

Submitted in partial fulfillment

of the requirements for the

DEGREE OF DOCTOR OF PHILOSOPHY

by

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Fall 1997

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Identification and Characterization of Bovine Herpesvirus 1 Minor Glycoproteins L, H and M

Bovine herpesvirus-1 (BHV-1) encodes a number of glycoproteins, which are present in the virion envelope and play a pivotal role in herpesvirus biology. Glycoproteins gL, gH, and gM are minor glycoproteins of BHV-1, which are well conserved among herpesviruses. The objective of this investigation was to isolate and identify the gene encoding glycoprotein gL and to identify and characterize the glycoprotein gL. In addition other minor glycoproteins, gH and gM, were identified and characterized.

Sequencing of 3113 nucleotides located at the right end of the HindIII L fragment of the BHV-1 genome, from map units 0.712 to 0.734 revealed four open reading frames (ORFs) designated as UL1, UL2, UL3, and UL3.5 based on their homology with proteins of other alphaherpesviruses. The UL1 ORF of 158 aminoacids exhibited limited homology with the UL1 (glycoprotein gL) homolog of herpes simplex virus (HSV-1) and pseudorabies virus (PRV). The UL2 ORF of 204 amino acids showed significant homology to the UL2 (uracil-DNA glycosylase) homolog of HSV-1 and PRV. The UL3 ORF of 204 amino acids showed significant homology to UL3 (nuclear phosphoprotein) of HSV-1 and PRV. The UL3.5 ORF of 126 amino acids showed limited homology to the UL3.5 ORF of PRV. The homolog of this gene is absent in HSV-1. Nucleotide sequence analyses also revealed potential TATA boxes located upstream of each ORF. However, only one polyadenylation signal was detected downstream of the UL3.5 ORF. Northern (RNA) blot analyses revealed four transcripts of 2.4, 1.9, 1.3, and 0.7 kb which are transcribed in the same direction and are 3'-coterminal transcripts. These mRNAs appear to yield proteins encoded by UL1 (2.4 kb), UL2 (1.9 kb), UL3 (1.3 kb) and UL3.5 (0.7kb) ORFs.

DNA sequence analysis of the bovine herpesvirus-1 (BHV-1) genome revealed the presence of an open reading frame named UL1 which exibited limited homology to glycoprotein gL of HSV-1. To identify the BHV-1 UL1 protein, rabbit antisera were prepared against two synthetic peptides that were predicted by computer analysis to encompass antigenic epitopes. Sera against both peptides immunoprecipitated a 16-17 kDa protein from *in-vitro* translated *in-vitro* transcribed mRNA, BHV-1 infected MDBK cells and purified virions. Enzymatic deglycosylation and lectin binding assays confirmed that the BHV-1 UL1 protein contains only O-linked oligosaccharides and was named glycoprotein gL. Sera against the UL22 protein immunoprecipitated a protein of 108

kDa from BHV-1 infected MDBK cells and purified virions which was modified only by N-linked oligosaccharides and was named glycoprotein gH. Glycoprotein gL expressed by recombinant vaccinia virus was properly processed and secreted into the medium. In contrast, glycoprotein gH expressed by recombinant vaccinia virus was found to be retained in the rough endoplasmic reticulum. However, gH coexpressed with gL by recombinant vaccinia viruses was properly processed and transported to the cell surface suggesting that complex formation between gH and gL is neccessary for the proper processing and transport of gH, but not gL. In addition, gH-gL complex formation is also required for induction of neutralizing antibody responses and anchoring of gL to the plasma membrane.

Previously, DNA sequence analysis of the BHV-1 genome revealed the presence of an open reading frame homologous to the UL10 gene of herpes simplex virus-1 (Vlcek et al., 1995). To identify the UL10 product of BHV-1, rabbit antiserum was raised against a UL10-GST fusion protein, constructed by fusing the C-terminal 80 amino acids of UL10 protein with the gene encoding the GST protein. Serum against the UL10-GST fusion protein immunoprecipitated a 36-37 kDa protein from *in-vitro* translated *in-vitro* transcribed mRNA, and a 43-44 kDa protein from BHV-1 infected MDBK cells and purified virions. Glucosamine labelling and enzymatic deglycosylation assays confirmed that the UL10 protein is glycosylated and was designated BHV-1 glycoprotein gM in accordance with the current herpesvirus glycoprotein nomenclature. However, compared to other herpesvirus gM homologs which do not have O-linked oligosaccharides, BHV-1 gM is post-translationally modified by the addition of both N- and O- linked oligosaccharides. In comparison to herpes simplex virus-1 gM, BHV-1 gM was found to be less hydrophobic. Immunofluorescence studies indicated that BHV-1 gM was present inside the cytoplasm and on the surface of infected cells.

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Dedicated to my parents

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ABSTRACT

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herpes simplex virus-1 gM, BHV-1 gM was found to be less hydrophobic. Immunofluorescence studies indicated that BHV-1 gM was present inside the cytoplasm and on the surface of infected cells.

ACKNOWLEDGEMENTS

This thesis could have not been completed without the contributions of many people.

First I would like to thank my supervisor, Dr. Lorne A. Babiuk, who accepted me as a Ph.D student and gave me the opportunity to work at the Veterinary Infectious Disease Organization (VIDO). He gave me his constant encouragement, guidance and helpful criticism during the course of my Ph.D degree.

I would like to thank Dr. Suresh K.Tikoo, who gave me the opportunity to work in his lab and introduced me to the realm of molecular biology and was always willing to help, listen and provide valuable suggestions.

I would also like to thank members of my advisory committee Dr. Sylvia van Drunen Littel-van den Hurk, Dr. John Gordon, Dr Lambert Loh and former member Dr. Henry Tabel for their suggestions and help.

I wish to thank Caron Pyne, Dr. P. Seshi Reddy, Neeraja Idamakanti, Dr. Sasha Zakhartchouk and Yan Chen for their help and for the happiness and working places which I shared with them in the laboratory. I am thankful to Dr. Sam Attah-Poku for his invaluable contributions to the synthesis and coupling of synthetic peptides; Dr. Jan van den Hurk for taking the pictures of my slides using the fluorescent microscope. I thank Neil Rawlyk, Betty Chow, and Brenda Karvonen for their help in providing reagents and technical support and Gordon Crockford for synthesis of oligopeptides.

I thank Joyce Sander, Wilf Finn, Dee Kirchmeier and other members of the front office and to the animal care and GMP staff for their help.

I express my thanks to Dr. Mohit Baxi and Dr. Radhey Shyam Kaushik for their help, friendship and a good sense of humour which kept me going during difficult times. Also I would like to thank all my fellow graduate students for their help.

I am grateful to my parents who taught me the values of good education and always helped me to pursue my carreer and higher studies.

Last but not the least I am thankful to my wife Neeraj for her unquestionable care and love and for her limitless support throughout my study in Canada. I would like to thank my daughter Aditi and my son Anish who were the major source of my happiness throughout my stay in Saskatoon.

My studies were supported by Canadian Commonwealth Scholarship and Fellowship Plan Award and Medical Research Council of Canada.

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ABBREVIATIONS USED IN THIS WORK

aa	amino acid
Asn	asparagine
α TIF	alpha gene transinducing factor
BICP	bovine infected cell protein
BHK cells	baby hamster kidney cells
BHV	bovine herpes virus
C-terminal	carboxy-terminal
DISC	disabled infectious single cycle
DMEM	Dulbecco's minimum essential medium
dUTPase	deoxyuridine triphosphatase
EBV	Epstein-Barr virus
EHV	equine herpes virus
ELISA	enzyme linked immunosorbent assay
endo H	endoglycosidase H
ER	endoplasmic reticulum
FBS	fetal bovine serum
FHV	feline herpesvirus
FITC	fluorescein isothiocyanate
Gal	galactose
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
GST	glutathione-S-transferase
HCMV	human cytomegalovirus
HHV	human herpesvirus
HS	heparan sulfate
HSPG	heparan sulfate proteoglycan
HSV	herpes simplex virus

HVS	herpes virus saimiri
HVT	herpesvirus of turkey
ILTV	infectious laryngotracheitis virus
IBR	infectious bovine rhinitracheitis
IPB	infectious pustular balanoposthitis
IPV	infectious pustular vulvovaginitis
IPTG	isopropyl β-D-thiogalactoside
kb	kilobases
kDa	kilodalton
Mab	monoclonal antibody
Man	mannose
MCMV	murine cytomegalovirus
MDBK cells	Madin Darby bovine kidney cells
MDV	Marek's disease virus
MEM	Eagle's minimum essential medium
m.w.	molecular weight
N-terminal	amino-terminal
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
pfu	plaque forming unit
PNGase F	peptide-N-glycosidase F
PRV	pseudorabies virus
RIPA	radio immunoprecipitation assay
RNR	ribonucleotide reductase
SA	sialic acid
SDS	sodium dodecyl sulphate
Ser	serine
SVV	simian varicella virus

syn	syncytia-forming
Thr	threonine
TK	thymidine kinase
ts	temperature sensitive
UL	unique long
US	unique short
VV	vaccinia virus
VZV	varicella-zoster virus

1.0 LITERATURE REVIEW

1.1 Glycoproteins

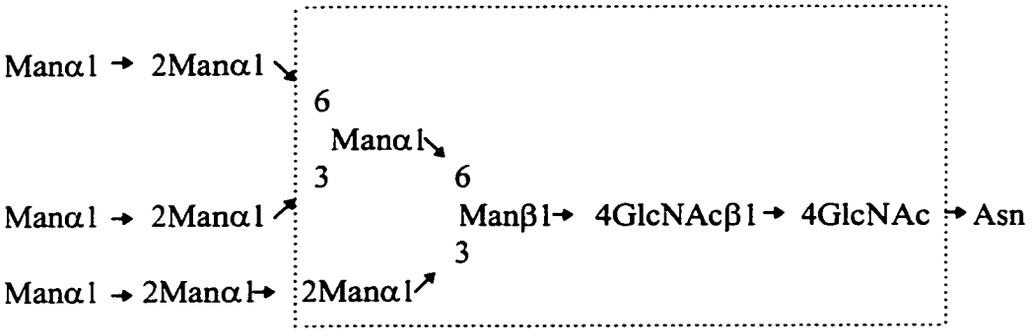
Among the molecular constituents of viruses, glycoproteins play an important role in the life cycle of viruses. Glycoproteins are widely distributed among enveloped viruses including herpesviruses. Integral membrane glycoproteins share certain common functional elements including a signal sequence at the amino terminal end of the protein This sequence is required for translocation of the polypeptide across the endoplasmic reticulum and may be cleaved during maturation. An external domain which may contain sites for the addition of oligosaccharides, a hydrophobic transmembrane domain usually near the carboxy terminus of the protein for anchoring the polypeptides in the membrane and a cytoplasmic domain are also features of these glycoproteins.

1.1.1 Structure

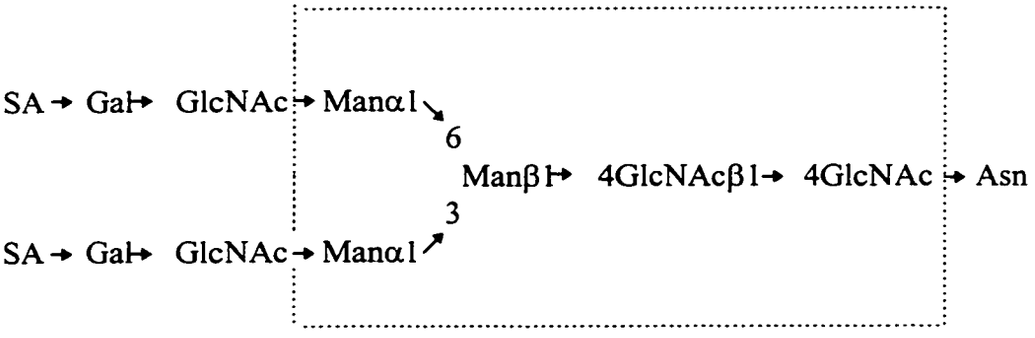
Generally, the carbohydrate content of viral glycoproteins varies from 5 to 40%. There is no evidence to suggest that viruses encode within their genomes enzymes required for biosynthesis of N-linked and O-linked oligosaccharides.Thus, it is assumed that glycosylation of viral envelope glycoproteins depends on the host cell's glycosylation apparatus. The fact that oligosaccharide moieties in viral glycoproteins are similar to those found in the host cells support this view (Yamashita et al., 1984). Two types of carbohydrate-peptide linkages have been identified in viral glycoproteins; namely N-glycosidic and O-glycosidic.

The N- or asparagine-linked oligosaccharides represent a diverse group of structures, that share a common biosynthetic pathway, and that contain a common core region of Manα1,6(Manα1,3)Manβ1,4-GlcNAcβ1,4-GlcNAc. Three major classes of N-linked oligosaccharides; high mannose, complex and hybrid types exist (Fig. 1.1). In

High mannose type



Complex type



Hybrid type

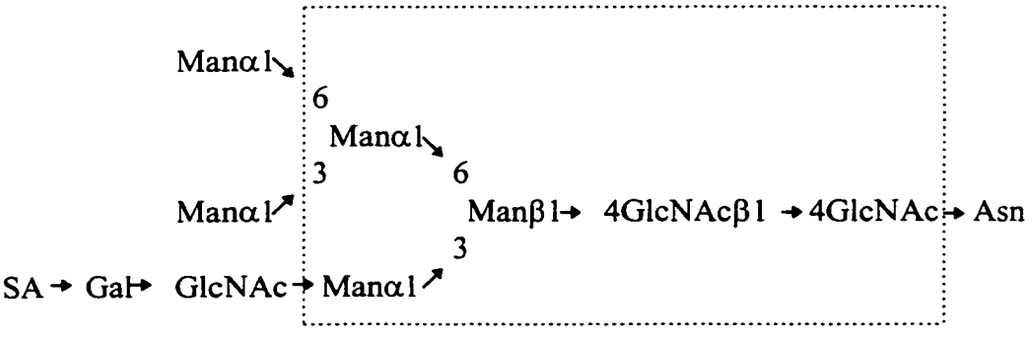


Fig. 1.1. Structures of the three types of N-linked oligosaccharides (Kornfeld and Kornfeld, 1985). A 'core' structure is boxed to indicate each of the various kinds of linked oligosaccharides found in these glycoproteins.
Asn=asparagine;Man=mannose;GlcNAc=N-acetylglucosamine;Gal=galactose;SA=sialic acid

each class, the common core structure (boxed) is lengthened by the addition of various other sugars.

For the high mannose chains, there may be as many as six additional α-linked mannoses, although some high mannose chains have fewer distal mannose residues (Kornfeld and Kornfeld, 1985). High mannose type oligosaccharides are most commonly found associated with immature glycoproteins (i.e. glycoproteins still in the endoplasmic reticulum during the process of biosynthesis), but they can also be found associated with some mature glycoproteins present in the virus envelope (Pan and Elbein, 1990).

In the biantennary (two side chains linked to the core structure) complex chain, the core region is lengthened by trisaccharide sequences composed of sialic acid-galactose-GlcNAc (Kornfeld and Kornfeld, 1985). However, other complex structures may have three (triantennary) or four (tetraantennary) of these trisaccharide units. In addition, some of them may have an α-linked fucosyl unit attached to the innermost GlcNAc, or they may be sulfated or phosphorylated as seen particularly in proteoglycans (Kornfeld and Kornfeld, 1985).

A much rarer group of oligosaccharides is represented by the hybrid type, which typically has one or two of the trisaccharide sequences, as well as partial high-mannose structure (Kornfeld and Kornfeld, 1985).

The O-linked oligosaccharides do not involve a lipid carrier (Berger et al., 1982). The sugar chains of O-linked oligosaccharides are generally simpler and less branched than those linked to asparagine (Fig. 1.2). The O-linked oligosaccharides are synthesized by the direct addition of carbohydrates to the polypeptide and is initiated by the transfer of N-acetylgalactosamine to the hydroxyl group of serine or threonine. Some of them are lengthened by the addition of sialic acid residues.

1.1.2 Synthesis and Processing

Generally herpesvirus glycoproteins are synthesized on polyribosomes with an amino terminal peptide extension called signal peptide. This signal peptide directs the translocation of the nascent polypeptide chain across the membrane of the endoplasmic

reticulum (ER) into the lumen. In most cases the signal peptide is cleaved by a cellular signal peptidase (Zwizinski and Wickner et al., 1980). In the lumen of the ER and simultaneously with cotranslational translocation, oligosaccharide chains are added to the emerging glycoproteins. First of all, biosynthesis of N-linked carbohydrates occurs

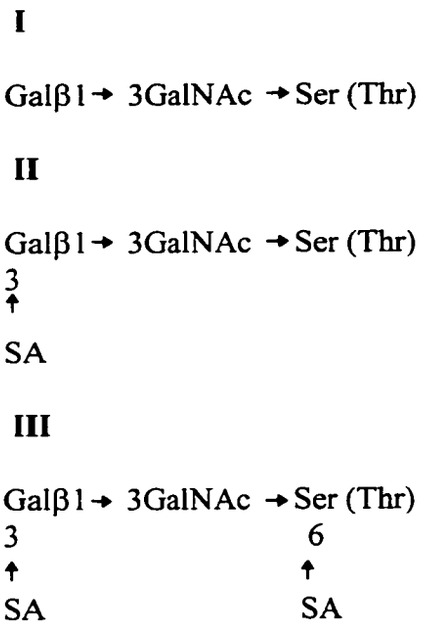


Fig. 1.2 Structures of three types of O-linked oligosaccharides (Berger et al., 1982). Gal=galactose;GalNAc=N-acetyl galactosamine;SA=Sialic acid;Ser=serine;Thr=threonine

via the formation of a mannose rich oligosaccharide precursor on a specific lipid carrier (dolichol phosphate) in the ER (Kornfeld and Kornfeld, 1985; Mononen and Karjalainen, 1984). All the N-linked oligosaccharides arise from the common intermediate Glc3Man9(Glc-NAc)2. The acceptor site on the protein is asparagine in the sub-sequence Asn-X-Ser/Thr where X can be any amino acid except proline and aspartic acid. It appears that to be glycosylated the Asn residue must be in an exposed region such as a β turn of the protein (Kornfeld and Kornfeld, 1985). The initial glycosylation reaction generally occurs as a co-translational event but in some cases it

occurs post-translationally. The precursor oligosaccharide is then modified by the removal of some sugars and addition of others to give rise to various classes of oligosaccharides. The outermost three glucose residues and one or two mannose residues are removed from the precursor oligosaccharide by glycosidase(s) and mannosidase(s) enzymes to give rise to a high mannose oligosaccharide (Man7-8(GlcNAc)2) (Hirschberg, 1987). In addition to glycosylation, fatty acids are also covalently attached to the proteins in the ER (Berger and Schmidt, 1985). The protein is then transferred to the Golgi apparatus. As the protein is transferred through the compartments of the Golgi, high mannose oligosaccharides are further processed by removal of mannose sugars and the addition of various other sugars to yield hybrid or complex oligosaccharides. All oligosaccharides are modified by cellular enzymes, which reside in the ER or the Golgi (Hirschberg, 1987). Other post-translational modifications of N-linked oligosaccharides include phosphorylation of mannose residues, sulphation of mannose and N-acetylhexosamine residues and O-acylation of sialic acid residues (Kornfeld and Kornfeld, 1985). Synthesis of O-linked oligosaccharides also occurs in the Golgi by covalent attachment of N-acetylgalactosamine to the hydroxyl group of serine and threonine residues (Hirschberg, 1987).

The oligosaccharide structure and processing of glycoproteins is usually studied by the use of glycosylation inhibitors, such as tunicamycin, monensin, swainsonine, castanospermine, 1-Deoxymannojirimycin and brefeldin A and digestion with glycosidases, such as endoglycosidase H, endoglycosidase F, N-glycosidase F (PNGase F), neuraminidase and O-glycosidase. Tunicamycin inhibits the addition of mannose-rich oligosaccharide precursor to dolichol phosphate and thus blocks the synthesis of N-linked oligosaccharides. Monensin blocks the processing of proteins in the Golgi and thus inhibits the synthesis of O-linked oligosaccharides and further processing of N-linked high mannose oligosaccharides. Swainsonine inhibits Golgi mannosidase II in the biosynthesis of complex glycoproteins. It is used to alter the oligosaccharide chain composition of glycoproteins for studies of the role of oligosaccharides in the stability and transportability of glycoproteins. Castanospermine inhibits glucosidase I (which

catalyses the first reaction in the processing pathway). 1-Deoxymannojirimycin blocks the activity of Golgi α-mannosidase I and inhibits the conversion of high mannose oligosaccharides to complex type oligosaccharides. Brefeldin A blocks reversibly the translocation of proteins from the endoplasmic reticulum to the Golgi. Endoglycosidase H cleaves only N-linked high mannose structures, endoglycosidase F hydrolyses N-linked high mannose and hybrid forms and N-glycosidase F hydrolyses all types of asparagine bound glycans. Neuaraminidase cleaves terminal sialic acid residues linked to N-acetylgalactosamine and O-glycosidase hydrolyses N-acetylgalactosamine linked to serine or threonine residues.

1.1.3 Functions

Glycoproteins are the major structural components of the envelope in herpesviruses. Due to the presence of viral glycoproteins on the surface of the virion, they play a major role in the interactions between viruses and their host cells. These glycoproteins are involved in several steps of viral multiplication. They are responsible for the initial attachment of the virus to cellular receptors (Herold et al., 1991), and subsequent virus and host cell membrane fusion and penetration of the host cell (Ligas and Johnson, 1988). They are involved in envelopment of the capsid, viral egress and cell-to-cell spread of the virus. Some glycoproteins are receptors for the Fc fraction of immunoglobulin G or the C3b factor of complement and thus help in immune evasion. The carbohydrate structure of viral glycoproteins plays an important role in maintaining the correct folding, stabilization of the native conformation (Olden et al., 1985; Olofsson, 1992), directing the intracellular transport (Von Figura and Hasilik, 1986) and protecting the molecule against proteolytic degradation (Olden et al., 1985; Elbein A.D., 1987). They help to maintain the physicochemical properties and to modulate the antigenicity of the glycoprotein. Carbohydrate moeities may also help the virus to escape from neutralising antibodies by masking the polypeptide (Skehel et al., 1984).

1.2 Herpesvirus Glycoprotein H and L Homologs

Every herpesvirus genome encodes a number of glycoproteins, most of which are found in the infected cell's membranes as well as the virion envelope, while some are present only in the infected cell's membrane (Roizman and Sears, 1996). Among herpesviruses, glycoproteins encoded by herpes simplex virus type 1 (HSV-1) have been well characterized and intensively studied. In the following section, the literature on HSV-1 glycoproteins gH and gL will be briefly reviewed. In additon, homologous glycoproteins of other herpesviruses will be discussed in the same section as the prototype HSV-1 molecules to emphasize comparative aspects.

1.2.1 Structure of Herpesvirus Glycoprotein H Homologs

The UL22 ORF of HSV-1 encoding glycoprotein gH, is located downstream from the gene encoding the viral thymidine kinase enzyme in the unique long region of the genome at map unit 0.27-0.312 (Gompels and Minson, 1986; McGeoch and Davison, 1986). Homologs of this gene have been detected in all the members of different herpesvirus subgroups including alpha, beta and gamma herpesviruses studied to-date. These include BHV-1 (Meyer et al., 1991), pseudorabies virus (PRV; Klupp and Mettenleiter, 1991), equine herpesvirus I (EHV-1; Robertson et al., 1991), equine herpesvirus 4 (EHV-4; Nicolson et al., 1990), feline herpesvirus-1 (FHV-1; Maeda et al., 1993), varicella zoster virus (VZV; Davison and Scott, 1986), simian varicella virus (SVV; Pumphrey and Gray, 1995), human cytomegalovirus (HCMV; Cranage et al., 1988), murine cytomegalovirus (MCMV; Xu et al., 1992), human herpesvirus 6 (HHV-6; Josephs et al., 1991), Epstein-Barr virus (EBV; Baer et al., 1984), herpesvirus saimiri (HVS; Gompels et al., 1988), Marek's disease virus (MDV, Scott et al., 1993) and herpesvirus of turkey (HVT, Scott et al., 1993). Among the herpesvirus glycoproteins, gH homologs comprise the second most highly conserved group surpassed only by the gB homologs (Fuller et al., 1989). The location of the gH gene is also conserved throughout the herpesviruses.

HSV-1 gH is an 838 amino acid type I integral membrane glycoprotein which contains a N-terminal hydrophobic signal sequence of 18 amino acids, a surface domain of 785 residues containing 7 N-linked glycosylation sites, a C-terminal membrane anchor region of 21 amino acids, from residues 803 to 824, and a positively charged small cytoplasmic domain of only 14 amino acids (Gompels and Minson, 1986). HSV-1 gH contains 8 cysteine residues, all of them are located in the extracellular domain. The characteristics of some of the selected herpesvirus gH glycoproteins are summarized in Table 1.1. Homologies between gH of HSV-1 and gH proteins of other herpesviruses vary. The HSV-1 gH has 36, 30, 28, 29, 17, 15, 15, 14, and 10% homology with PRV, BHV-1, EHV-4, VZV, EBV, HCMV, HHV-6, MCMV, and HVS

Table 1.1 Diverse Characteristics of gH among Herpesviruses

	HSV-1	BHV-1	PRV	EHV-1	VZV	HCMV	EBV	HHV-6
No of amino acids	838	842	686	848	841	743	706	694
Size of gH (kDa)	110	108	95	116	118	86	85	110
No of N-linked glycosylation sites	7	6	3	11	10	6	5	12
No of cysteine residues	8	12	10	11	8	13	13	13
Contain signal sequence and transmembrane anchor sequence	+	+	+	+	+	+	+	+
Dependent upon gL for transport to cell membrane	+	+	+	U	+	+	+	+

U: Unknown

References: Baer et al., 1984; Baranowski et al., 1995; Cranage et al., 1988; Davison and Scott, 1986; Gompels and Minson, 1986; Hutchinson et al., 1992b; Josephs et al., 1991; Kaye et al., 1992; Klupp and Mettenleiter, 1991; Klupp et al., 1992; Klupp et al., 1994; Liu et al., 1993b; McGeoch and Davison, 1986; Meyer et al., 1991; Montalvo and Grose, 1986; Robertson et al., 1991; Stokes et al., 1996; Yaswen et al., 1993

gH proteins, respectively. (Baer et al., 1984; Cranage et al., 1988; Davison and Scott, 1986; Gompels et al., 1988; Josephs et al., 1991; Klupp and Mettenleiter, 1991; Meyer et al.,1991; Nicolson et al., 1990; Xu et al., 1992). The highest degree of homology regarding the location of N-linked glycosylation sites and cysteine residues resides in the C-terminal part of the molecule. Out of the 8 cysteine residues present in HSV-1 gH, 4 are found at colinear positions in gH of BHV-1, PRV, VZV, EHV-4, HCMV, EBV, and HVS. Similarly, out of 7 N-linked glycosylation sites, 2 can be aligned with corresponding sites in gH of BHV-1, EHV-4, EBV, and HVS. One C-terminal N-linked glycosylation site has been conserved throughout herpesviral gH proteins. The strong conservation of the position of cysteine residues strongly implies a significant degree of conservation of the secondary and tertiary structure of these proteins, presumably involving disulfide bonding (Wilcox et al., 1988).

The C-terminus of gH contains two of the most conserved regions. Region 1 consists of an 11 amino acid stretch, SPCAASLRFDL encompassing a conserved cysteine residue. The three amino acids SPC are strictly conserved throughout herpesviral gH proteins, except in HCMV and MDV, where serine had been substituted by threonine. Serine at position 6, except in MDV, arginine at position 8, and aspartic acid at position 10 are also strictly conserved. Region 2 contains seven highly conserved amino acids, LFPNGTV, which includes an N-linked glycosylation sequence. This region is located in the membrane spanning domain and is identical in all the alpha herpesvirus homologs, except EHV-4 where the first amino acid, leucine, is substituted by methionine. In the beta and gamma herpesviruses, four identical amino acids, NGTV are found, except in MDV where valine is substituted by isoleucine.

1.2.2 Structure of Herpesvirus Glycoprotein L Homologs

The gene coding for glycoprotein gL has been detected in all the members of different herpesvirus subgroups, e.g alpha, beta, and gamma herpesviruses, including HSV-1 (McGeoch et al., 1988), PRV (Dean and Cheung, 1993; Klupp et al., 1994), EHV-1 (Telford et al., 1992), infectious laryngotracheitis virus (ILTV; Fuchs and Mettenleiter, 1996), VZV (Davison and Scott, 1986), HCMV (Chee et al., 1990),

MCMV (Xu et al., 1994), MDV (Yoshida et al., 1994), EBV (Baer et al., 1984), and HHV-6 (Liu et al., 1993b). In all herpesviruses the gene coding for glycoprotein gL lies immediately 5' of the gene coding for uracil DNA glycosylase. Despite the conservation of genomic location, glycoprotein gL encoded by different herpesviruses does not show significant amino acid sequence homology.

The gene coding for HSV-1 glycoprotein gL is located in the unique long region of the genome and designated as UL1 (McGeoch et al., 1988).The ORF of HSV-1 gL codes for a protein of 224 amino acids with a single hydrophobic domain at the amino-terminus (McGeoch et al., 1988). The amino-terminal methionine residue of gL is cleaved during post translational processing (Hutchinson et al., 1992b), which suggests that the hydrophobic domain of gL functions as a signal peptide. There are no additional hydrophobic domains present in gL, which could act as a putative transmembrane domain. HSV-1 gL contains a single site for the attachment of N-linked oligosaccharides and 5 cysteine residues (McGeoch et al., 1988). In Table 1.2 the characteristics of the identified gL glycoproteins of some selected herpesviruses are summarized. The overall amino acid sequence homology between HSV-1 UL1 and other herpesviral UL1 gene products is not significant. However, comparison of HSV-1 gL with MDV, VZV and EHV-1 gL homologs reveals a highly conserved region extending from amino acids 79 to 106 in HSV-1 gL (Yoshida et al., 1994). Of the 28 residues in this region, 8 residues, including cysteine at position 8 are conserved among all four gL homologs.

1.2.3 Synthesis, Processing and Transport of Herpesvirus Glycoprotein H and L Homologs

HSV-1 gH is a 110 kDa glycoprotein which is present at relatively low levels in the viral envelope and on the surface of infected cells (Buckmaster et al., 1984; Gompels and Minson, 1986; Showalter et al., 1981). The size of the unprocessed gH polypeptide is 90 kDa, which is modified post-translationally by the addition of N-linked glycans into a mature protein of 110 kDa in HSV-1 infected cells (Buckmaster et

Table 1.2 Diverse Characteristics of gL among Herpesviruses

	HSV-1	PRV	VZV	HCMV	HHV-6	EBV	MDV
No of amino acids	224	156	158	278	250	137	195
Size of gL (kDa)	40	20	20	31	40	25	25
No of N-linked glycosylation sites	1	N	1	1	1	3	2
No of cysteine residues	5	2	5	6	6	5	5
Contains signal sequence	+	+	-	+	+	+	+
Associates with mature form of gH	+	+	-	+	+	+	U
Linked covalently to gH	-	U	-	+	+	-	U

U: Unknown.

N: None

References: Davison and Scott, 1986; Dean and Cheung, 1993; Forghani and Grose, 1994; Hutchinson et al., 1992b; Kaye et al., 1992; Klupp et al., 1994; Liu et al., 1993b; Spaete et al., 1993;Yaswen et al., 1993 Yoshida et al., 1994

al., 1984). Compared to the expression of mature glycosylated gH protein in infected BHK cells, recombinant gH produced in transiently transfected COS-1 cells has been found to be smaller, misfolded, aggregated, was neither processed nor transported to the cell surface and did not possess all of the conformational epitopes. (Gompels and Minson, 1989; Roberts et al., 1991). Foa-Tomasi et al. (1991) has also demonstrated that gH constitutively expressed in cells is retained intracellularly and that the oligosaccharides are not fully processed to complex forms. However, gH was transported to the cell surface and contained complex carbohydrates when transfected cells were superinfected with HSV-2 (Foa-Tomasi et al., 1991; Gompels and Minson, 1989;). In additon, HSV-1 gH expressed by vaccinia virus recombinants did not induce a protective immune response in mice (Forrester et al., 1991). Similarly, HCMV and EBV gH homologs expressed in transiently transfected cells or in recombinant vaccinia

virus infected cells were retained intracellularly (Cranage et al., 1988; Heineman et al., 1988). These results suggested that other herpesvirus proteins may be required during or after synthesis of gH to assist in the proper folding and transport of gH.

Using HSV-1 glycoprotein deletion mutants, it was shown that glycoproteins gB, gC, gD, gE and gI were not required for maturation of gH (Foa-Tomasi et al., 1991; Roberts et al., 1991). Recently, an accessory molecule named glycoprotein gL, encoded by the UL1 gene of HSV-1, was identified as the viral protein that forms a complex with gH (Hutchinson et al., 1992b; Roop et al., 1993). Using vaccinia virus recombinants (Hutchinson et al., 1992b), gL negative HSV-1 mutants (Roop et al., 1993) or using baculovirus expressed gL and gH (Westra et al., 1997), it was shown that gL is required for proper processing and transport of gH. Similarly, using vaccinia virus recombinants, gL was found to be dependent on gH for proper post-translational processing and cell surface expression (Hutchinson et al., 1992b), suggesting that gH and gL form a hetero-oligomer. This heterodimer is transported to the cell surface and also incorporated into virions. The formation of a gH-gL complex occurs in members of all three subfamilies of herpesviruses (Duus et al., 1995; Forghani and Grose, 1994; Hutchinson et al., 1992b; Kaye et al., 1992; Klupp et al., 1994; Roop et al., 1993; Spaete et al., 1993; Yaswen et al., 1993; Westra et al., 1997). However, recently it has been shown that in HCMV, a third glycoprotein, gp125, is also associated with gH and gL and all three glycoproteins form a gCIII complex in the virion envelope (Li L. et al., 1997).

Although glycoproteins gH and gL are structural components of all herpesviruses examined so far, data from HSV-1, HCMV and HHV-6 studies indicate that there are separate domains in gH for interaction with gL and for mediating cell fusion. Only the C-terminal cysteine containing domain of gH has been implicated in cell fusion (Gompels et al., 1988). Analysis of gH antibody resistant HSV-1 mutants has shown that the non-conserved N-terminal domain may interact with gL (Gompels et al., 1991). In this study, antibody recognition was dependent on the interaction of gH with another virus protein (subsequently shown to be gL). The recognition site was shown to map to a conformation-dependent epitope spanning the N-terminal 320 amino acids (Gompels et al., 1991). Similarly, by site-directed mutagenesis and transient

cellular expression studies in HHV-6, it has been shown that the 230 amino acid domain present in the N-terminus of HHV-6 gH is required for interaction with HHV-6 gL (Anderson et al., 1996). Further, it has been shown that HCMV gL can functionally substitute for HHV-6 gL (Anderson et al., 1996) and that the gL glycoproteins of EBV and VZV are functionally interchangeable (Li L. et al., 1997). Although there may be similarity in domains of gH homologs interacting with gL, there appear to be differences in strategies for complex assembly (Table 1.2). The mechanism for HSV-1 gH and gL complex formation is not known, but it does not appear to involve disulphide bonds (Hutchinson et al., 1992b). In contrast, disulphide linkages are an important feature of gH/gL complex formation in betaherpesviruses examined to-date (Table 1.2). Under non-reducing conditions HCMV gH and gL form a high molecular-weight complex that is composed of gH and gL monomers (Kaye et al., 1992). Similarly, interaction between HHV-6 gH and gL results in the formation of a large complex involving intermolecular disulphide bridges (Anderson et al., 1996). However, within the betaherpesviruses, there appear to be differences in HHV-6 gH/gL complex assembly. As compared to intra-and inter-molecular dimer formation between gH and gL in HHV-6, only inter-molecular dimer formation has been observed between gH and gL in HCMV (Anderson et al., 1996).

Studies to elucidate the mechanism of association between the gH-gL complexes and plasma membranes have been done with HSV-1, HCMV, EBV and VZV. Although gH homologs of all the members of the alpha, beta, and gamma herpesviruses have a carboxy-terminal hydrophobic transmembrane anchor (Table 1.1), there is only a single hydrophobic domain at the amino terminus of glycoprotein gL characteristic of a signal sequence, except in VZV gL (Table 1.2). Since there are no additional hydrophobic regions in gL that might serve as a membrane anchor, it has been postulated that gL may be retained at the membrane by association with gH (Hutchinson et al., 1992b; Roop et al., 1993). In the absence of gH, the gL proteins are either secreted, as is the case for herpes simplex virus-1 (Dubin and Jiang, 1995) and cytomegalovirus gL (Spaete et al., 1993), or expressed as a type 2 membrane protein, as is the case for EBV gL (Li Q. et al., 1995). The predicted sequence of VZV gL does not contain a cleaveable signal

peptide but does contain a 16 residues hydrophobic endoplasmic reticulum-targeting sequence (residues 72 to 87) that may serve to anchor the protein in the membrane (Duus et al., 1995). The ER targeting sequence would provide a mechanism for gL to enter the ER without an ER signal peptide and allow it to be processed independently of gH (Duus et al., 1995).

1.2.4 Functions of Herpesvirus Glycoprotein H and L Homologs

Although glycoprotein gH is a minor component of the infected cell surface and virion envelope (Buckmaster et al., 1984; Richman et al., 1986), there is evidence to suggest that it plays an important role in virus replication and pathogenesis. This evidence is based on the following (i) Coding sequences homologous to those of HSV-1 gH have been identified in all members of the alpha, beta, and gamma herpesviruses studied so far. Thus like gB, gH appears to be a member of a subset of conserved genes in all herpesviruses (Cranage et al., 1988; Davison and Taylor, 1987; Gompels et al., 1988; McGeoch et al., 1988). (ii) Viruses which lack gH are not infectious. Using the temperature sensitive mutant, tsQ26, it has been shown that at the non permissive temperature extracellular virus lacks gH and is noninfectious (Desai et al., 1988). Thus like gD or gB (Cai et al., 1988; Ligas and Johnson, 1988), gH appears to be essential for infectivity. Glycoprotein H was also shown to be essential for infectivity in PRV gH null mutants (Peeters et al., 1992) (iii) Monoclonal antibodies (MAbs), specific for HSV-1 gH, can neutralize virus infectivity in the absence of complement (Gompels and Minson, 1986). Similarly, antibodies to gH homologs of HCMV or EBV also neutralize the respective viruses in the absence of complement (Cranage et al., 1988; Gompels et al., 1988; Pachl et al., 1989; Rasmussen et al., 1984; Strnad et al., 1982).

HSV-1 glycoprotein gH is involved in virus penetration (Fuller et al., 1989; Fuller and Lee, 1992). Biochemical approaches combined with qualitative and quantitative electron microscopy have shown that anti gH MAbs inhibit virus penetration without having a significant effect on virus adsorption (Fuller and Lee, 1992; Fuller et al., 1989). A model of virus entry has been proposed in which gD

mediates a stable attachment to host cells that is likely required for penetration, with gH participating in fusion (Fuller and Lee, 1992).

A number of observations suggest that glycoprotein gH plays a role in cell fusion: (i) Studies with HSV-1 gH null mutants showed that the defect in the gH$^-$ virions could be partially overcome by enhancing membrane fusion with polyethylene glycol (PEG), and secondly, gH-virions could block adsorption of superinfecting wild type virions (Forrester et al., 1992). Similar findings have been reported for PRV (Babic et al., 1996). (ii) MAbs to HSV-1 gH inhibit the transfer of virus from infected to uninfected cells during plaque formation by syncytial or nonsyncytial virus strains (Gompels and Minson, 1986). Similar results have been shown using antibodies against the gH homolog of VZV (Keller et al., 1987). (iii) Deletion of the gH gene from a syncytial genetic background (syncytial strain of HSV-1 which contains a substitution of valine for alanine in the gB gene) abolishes cell-cell fusion (Davis-Poynter et al., 1994).

Attempts have been made to investigate the functional domains of glycoprotein gH of HSV-1 which are required for the entry of the virus and for cell fusion (i) Using a COS7 cell transient transfection and complementation system, it has been reported that certain mutations in the C-terminal tail, which is composed of a 14 amino acid residue KVLRTSVPFFWRRE, abolished the ability of a syncytial strain of HSV-1 to induce polykaryocyte formation without any significant effect on the rate at which mutant gH containing envelopes fuse with the cellular membranes (Wilson et al., 1994). It has been concluded that the SVP motif and particularly the valine residue of the cytoplasmic tail of gH is essential for mediating syncytium formation but is not important for the fusion event which permits viral entry (Wilson et al., 1994). (ii) Construction of HSV-1 recombinants that express gH molecules with mutations in the cytoplasmic tail, deletion of the SVP motif, or replacement of valine residue of this triplet by alanine, led to reduced syncytium formation (Browne et al., 1996). These mutants also entered cells less efficiently than wild type virus. (iii) By constructing a set of linker insertion mutants in HSV-1 gH (Galdiero et al., 1997), amino acid residues

691/692, 791, and 799 in the C-terminal third of the external domain were shown to affect the ability of gH to function in cell fusion and virus entry.

As discussed earlier, glycoprotein gL associates with gH either by covalent or by noncovalent interactions, so some of the functions for gH mentioned above are common to gL. Similar to gH, gL plays an important role in the replicaton of herpesviruses. A HSV-1 gL deletion mutant failed to incorporate gH into the virion envelope and was unable to enter cells, although the mutant could adsorb to the cell surface (Roop et al., 1993). By constructing a double mutant (carrying a syn allele at the gB locus and deletion of gH/gL), these mutants could bind to cells but failed to enter and induce cell-cell fusion (Davis-Poynter et al., 1994). Although attempts to achieve HSV-1 induced cell fusion by co-expression of gH and gL or by expression of various combinations of gH and gL with other glycoproteins have not succeeded (Davis-Poynter et al., 1994; Spear, 1993), co-expression of VZV gH and gL in a vaccinia virus expression system resulted in cell fusion (Duus et al., 1995).

Studies have also been conducted to identify the role of gL in viral infectivity or cell fusion and to determine the antigenic structure and functional domains of gL (Novotny et al., 1996). By raising polyclonal and monoclonal anti-gL antibodies against the KOS strain of HSV-1, antigenic determinants were localised to the C-terminal 55 amino acid region of HSV-1 gL. The antibodies specific for these epitopes inhibited virus induced cell fusion, provided that the virus strain expressed the relevant antigenic determinants, but failed to neutralize viral infectivity. These results suggest that there are strain dependent differences in the structure and antigenic conformation of HSV-1 gL and the roles of gL in cell fusion and viral entry may be different for different strains. (Novotny et al., 1996).

1.3 Herpesvirus Glycoprotein M Homologs

1.3.1 Structure of Herpesvirus Glycoprotein M Homologs

Glycoprotein gM is the one of the nonessential glycoproteins which is conserved in all herpesvirus subfamilies. The gene coding for glycoprotein gM has been identified

in HSV-1 (McGeoch et al., 1988), BHV-1 (Vlcek et al., 1995), EHV-1 (Telford et al., 1992), PRV (Dijkstra et al., 1996), VZV (Davison and Scott, 1986), HCMV (Chee et al., 1990; Lehner et al., 1989), MCMV (Scalzo et al., 1995), HHV-6 (Lawrence et al., 1995), EBV (Baer et al., 1984), and HVS (Albrecht et al., 1992).

Glycoprotein gM of HSV-1 is encoded by the UL10 gene present in the unique long region of the genome (McGeoch et al., 1988). There are two potential in frame translation initiation codons for UL10 (McGeoch et al., 1986). The initiation from the first methionine codon should produce a hydrophilic N terminus and may encode a polypeptide of 473 amino acids, whereas the initiation from the second in-frame methionine should produce a potential cleaveable N-terminal signal sequence and may encode a polypeptide of 455 amino acids (McGeoch, 1985; McGeoch et al., 1986). By constructing viruses containing mutations in these methionine codons, it has been shown that UL10 does not specify an amino-terminal cleavable signal sequence and that the translation initiation site is at the first methionine codon (Baines and Roizman, 1993). Amino acid sequence analysis of the UL10 ORF reveals two potential sites for the addition of N-linked oligosaccharides. One site is present within the first hydrophilic region following a hydrophobic domain at amino acid 71 and another site is present at amino acid 247 (McGeoch et al., 1988). The position of the first N-linked glycosylation site is conserved in all the UL10 homologs studied so far. The protein encoded by the UL10 gene has the characteristics of a type III membrane protein with eight potential hydrophobic transmembrane domains (McGeoch et al., 1988). Comparison of HSV-1 gM with other gM homologs shows conservation of (i) a short hydrophilic region at the amino terminus (ii) eight stretches of hydrophobic transmembrane domains and (iii) a hydrophilic and charged carboxy terminus varying in length between 29 (HHV-6) and 134 amino acids (HSV-1). Among the alphaherpesviruses, gM proteins exhibit homologies between 32 and 40% (Dijkstra et al., 1996).

Recently, glycoprotein gM of HSV-1 has been analysed in detail. Antiserum raised against the carboxy terminal region of UL10 immunoprecipitated a protein of 47 kDa from HSV-1 infected cells and from purified virions (MacLean et al., 1993). Glycoprotein gM represents a minor component of the virions in HSV-1 (Baines and

Roizman, 1993; MacLean et al., 1993) compared to being a major component of virions in EHV-1 (Pilling et al., 1994) and PRV (Dijkstra et al., 1996). Enzymatic deglycosylation studies have shown that HSV-1 gM is modified by the addition of only N-linked glycans (Baines and Roizman 1993; MacLean et al., 1993). While PRV and EHV-1 gM homologs have been shown to form dimers (Dijkstra et al., 1996; Osterrieder et al., 1997), oligomerization has not been reported in HSV-1 (Baines and Roizman, 1993). Like HSV-1 glycoprotein gK (Hutchinson et al., 1992a), HSV-1 gM (Baines and Roizman, 1993) differs from other HSV-1 glycoproteins in that it is highly hydrophobic and aggregates when boiled in buffers containing SDS. This aggregation has also been documented for glycoprotein gM of EHV-1 (Pilling et al., 1994) and PRV (Dijkstra, 1996).

1.3.2 Functions of Herpesvirus Glycoprotein M Homologs

Studies have been performed to identify the role of glycoprotein gM in the life cycle of herpesviruses. By constructing HSV-1 UL10 Lac Z insertion mutants, it has been shown that the gM negative mutants were slightly impaired in growth in cell culture compared to wild type and revertant viruses (Baines and Roizmann, 1991; MacLean et al., 1991; MacLean et al., 1993). In vivo, gM deletion mutants showed reduced virulence in a mouse model (MacLean et al., 1993). The deletion of gM obviated the syncytial phenotype of a viral gB mutant, indicating a role of gM in cell to cell fusion (Davis-Poynter et al., 1994). Similarly EHV-1 (Osterrieder et al., 1996) and PRV (Dijkstra et al., 1996) gM homologs have been implicated in cell fusion and may contribute to the spread of virus in cultured cells. The gM homolog of HCMV is part of the gC-II glycoprotein complex, which has been shown to bind heparin and therefore may play a role in attachment of virus to cells (Kari et al., 1994).

1.4 Bovine Herpesvirus Type I

1.4.1 Classification

Bovine herpesvirus has been classified as a member of the family Herpesviridae on the basis of morphological features and physicochemical properties (Armstrong et al., 1961; Madin et al., 1956). On the basis of biological properties such as host range, duration of reproductive cycle, cytopathology, latent infection, genome structure, and sequence homology, it has been classified as a member of the subfamily Alphaherpesvirinae (Roizman et al., 1982). According to the sequence arrangement of the viral genome, BHV-1 is subclassified in the genus Varicellovirus (VZV like viruses) rather than with BHV-2 in the genus Simplexvirus (Brown, 1989). Only one serotype of BHV-1 has been reported by cross neutralisation tests (Nyaga and McKercher, 1979). However, based on their molecular properties such as restriction endonuclease analysis and selective reactivity with monoclonal antibodies and viral protein patterns, BHV-1 strains are subdivided into five subtypes 1, 2a, 2b, 3a and 3b (Wyler et al., 1989). It is impossible to establish a strict correlation between clinical origin of the BHV-1 isolates and their molecular subtypes, however, a partial correlation seems possible in that subtype 1.1 contains infectious bovine rhinotracheitis (IBR) like strains, subtype 1.2a and 1.2b contain mostly infectious pustular vulvovaginitis (IPV) like strains, and subtype 1.3a and 1.3b contain strains exhibiting neuropathogenic potential although subtype 1 strains may also occassionally exhibit this property (Wyler et al., 1989). Antigenically and by restriction endonuclease DNA fingerprinting, the neurological strain was found to be different from BHV-1. So the neurological strain was designated as bovine encephalitis herpesvirus (BEHV) (Bulach and Studdert, 1990; Studdert, 1989) Recently bovine encephalitis herpesvirus has been classified as bovine herpesvirus 5 (BHV-5) (Roizman et al., 1992).

1.4.2 Clinical Syndromes

Diseases associated with BHV-1 are of considerable importance in North America (Gibbs and Rweyemamu, 1977; Yates, 1982). BHV-1 is associated with a

number of clinical syndromes in cattle which may affect animals of various ages (Blood and Radostits, 1989).

1.4.2.1 Respiratory Diseases

Infectious bovine rhinotracheitis (IBR) is by far the most significant and economically important manifestation of BHV-1 infection, specifically in feedlots. Virus entry into the respiratory tract generally occurs by aerosol route or by direct contact with virus present in nasal secretions. Principally, infection involves the upper respiratory tract, but sometimes also affects the lower parts of the lung. After an incubation period of 2-3 days, animals develop a fever, followed by increased respiratory rate, persisting harsh cough, anorexia, depression, and in milking cows there is a severe drop in milk production and emaciation. Within a day or two, a clear bilateral nasal discharge develops and the mucosa of the nares becomes hyperemic (red nose) (Gibbs and Rweyemamu, 1977). In the later stages of the disease, nasal discharge changes from clear to mucopurulent. This acute stage of the disease lasts from 5 to 10 days after which animals recover rapidly. The severity of the clinical disease depends on the strain of the virus, the immunological status of the animal, environmental stressors, secondary infections and the age of the animal. These factors can cause a complex respiratory syndrome known as "shipping fever". In this syndrome, initial BHV-1 infection is followed by secondary bacterial infection (often *Pasteurella haemolytica*) which may produce a potentially fatal pneumonia (Babiuk et al., 1988; Yates, 1982). Mortality in both beef and dairy cattle are primarily due to secondary bacterial infection.

Although infrequent, IBR can occur in young calves and cause either severe respiratory disease or fatal systemic disease and rapid death. Neonatal IBR infections probably occur due to lack of maternal immunity and are complicated by a number of management factors (Mechor et al., 1987).

When the respiratory form of IBR develops in a herd that includes pregnant cattle, 25% of pregnant cattle may abort. Abortions, if they occur, do so after an incubation period of 3-6 weeks and are most common between the 5th and 8th month of pregnancy.

1.4.2.2 Reproductive Diseases

Infectious pustular vulvovaginitis (IPV) is a vaginal and vulvar infection characterized by frequent micturation and tail not returning to normal position. Later on pustules (1-2 mm in diameter) appear which disseminate over the mucosal surface and are sometimes accompanied by mucopurulent discharge. Old pustules break down leaving shallow pink eroded areas. In IPV, nasal discharge is not evident. The acute stage of disease lasts from 2-4 days and the lesions heal 10-14 days after the onset of disease. If systemic infection of calves occurs, abortions are possible (Snowdon, 1965).

During breeding, IPV infected cows can infect bulls, resulting in infectious pustular balanoposthitis (IPB). IPB develops after an incubation period of 1-3 days and is characterized by multiple pustular lesions on the penis, small amounts of exudate and fever (Snowdon, 1965). Infected bulls can transmit IPV to cows even in the absence of visible lesions. This is one of the reasons that all the bulls at artificial insemination units must be seronegative for BHV-1.

1.4.2.3 Encephalitis

Despite BHV-1 neurotropism, encephalitis rarely occurs in cattle. Encephalitis is presumed to occur as an unpredictable sequel to either acute respiratory infection or reactivation of latent virus from trigeminal ganglia and centripetal spread to the brain. The neurological signs are characterized by incoordination, aimless circling, muscular tremors, recumbency, ataxia, blindness, licking of the flanks and eventually death (Kahrs, 1977). Sporadic cases of BHV-1 encephalitis are more prevalent in Australia and Argentina. BHV-1 strains exhibiting neuropathogenic potential represent an antigenic variant and are designated as BEHV or BHV-5.

Additional clinical problems associated with BHV-1 include conjuctivitis, mastitis, metritis, enteritis and dermititis.

1.4.3 Replication

BHV-1 infection of permissive cells is initiated by attachment of envelope glycoproteins gB and or/gD and/or gC (Li Y. et al., 1995; Liang et al., 1991) to cell

surface receptors. Heparan sulphate proteoglycan (HS, HSPG) present on the cell surface has been demonstrated to be the initial receptor for interaction with BHV-1 (Liang et al., 1991, 1993a; Okazaki et al., 1987, 1991). However, some other non-HS receptors may also be important for high affinity binding of BHV-1 (Li Y. et al., 1995). Stable attachment of BHV-1 to the cell surface is followed by penetration into the cell by direct fusion between envelope and plasma membrane. The nucleocapsid and tegument are released into the cytoplasm of the cell and transported to the nucleus through nuclear pores. During productive infection, the viral genes are transcribed in a coordinately regulated and sequentially ordered cascade manner (Fig. 1.3). The viral genes are grouped into three categories named α (which encode immediate early proteins), β (which encode early proteins), and γ (which encode late proteins). The γ gene products can be divided further into $\gamma 1$ and $\gamma 2$ subclasses. The $\gamma 1$ genes are expressed earlier in infection than $\gamma 2$ genes and are affected minimally if viral DNA replication is interrupted, whereas $\gamma 2$ genes are expressed later in infection and are not expressed at all if viral DNA synthesis is blocked. Briefly, the transcription of α genes is induced by tegument proteins such as Btif and VP8 (Misra et al. 1981). The translation of these early genes results in the production of immediate early proteins BICP0, BICP4 and BICP22 (Schwyzer et al., 1993; Wirth et al., 1992). These proteins are necessary for transcription and translation of β genes. Translation of β genes results in the production of early proteins, mainly enzymes such as thymidine kinase and DNA polymerase. A new round of transcription and translation initiated by early proteins yields γ proteins, consisting primarily the structural proteins of the virus. Viral DNA is replicated in the nucleus of the infected cell, with the involvement of cellular and viral proteins, by a rolling *circ*le mechanism that yields head to tail concatemers of unit length DNA (Ben-Porat et al., 1976; Hammerschmidt et al., 1988). Viral DNA, some β proteins and the majority of γ proteins assemble into the nucleocapsids within the nucleus of the infected cell (Ludwig, 1983; Rixon, 1993). The viral envelope is acquired by budding through the nuclear membrane. Release of virions takes place by rupture of infected cells or by fusion of infected cells with non-infected cells (Roizman and Sears, 1996; Ruyechanetal., 1979).

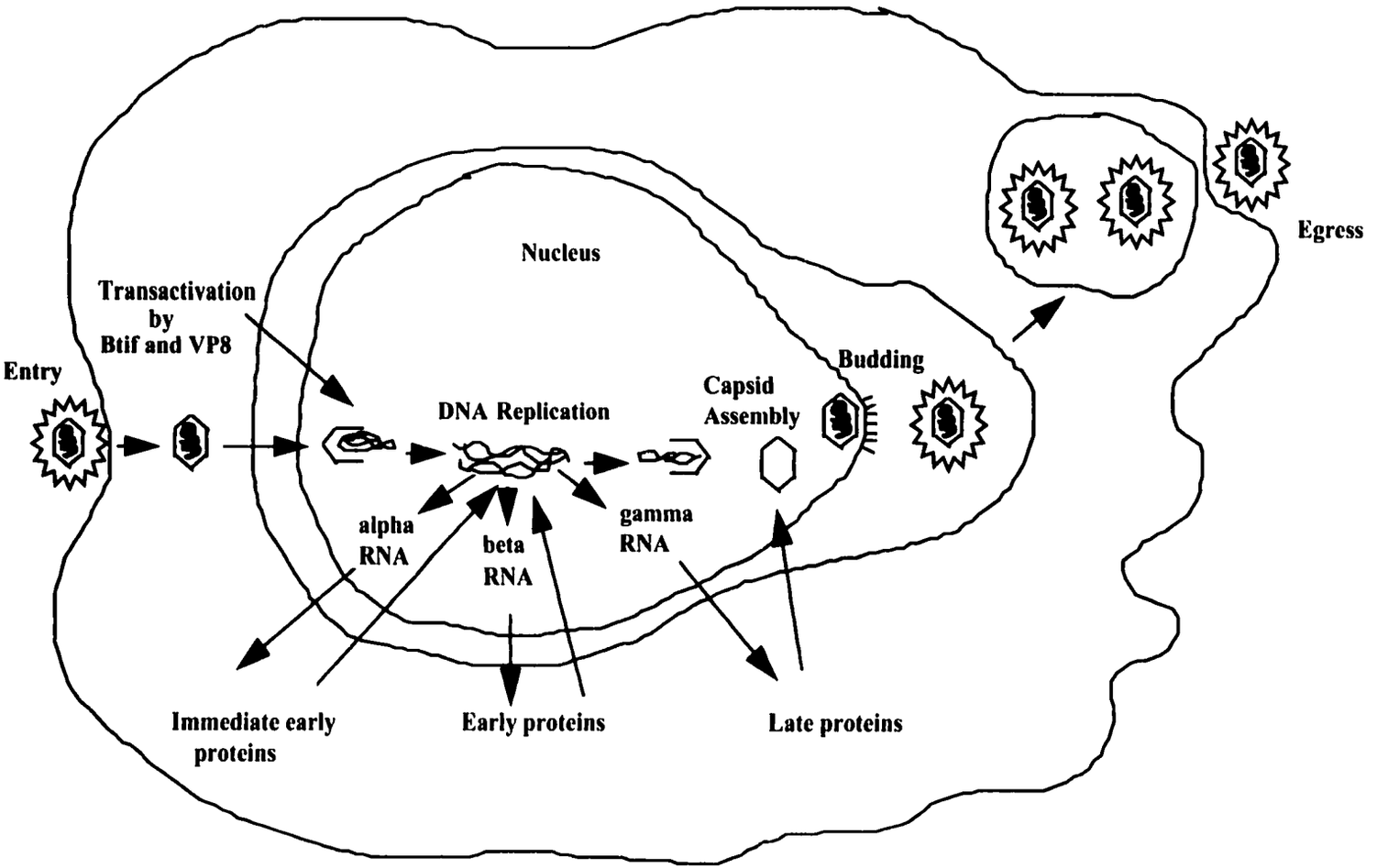


Fig. 1.3 Replication of BHV-1 (adapted from Roizman and Sears, 1990)

1.4.4 Structure of the Virus

BHV-1 appears to have a similar morphology to the other herpesviruses, as studied by electron microscopy using thin sections and negative staining (Armstrong et al., 1961). The general chemical and physical properties of BHV-1 are summarized in Table 1.3.

Table 1.3. Chemical and Physical properties of BHV-1[a]

Diameter (nm)	150-200 (virion) ; 95-110 (nucleocapsid)
Symmetry of capsid	Icosahedral with axial symmetry of capsomeres 5:3:2
Number of capsomeres	162 (150 hexameric and 12 pentameric)
DNA	Linear, double stranded with a molecular weight of 88 Mda and G+C ratio of 72%
Buoyant density in CsCl	1.249-1.254 g/cm3 (virion) 1.730-1.732 g/cm3 (DNA)
Sedimentation coefficient	1680-1830s (virion)
Physical : Infectious particle ratio	200:1

a Gibbs and Rweyemamu, 1977

The virion has four main structural components: envelope, tegument, capsid, and core.

(a) Envelope: The envelope of BHV-1 is similar in structure to the envelope of other herpesviruses (Bocciarelli et al., 1966; Darlington and Moss, 1969) and consists of a double membrane of lipid which is morphologically similar to the cell membrane. The envelope is acquired from the inner lamella of the nuclear membrane, from the cytoplasmic membrane, or from the plasma membrane. Spike-like projections protrude from the envelope (Valicek and Smid, 1976). The spikes are viral glycoproteins that are

involved in various interactions with host cells during virus entry and spread and in the induction of virus-neutralising antibodies.

(b) Tegument: Between the envelope and the capsid is an amorphous proteinaceaous layer known as the tegument (Valicek and Smid, 1976). The proteins present in the tegument play a role in transcription during productive infection.

(c) Capsid: As seen in negatively stained preparations, the capsid of BHV-1 has a distinct hexagonal profile and is composed of capsomeres arranged in an ordered pattern of equilateral triangular facets with five capsomeres on each edge of the facet (Watrach and Bahnemann, 1966). The icosahedral capsid is made up of 162 capsomeres (Caspar and Klug, 1962). The capsomeres are hollow structures of polygonal cross section; each capsomer is 12 nm long, 11.5 nm wide with an axial hole of 3.5 nm (Watrach and Bahnemann, 1966).

(d) The core: The outline of the core, as seen in negatively stained preparations of the virus, is polygonal, usually hexagonal in shape and similar to that of other herpesviruses. Within the core, DNA is packed to form a centrally located electron dense mass.

1.4.5 Structure of the Genome

The genome of BHV-1 is a single linear molecule of double stranded DNA of 135-140 kilobase pairs (kbp) (Mayfield et al., 1983; Wyler et al., 1989). As shown in Fig.1.4, the BHV-1 genome exhibits the typical arrangement of group D herpesviruses. The DNA is composed of a unique long (U_L) segment of approximately 102-104 kbp and a unique short (U_S) segment of approximately 10.5 to 11 kbp flanked by inverted repeat (IRS and TRS) regions of 12 kbp each (Mayfield et al., 1983; Wyler et al., 1989). The inverted repeat region allows the U_S region to invert relative to the U_L region yielding two linear isomers.

Northern (RNA) blot analysis was used to determine the spatial and temporal distribution of BHV-1 transcripts (Wirth et al., 1989). During productive BHV-1

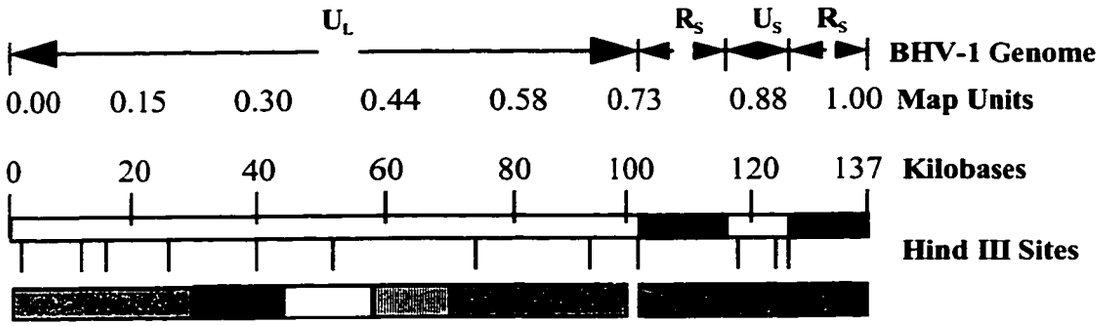


Fig. 1.4 Structure of the BHV-1 Genome. A map of the genome (Cooper strain) as adapted from Mayfield et al., 1983. The long segment extends from map units 0.00 to 0.74. The short segment extends from map units 0.75 to 1.00. The entire genome has been sequenced by international collaboration. Sequencing has been done mainly by Schwyzer et al., 1996 ▨; in data base but not yet published ■ ; Meyer et al., 1997 ▭ ; published by several authors ▥ ;Vlcek et al., 1995 ▧ ; Schwyzer et al., 1994 and preceeding articles ▦ ; Leung-Tack et al., 1994 ▥ .

infection, 54 transcripts ranging from 0.4 to larger than 8 kb have been identified. Of these 54 transcripts, 4 were classified as IE by using the protein synthesis inhibitor cycloheximide. Using the DNA synthesis inhibitor cytosine arabinoside (Ara C), 21 transcripts have been classified as early and 12 transcripts as late, whereas the remaining 17 transcripts could not be classified as early or late. Seal et al., (1991) have identified 59 transcripts ranging in size from 0.6 to 10 kb during productive infection of bovine cells by the BHV-1 Cooper isolate. The additonal transcripts reported by these authors is due to the use of the terminal EcoRI F fragment in northern blotting.

To identify and characterize virus specified proteins, to explain their concerted action, to construct recombinant viruses, and to manipulate the virus for vaccine production or gene therapy, it is necessary to determine the genomic sequence of the BHV-1 virus. Previously, the complete nucleotide sequences of the genomes of VZV (Davison and Scott, 1986), HSV-1 (McGeoch et al., 1988) and EHV-1 (Telford et al., 1992) have been determined. Analysis of the BHV-1 genome started with a mapping of

cloned HindIII fragments which was established twelve years ago (Mayfield et al., 1983). Sequence analysis has progressed gradually from 1987 when the structure of the thymidine kinase (TK) gene was first determined (Bello et al., 1992). Recently, the complete nucleotide sequence of the BHV-1 genome (136 kb) has been completed by international cooperation (Schwyzer, 1995) (Fig. 1.4).

Based on electrophoretic analysis of radiolabeled virions by sodium dodecyl sulphate-polyacrylamide gels or virus encoded proteins by linear gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 33 structural and 15 non-structural proteins have been identified in productive BHV-1 infection (Misra et al., 1981; Bolton et al., 1983). With the availability of the complete BHV-1 sequence, it has become possible to identify and predict all the potential open reading frames (ORFs) (Schwyzer and Ackermann, 1996). Analysis of the sequence revealed 67 unique genes and two genes, both duplicated in the inverted repeats. Thus, BHV-1 encodes at least 69 proteins (Table 1.4). Most of the BHV-1 ORFs show sequence homology with counterparts in HSV-1 and occur in the inverted prototypic orientation compared to the orientation of the genes present in HSV-1 (Mayfield et al; 1983; Tikoo et al., 1995; Schwyzer and Ackerman, 1996). As most of the proteins and glycoproteins of BHV-1 are homologous in structure and function to their HSV-1 homologs, the ORFs of BHV-1 are named after their HSV-1 homologs (Tikoo et al., 1995; Schwyzer and Ackermann, 1996). Protein products for many of the ORFs have been identified by physical methods,whereas the functions of the proteins encoded by other ORFs were deduced from sequence homology with well characterized proteins of other herpesviruses (McGeoch and Cook, 1994).

1.4.6 Proteins

1.4.6.1 Regulatory Proteins

(a) Regulatory proteins during lytic infection: Like other herpesviruses, the BHV-1 genes are expressed in a coordinately regulated manner during the lytic cycle of

Table 1.4.BHV-1 Genes and their Products[a]

Function/Location [b]	Name [c]	Remarks [d]
Glycoprotein	gB, gC, gD, gE, gI, gH, gG, gK, gM	gB, gC, gD, gE, gI, gH, gG, gK have been characterized.
Envelope	UL20, UL34, UL43, UL49.5	UL49.5 has been characterized.
Tegument	UL11 (myristylated), UL36, UL37, UL41 (virion host shutoff protein), UL46, UL47, UL48, UL49, US9	UL47 (VP8), UL48 (BTIF) and UL49 (VP22) have been characterized. UL47 and UL48 are also regulatory.
Capsid	UL18, UL19 (major capsid protein), UL26/UL26.5 (serine protease and its substrate), UL35, UL38	
Other virion	UL4, UL21, UL24	UL24 has been characterized.
Cleavage/Packaging	UL6, UL15, UL25, UL28, UL32, UL33	
DNA replication	UL5, UL8, UL9 (origin binding protein), UL29 (major DNA binding protein), UL30, UL42 (DNA polymerase and accessory protein), UL52 (helicase/primase).	BHV-1 has a functional ori_S(Geng et al., 1995) but no ori_L.
Enzyme	UL2 (uracil DNA glycosylase); UL12 (DNase); UL23 (thymidine kinase); UL39, UL40 (subunits 1 and 2 of ribonucleotide reductase); UL50 (dUTPase); UL13, US3 (protein kinase)	Proven enzyme activity only for UL23 and UL50. Although, the proteins coded by UL39 and UL40 ORFs have been identified, however, the enzyme activity of these proteins have not been proved yet.
Regulatory	BICP0, BICP4, BICP22, BICP27	All these proteins have been characterized.
Unknown	UL3, UL7, UL14, UL16, UL17, UL31, UL51, US2	
BHV-1 specific	UL0.5, UL3.5, *circ*, US1.5	These proteins are absent in HSV-1. *Circ* protein in BHV-1 has been characterized.
Absent in BHV-1	gJ, UL8.5, UL9.5, UL45, UL55, UL56, ORF P, γ34.5, US8.5, US10, US11, and ICP47	These proteins are present in HSV-1

a. Adapted from: 'Molecular virology of ruminant herpesviruses' by Schwyzer, M and Ackermann, M in: Veterinary Microbiology vol. 53. 1996.

b Proteins are grouped according to their presumed function or location in the virion.

c The nomenclature for BHV-1 proteins closely follows that for HSV-1, where proteins are designated by the numbers of the open reading frames encoding them (except for the glycoproteins and regulatory proteins)

d.About 22 proteins of BHV-1 have been characterized.

BHV-1 infection. The proteins encoded by BHV-1 genes can be categorised as immediate early (IE or α), early (E or β) or late (L or γ) depending upon the order of their synthesis in infected cells (Misra et al., 1981; Wirth et al., 1989).The IE genes encoding these proteins are clustered in the inverted and terminal repeat and adjacent region. To date, three major IE proteins BICP0, BICP4, and BICP22 have been characterized. In addition, IE protein *circ*, E protein BICP27, and IE gene trans-inducing factor (α TIF) have been characterized.

(i) BICP0: BHV-1 BICP0, a homolog of HSV-1 ICP0 protein and protein 61 of varicella-zoster virus, is encoded by an unspliced early 2.6 kb RNA (ER2.6) and spliced 3' coterminal 2.9 kb immediate-early RNA (IER 2.9) (Wirth et al., 1992). The gene is located at the junction of the right end of the U_L and inverted repeats (IRs) and encodes an open reading frame of 676 amino acids with a predicted Mr of 68 kDa (Wirth et al., 1992). BICP0 contains a cysteine rich zinc finger domain bearing homology to similar domains found in ICP0 of HSV-1 and protein 61 of VZV (Wirth et al., 1992). BICP0 acts as a strong activator of a variety of homologous and heterologous promoters, including the IER4.2/2.9 of BICP4/BICP0 proteins and ER2.6 promoter of BICP0 protein, and as a repressor of the IER1.7 promoter of BICP22 protein (Wirth et al., 1992). Rabbit sera raised against synthetic oligopeptides of BICP0 identified a protein of 97 kDa by western blots, during the IE phase and later periods of BHV-1 infection, indicating a switch of BICP0 synthesis from IER2.9 to ER2.6 (Fraefel et al., 1994b). BICP0 protein synthesized by baculovirus recombinants produced a band of the same size (97 kDa) as in mammalian cells. Immunoflourescent staining of BHV-1 infected cells revealed nuclear localization of BICP0 (Fraefel et al., 1994b). Using a transient expression assay, it was shown that zinc is important for BICP0-induced transactivation of the IER4.2/2.9 promoter (Fraefel et al., 1994b). Recently, it has been shown that BICP0 is required for the synthesis of glycoprotein gC in MDBK cells as glycoprotein gC failed to appear during infection of MDBK cells with recombinant BHV-1 containing a β-gal gene inserted into the BICP0 coding sequence (Koppel and Schwyzer, 1996)

(ii) BICP4: BICP4, a homolog of HSV-1 ICP4 protein is encoded by an immediate early 4.2 kb RNA (IER4.2) present in the inverted repeats (IRs and TRs) (Schwyzer et al., 1993). It is the largest IE protein of 1343 amino acids with a predicted Mr of 136 kDa (Schwyzer et al., 1993). By transient expression assays it has been shown that BICP4 can cause transrepression of IER4.2/2.9/1.5/1.7 of BICP4/BICP0/*circ*/BICP22 proteins and transactivation of ER2.6 of BICP0 protein and latency associated transcript (LAT) (Schwyzer et al., 1993; Gstaiger and Schaffner, 1994). Antipeptide sera raised against N- and C- terminal oligopeptides of the BICP4 protein recognised a protein of 180 kDa, which is post translationally modified by phosphorylation, poly(ADP)ribosylation, adenylation, and guanylation (Koppel et al., 1995). Immunofluorescent staining of BHV-1 infected cells indicated the presence of the BICP4 protein in the nucleus of the infected cell (Koppel et al., 1995).

(iii) BICP22: BICP22, a homolog of HSV-1 ICP22 protein is synthesized by identical spliced immediate early transcripts (IER1.7) throughout infection (Schwyzer et al., 1994). The BICP22 gene, which is present in inverted repeats (IRs and TRs), is expressed with IE and late kinetics under the control of a single promoter (Schwyzer et al., 1994). Antisera directed against a C-terminal oligopeptide of BICP22 detected a major band of 50 kDa and a minor band of 35 kDa (Koppel et al., 1995). BICP22 protein has been detected in the nucleus by immunofluorescence and plays a role in IE regulation, acting as a trancriptional repressor (Koppel et al., 1995).

(iv) BICP27: BHV-1 BICP27, a homolog of HSV-1 ICP27 (also called Vmw63), is encoded by an early 1.7 kb RNA (ER1.7) located in the left genomic terminus of BHV-1 (Singh et al., 1996). The BICP27 gene encodes a protein of 400 amino acids with a predicted m.w. of 43 kDa (Singh et al., 1996). Rabbit antisera raised against an amino terminal oligopeptide recognised a protein of 50 kDa and the protein of the same m.w. was also seen by expression in baculovirus (Singh et al., 1996). BICP27 has been shown to accumulate in the nuclei of BHV-1 infected cells (Koppel et al., 1995; Singh et al., 1996). Transient expression assays using target genes with different poly(A) sites showed that BICP27 may be involved in 3' processing of mRNA (Singh et al., 1996).

(v) Circ: *Circ* protein, a homolog of VZV ORF2 and EHV-1 ORF3 but without any homolog in HSV-1, is synthesized by a coterminal spliced immediate early 1.5 kb RNA (IER1.5) and unspliced 1.1 kb late RNA (LR1.1) located in the left end of the genome (Fraefel et al., 1993). The *circ* gene encodes a protein of 247 amino acids with a predicted m.w. of 43 kDa (Fraefel et al., 1993). Rabbit antisera raised against a carboxy terminal oligopeptide recognised a virion associated protein of 34 kDa, which accumulated in the cytoplasm of infected cells, whereas expression of the *circ* gene by a recombinant baculovirus resulted in three recombinant proteins of 32, 34, and 35 kDa (Fraefel et al., 1994a). The recombinant *circ* proteins and the *circ* protein specified by BHV-1 were both myristylated. The *circ* gene could be deleted from the BHV-1 genome without affecting virus replication in cell culture (Fraefel et al., 1994a). Recently, it has been shown that the *circ* gene is non-essential for infection, replication, establishment of latency, and reactivation of BHV-1 in calves (Engels et al., 1996).

(vi) BTIF: BHV-1 α TIF (BTIF), a homolog of HSV-1 α TIF (also known as VP16, Vmw65, ICP25), is a gene product of gene UL48 (Carpenter and Misra, 1992). Sequence analysis of the gene has identified a 1512 bp ORF encoding a protein of 504 amino acids (Carpenter and Misra, 1992). BTIF has been shown to be part of the virion tegument and is synthesized in the later stages of infection (Misra et al., 1994). By transient expression assays, it has been shown that BTIF can transactivate and stimulate the activity of the IE1 promoter and this process requires recognition of a TAATGARAT-like motif by BTIF and cellular factors (Misra et al., 1994). However, unlike HSV-1 α TIF, its carboxy terminal region is insufficient for efficient transactivation but it requires cooperative determinants in both its amino terminal and carboxy terminal regions and further it has been shown that HSV-1 α TIF and BTIF transactivate transcription by using different cellular targets (Misra et al., 1994; Misra et al., 1995).

(vii) VP8: VP8, a homolog of HSV-1 protein VP13/14 (coded for by the UL47 gene), is the most abundant viral protein found in the tegument of the virions and in virus infected cells (Marshal et al., 1986; Misra et al., 1981). The gene coding for VP8 is located between 0.088 and 0.108 map units on the BHV-1 genome and contains a 2226

bp ORF encoding a 742 amino acid protein (Carpenter and Misra, 1991). Using VP8 specific antiserum, the VP8 protein has been detected in virions and in virus infected cells as a 96 kDa protein containing O-linked oligosaccharides (van Drunen Littel-van den Hurk et al., 1995). VP8 has been detected in the nucleus of BHV-1 infected cells as early as 2 hr postinfection, which supports the hypothesis that VP8 may modulate the transactivation of immediate early genes by BTIF gene products, analogous to HSV-1 VP13/14 protein (Carpenter, 1992; van Drunen Littel-van den Hurk et al., 1995). VP8 has been shown to be a good immunogen in cattle, which induces humoral and specifically cell mediated immunity to BHV-1 (van Drunen Littel-van den Hurk et al., 1995).

(b) Regulatory protein during latent infection: BHV-1 establishes life long latent infection in sensory ganglionic neurons of infected cattle (Rock, 1994). The molecular mechanisms underlying BHV-1 latency and reactivation are poorly understood. In contrast to the 70 to 80 viral genes expressed during a lytic infection of bovine cells, gene expression is strictly limited during latent infection. Only a small region of the genome designated as the latency related gene (LRG) is transcriptionally active in latent infection of neurons which is controlled by a 980 nucleotide promoter. This promoter controls transcription in the opposite sense to the BICP0 gene (Bratanich and Jones, 1992; Bratanich et al., 1992; Kutish et al., 1990). DNA sequence analysis of the LRG revealed two major ORFs (Kutish et al., 1990). Antibodies directed against a synthetic oligopeptide present on the amino terminus of ORF 2, detected a 41 kDa protein designated as latency related protein (LRP) in mammalian cells transfected with LRG expression vectors (Hossain et al., 1995). Using reverse trancriptase-mediated PCR (RT-PCR) it was shown that LR RNA is spliced near the C-terminus of ORF 2 and a fraction of LR RNA is poly (A)+, which is translated into a 41 kDa protein (Hossain et al., 1995). Analysis of nuclear and cytoplasmic fractions of cells transfected with LRG expression vectors indicated that LRP is predominantly located in the nucleus (Hossain et al., 1996). Also it has been shown that LRP exerts its inhibitory effect on cell cycle progression by forming a complex with cyclin A and this activity may neutralize the

deleterious effect of cyclin A and enhance the survivability of infected neurons during latency (Schang et al., 1996).

1.4.6.2 Virus encoded Enzymes

The genome of alphaherpesviruses is capable of encoding a large array of enzymes involved in nucleic acid metabolism and DNA synthesis. Generally, these enzymes are not essential for productive infection in cell culture. However, enzymes regulating nucleic acid metabolism constitute important virulence determinants since mutants defective for enzyme synthesis are often impaired in infectivity *in-vivo*. Some of these enzymes like thymidine kinase, deoxyuridine triphosphatase, and ribonucleotide reductase encoded by the BHV-1 genome have been sequenced and the enzyme activity has been demonstrated for thymidine kinase and deoxyuridine triphosphatase.

(i) Thymidine kinase (TK): TK is one of the most studied viral proteins due to its importance in the virulence of herpesviruses and development of antiherpetic drugs. It has been shown that thymidine kinase activity induced in BHV-1 infected cells differed from those of host cell enzyme activity in substrate specificity, thermostability, and in its ability to use different nucleotide triphosphates as phosphate donors (Weinmaster et al., 1982). In addition to confirming the virus specific nature of this enzyme activity, isolation of a BHV-1 mutant lacking this enzyme activity suggested that the TK activity is not essential for viral replication (Kit and Qavi, 1983; Weinmaster et al., 1982). Deletion of the TK gene reduces the virulence of virus in vivo (Kit et al., 1986) and also reduces the abortifacient activity of BHV-1 in heifers (Miller et al., 1991; Miller et al., 1995). It has also been shown that due to serological differentiation of TK- mutants, it can be used as a marker vaccine (Kaashoek et al., 1996b).

The BHV-1 TK gene has been mapped (Bello et al., 1987) and the sequence of the TK gene of the Cooper strain of BHV-1 has been determined (Bello et al., 1992). The TK gene sequence of the Cooper strain differed from the TK gene sequences of other BHV-1 strains (Kit and Kit., 1987; Mittal and Field, 1989). A TK open reading frame of 1077 bp coding for a protein of 359 amino acids has been identified on the left

hand of the BHV-1 gH gene (Bello et al., 1992). Northern blot analysis indicated that the BHV-1 TK transcript was 4.3 kb in size and 3' coterminal with the downstream 3.1 kb transcript of the BHV-1 glycoprotein gH (Bello et al., 1992). The 5' end of the TK transcript was found to overlap a 5.2 kb transcript with opposite polarity to the TK mRNA (Bello et al., 1992).

(ii) Deoxyuridine triphosphatase (dUTPase): This enzyme is conserved among alphaherpesviruses. The main function of this enzyme is to hydrolyse dUTP to dUMP, providing both a mechanism to prevent incorporation of dUTP into DNA and a pool of dUMP for conversion to dTMP by thymidylate synthetase. It, therefore, plays a vital role in maintaining cell viability by preventing extensive uracil substitution, which could lead to DNA fragmentation.

The gene coding for BHV-1 dUTPase is colinear with the HSV-1 UL50 gene (the HSV-1 dUTPase gene) and sequence analysis revealed an ORF of 975 bp capable of encoding a protein of 325 amino acids (Liang et al., 1993b). The enzyme activity of BHV-1 dUTPase has been proved by partial deletion of the putative dUTPase gene and this mutant was found to be fully viable for virus growth in cell culture (Liang et al., 1993b).

(iii) Ribonucleotide reductase (RNR): RNR is one of the key enzymes required for efficient viral growth and DNA replication in non dividing cells. The enzyme is made up of two proteins, the large subunit and the small subunit, which are tightly associated in an $\alpha 2\beta 2$ complex and both subunits are required for activity. RNR catalyses the reduction of ribonucleoside diphosphates to their corresponding 2'-deoxy forms and thus creating a pool of substrates for DNA synthesis.Although the enzyme activity of RNR encoded by BHV-1 has not been proved yet, both subunits of bovine RNR have been sequenced and the proteins coding by them have been identified. The gene coding for the small subunit (R2) of RNR is a homolog of the HSV-1 UL40 gene and codes for an ORF of 314 amino acids (Simard et al., 1992) whereas the gene coding for the large subunit (R1) is a homolog of HSV-1 UL39 and codes for an ORF of 787 amino acids (Simard et al., 1995). It has been shown that R1-specific antiserum reacted with a polypeptide of 85 kDa and R2-specific antiserum reacted with a polypeptide of 34 kDa

in BHV-1 infected cells (Simard et al., 1995). Further it has been shown that the R1 protein was synthesized earlier than the R2 protein and R1 protein accumulated to a higher level than R2 protein in contrast to the transcript level of R2, which was shown to be higher than R1 (Simard et al., 1995).

Although the DNA sequence for the BHV-1 genes UL30; UL42; UL5, UL8 and UL52; UL12; UL13 and US3 coding for their respective HSV-1 counterparts DNA polymerase; DNA polymerase accessory protein; helicase/primase complex; DNase; protein kinase (Leung-Tack et al., 1994; Meyer et al., 1997; Schwyzer et al., 1996; Vlcek et al., 1995) has been determined, these BHV-1 enzymes have not been isolated and functionally tested yet.

1.4.6.3 Glycoproteins

Sequencing of the BHV-1 genome has led to the identification of at least 9 genes with the potential to encode glycoproteins (Fitzpatrick et al., 1989; Khadr et al., 1996; Leung-Tack et al., 1994; Meyer et al., 1991; Rebrodosa et al., 1994; Schwyzer and Ackermann, 1996; Schwyzer et al., 1996; Tikoo et al., 1990; Vlcek et al., 1995; Whitbeck et al., 1988). These glycoproteins were named gB, gC, gD, gE, gG, gH, gI, gK, and gM, in accordance with their homology to the sequences of herpes simplex virus 1 (HSV-1) which is considered to be the prototype of the alphaherpesvirinae subfamily (Tikoo et al., 1995).

SDS-PAGE analysis of [^{3}H] glucosamine labeled BHV-1 proteins has revealed the presence of 11 glycosylated proteins associated with the viral envelope (Bolton et al., 1983; Marshall et al., 1986; Misra et al., 1981). Production of a series of monoclonal antibodies against purified BHV-1, has allowed the identification of 10 glycoproteins with molecular weights ranging from 42 to 180 kd (Baranowski et al., 1993; Marshall et al., 1986; van Drunen Littel-van den Hurk et al., 1984a). Immunoprecipitation under reducing and non reducing, and peptide mapping studies indicated that these 10 glycoproteins are derived from 6 glycoproteins namely gI, gII, gIII, gIV, gp93, and gp42 (Baranowski et al., 1993; Marshall et al., 1986; van Drunen Littel-van den Hurk and Babiuk, 1986a). According to their analogous counterparts in

HSV-1 and the herpesvirus nomenclature, glycoproteins gI, gII, gIII and gIV are now called gB, gH, gC and gD, respectively. Glycoproteins gB, gC and gD have been well characterized (Collins et al., 1984; Marshal et al., 1986; van Drunen Littel-van den Hurk et al., 1984; van Drunen Littel-van den Hurk and Babiuk, 1986a), and their genes have been mapped and sequenced (Fitzpatrick et al., 1989; Misra et al., 1988; Tikoo et al., 1990; Whitbeck et al., 1988; Zamb, 1987). Recently, several minor glycoproteins of BHV-1 gE, gI, gG and gK have been identified. Some of the properties of glycoproteins gB, gC, gD, gE, gI, gG and gK are discussed below:

(i) Glycoprotein gB: BHV-1 gB is one of the major glycoprotein found in the virion envelope and plasma membrane of virus infected cells (Marshall et al., 1986; van Drunen Littel-van den Hurk et al., 1984). DNA sequence analysis has shown that BHV-1 gB is a 933 amino acid molecule located in the U_L region of the genome. Glycoprotein B is a type III integral membrane protein, which includes an unusually long putative amino terminal signal sequence located between residues 50 to 66, the large hydrophobic transmembrane domain between residues 759 to 828 with multiple alpha helices stretching alternately back and forth across a lipid bilaminar membrane, and a 104 amino acid cytoplasmic tail (Misra et al., 1988; van Drunen Littel-van den Hurk et al., 1992; Whitbeck et al., 1988; Zamb, 1987). The amino acid sequence contains five potential sites for the addition of N-linked oligosaccharides and 10 cysteine residues (Misra et al., 1988; Whitbeck et al., 1988). The ectodomain region of all gB homologs is well conserved especially the 10 cysteine residues and many of the N-linked glycosylation sites. BHV-1 gB is synthesized on the ER where high mannose type oligosaccharides are added co-translationally (van Drunen Littel-van den Hurk and Babiuk, 1986a). The polypeptide backbone is a 105 kD protein which is glycosylated to a 117 kD intermediate precursor. This intermediate precursor undergoes further processing in the Golgi to form a mature protein of 130 kD containing N-linked complex and high mannose type oligosaccharides. The majority of this mature form of gB is cleaved to form proteins of 74 and 55 kD, which are linked to each other by disulfide bonds to form heterodimers.

BHV-1 gB is one of the essential viral glycoproteins (Whitbeck et al., 1988; Wyler et al., 1989) playing an important role in initial virus attachment (Liang et al., 1991). It has been suggested that BHV-1 gB interacts with cells in a two step fashion, with initial binding to cellular heparan sulfate (HS) receptors followed by secondary high affinity binding to a non -HS receptor. The heparan binding domain was localized to the N-terminal region of gB (Li et al., 1995; Li et al., 1996). Following initial interaction with the cell, gB is involved in cell fusion and virus penetration (Dubuisson et al., 1992; Fitzpatrick et al., 1990). It has been shown that cleavage of gB may be necessary for cell to cell spread of the virus (Blewett and Misra, 1991; Kopp et al., 1994).

In addition to being involved in virus cell interactions, gB also induces a strong neutralising antibody response and is recognised by CD4+ helper T lymphocytes. Immunization of cattle with purified gB elicited significant protection from BHV-1 challenge (Babiuk et al., 1987; Gao et al., 1994; Israel et al., 1992; van Drunen Littel-van den Hurk et al., 1990). Due to its immunogenicity, it has been suggested that gB may be an important candidate for developing a BHV-1 subunit vaccine.

(ii) Glycoprotein gC: BHV-1 gC is one of the major glycoproteins present on the envelope of the virions and the plasma membrane of virus infected cells (Marshall et al., 1986; van Drunen Littel-van den Hurk et al., 1984). DNA sequence analysis has shown that BHV-1 gC is a 521 amino acid molecule coded for by the U_L region of the genome. Glycoprotein C is a type I integral membrane protein which includes an amino-terminal signal sequence between residues 7 to 21, a carboxy terminal hydrophobic transmembrane domain between residues 467 to 500, and a cytoplasmic tail located between residues 501 to 521 (Fitzpatrick et al., 1989). It contains an R-G-D motif between residues 141 to 260 typical of cellular adhesion glycoproteins, a region homologous to class II MHC antigen between residues 261 to 370, and a region homologous to C2-set members of the immunoglobulin superfamily (Fitzpatrick et al., 1989). It contains 4 potential N-linked glycosylation sites in the amino-terminal region and a serine and threonine rich region of 60 aminoacids between residues 32 and 92 for the addition of O-linked oligosaccharides (Fitzpatrick et al., 1989). Glycoprotein gC is

found as a 180/97 kDa dimeric glycoprotein which contains both N-linked complex-type and O-linked oligosaccharides (Marshall et al., 1986; Okazaki et al., 1987; van Drunen Littel-van den Hurk and Babiuk, 1986a).

BHV-1 gC is a target for neutralizing monoclonal antibodies and complement mediated lysis (Collins et al., 1984; Fitzpatrick et al., 1990; Marshall et al., 1988; van Drunen Littel-van den Hurk et al., 1985). Immunization of cattle with affinity purified BHV-1 glycoprotein gC results in protection from a lethal challenge of BHV-1 and *P. haemolytica* (Babiuk et al., 1987). In addition, cattle vaccinated with vaccinia virus expressing BHV-1 gC developed neutralizing antibodies at levels that were protective against lethal challenge (van Drunen Littel-van den Hurk et al., 1989). BHV-1 gC is also recognized by CD4+ and CD8+ T lymphocytes (Denis et al., 1993; Hutchings et al., 1990; Leary and Splitter, 1990a). B cell epitopes (Fitzpatrick et al., 1990) and T cell epitopes (Leary and Splitter 1990a) have been identified on this glycoprotein. BHV-1 gC is not an essential protein for replication in cell culture but gC- mutants have lower replication efficiencies and shorter shedding periods during experimental *in vivo* infections (Liang et al., 1993a). Glycoprotein C is the primary attachment protein for BHV-1 and has been shown to mediate attachment to tissue culture cells in a heparin dependent manner (Liang et al., 1991; Okazaki et al., 1987, 1991).

(iii) Glycoprotein gD: BHV-1 gD is one of the major glycoproteins found on the surface of the virion and virus infected cells (Marshall et al., 1986; van Drunen Littel-van den Hurk et al., 1984). It appears to be the first glycoprotein synthesized by BHV-1 during a productive infection (Hughes et al., 1988; Misra et al., 1981, 1982; Nelson et al., 1989). The gene coding for BHV-1 gD is located in the U_S region of the genome (Tikoo et al., 1990). The ORF of gD codes for a polypeptide of 417 amino acids, which contains an amino terminal signal sequence of 18 amino acids, a large amino-terminal extracellular domain of 343 amino acids, a hydrophobic transmembrane domain of 29 amino acids and a highly charged cytoplasmic domain of 29 amino acids at the carboxy terminus (Tikoo et al., 1990). BHV-1 gD contains three potential sites for the addition of N-linked oligosaccharides (Tikoo et al., 1990), two of which are actually utilized (Tikoo et al., 1993a).Glycoprotein D occurs both as a 71-77 kDa monomer and a non-

covalent homodimer of 140-150 kDa. (Hughes et al., 1988; Marshall et al., 1986; Okazaki et al., 1987). BHV-1 gD contains both N-linked complex and O-linked oligosaccharides (van Drunen Littel-van den Hurk and Babiuk, 1986a). The addition of N-linked oligosaccharides is not necessary for processing and transport of the glycoproteins to the cell surface, however it is important for the formation of discontinuous epitopes (Tikoo et al., 1993b).

Glycoprotein D is an essential protein for viral replication in cell culture (Fehler et al., 1992) and is involved in a number of important functions. Glycoprotein D is suggested to play an important role in virus attachment as it has been shown that after initial binding of gC and gB to cellular HS receptors followed by high affinity binding of gB to non HS receptors, gD binds to non HS receptors (Li et al., 1995; Liang et al., 1993a; Okazaki et al., 1991). Using gD deletion mutants, it has been shown that gD is essential for virus penetration (Fehler et al., 1992). In addition, BHV-1 gD is also required for direct cell to cell spread of the virus (Fehler et al., 1992). BHV-1 gD expressed at high levels in mammalian cells can cause membrane fusion (Tikoo et al., 1990). However, recently it has been demonstrated that the function of gD for virus entry into cells can be taken over by some other constituent(s) of BHV-1 particles and that the major role of gD may be to ensure penetration-competent conformation of other glycoproteins in the virus envelope rather than directly causing membrane fusion (Schroder et al., 1997).

Antibodies against gD are the most effective at neutralizing BHV-1 virus, compared to antibodies against BHV-1 gB and gC (Marshall et al., 1988). Immunization of cattle with BHV-1 gD results in protection from a lethal challenge of BHV-1 and *P. haemolytica* (Babiuk et al., 1987; van Drunen Littel-van den Hurk et al., 1993a). BHV-1 gD is also recognised by CD4+ helper and CD8+ cytotoxic T cells (Denis et al., 1993; Hutchings et al., 1990). B cell (Tikoo et al., 1993b) and T cell epitopes (Leary and Splitter, 1990b; Tikoo et al., 1995a) have been identified for this glycoprotein.

(iv) Glycoprotein gE: The homologs of BHV-1 gE have been found within all members of the alphaherpesvirinae subfamily indicating an important and conserved role of this

glycoprotein in the biology of the alphaherpesvirinae subfamily (Leung-Tack et al., 1994; Rebrodosa et al., 1994). The BHV-1 gene homologous to HSV-1 gE is located in the unique short region of the genome and codes for an open reading frame of 575 amino acids (Leung-Tack et al., 1994; Rebrodosa et al., 1994). The amino acid sequence contains two highly conserved domains (amino acid 275 to 283 and 358 to 370), two conserved cysteine clusters C1 and C2 (the C1 cluster contains 4 conserved cysteine residues and the C2 cluster contains 6 cysteine residues) and a highly conserved γ -Fc binding motif (Bienkowska-Szewczyk et al., 1996; Leung-Tack et al., 1994; Rebrodosa et al., 1994). The BHV-1 gE has characteristics of a membrane glycoprotein including an amino terminal hydrophobic domain resembling a signal sequence, a carboxy terminal hydrophobic transmembrane domain, two putative N-glycosylation and two putative O-glycosylation sites (Rebrodosa et al., 1994). Glycoprotein gE is processed in the endoplasmic reticulum by the addition of N-linked oligosaccharides to the 84-86 kDa polypeptide chain, which is further processed in the Golgi to an Endo H resistant form (Baranowski et al., 1996b; Whitbeck et al., 1996). Recently, BHV-1 glycoprotein gE has been shown to form a non-covalent oligomer with another BHV-1 glycoprotein, gI in infected and transfected cells and the formation of this complex is necessary for efficient processing of the gE precursor to its mature form (Whitbeck et al., 1996). Using a baculovirus expression system, it was shown that BHV-1 glycoproteins gE and gI can form a complex. For the formation of this complex, a cystine noose motif present in the conserved C1 cluster at the N-terminal part of glycoprotein E is essential (Bienkowska-Szewczyk et al., 1996).

BHV-1 gE is a non-essential glycoprotein which is dispensable for virus replication in cell culture (Rijsewijk et al., 1995). However, a glycoprotein gE deletion mutant of BHV-1 showed reduced virulence and is considered to be a good candidate for a modified live or killed BHV-1 marker vaccine (Kaashoek et al., 1993; van Engelenburg et al., 1994; van Engelenburg et al., 1995). Like the HSV-1 and PRV homologs, BHV-1 gE may be required for cell to cell spread of the virus (Rebrodosa et al., 1995; Rijsewijk et al., 1995). BHV-1 gE is not crucial for the establishment of BHV-1 latent infection. However, BHV-1 gE deleted mutants establish a latent state

less efficiently, and are less likely to be reactivated than wild type virus, suggesting its role in mediating neuronal spread of the virus (Kaashoek et al., 1996a).

(v) Glycoprotein gI: The BHV-1 gene homologous to HSV-1 gI is located in the unique short region of the genome and codes for an open reading frame of 380 amino acids with features characteristics of a membrane associated glycoprotein (Leung-Tack et al., 1994). Alignment analysis of BHV-1 gI with HSV-1 gI counterparts revealed several conserved domains located in the first half of the protein, suggesting a common biological function of gI like proteins (Leung-Tack et al., 1994). The polypeptide backbone of BHV-1 gI is 36-40 kDa which is processed in the endoplasmic reticulum and Golgi apparatus by N-glycosylation to an endo H resistant mature glycoprotein of 61-63 kDa (Baranowski et al., 1996b; Whitbeck et al., 1996). It has also been shown that the mature product of BHV-1 gI is further processed into two smaller fragments of 45 and 16 kDa and that the 45 kDa fragment associates with glycoprotein gE. (Whitbeck et al., 1996). BHV-1 gI is a non essential glycoprotein and is dispensable for virus multiplication in cell culture (Rijsewijk et al., 1995). In vitro phentypic analysis of gI deleted BHV-1 mutants indicated a role for BHV-1 gI in cell to cell spread of the virus (Rijsewijk et al., 1995). Deletion of the gI gene reduced BHV-1 virulence in calves, suggesting its use as a marker vaccine (Kaashoek et al., 1996a). Although the HSV-1 gI-gE complex can bind to the Fc region of immunoglobulin G (Bell et al., 1990; Hanke et al., 1990; Johnson et al., 1988), the BHV-1 gI-gE complex does not function as a Fc receptor (Whitbeck et al., 1996).

(vi) Glycoprotein gG: The BHV-1 gene homologous to HSV-1 gG is located in the unique short region of the genome and codes for an open reading frame of 444 amino acids with features characteristic of membrane glycoproteins (Leung-Tack et al., 1994). Recently, the protein products of the gG gene have been identified and they have been shown to be secreted in culture media of BHV-1 infected cells as a 65 kDa protein band and very diffusely migrating proteoglycans with an apparent molecular weight between 90 and greater than 280 kDa, the later constituting glycoproteoglycan G (gpgG) (Keil et al., 1996). Keil et al. (1996) showed that glycoprotein gG contains both N- and O-linked oligosaccharides. It has been demonstrated, for the first time for herpesvirus

proteins, that BHV-1 gG protein is further post-translationally modified by the addition of glycosaminoglycans. Glycoprotein gG is nonessential for replication of BHV-1 (Kaashoek et al., 1996a). In contrast, plaque size formation is affected by deletion of the BHV-1 gG encoding gene and the BHV-1 gG negative mutant has reduced virulence in calves (Kaashoek et al., 1996a; Rijsewijk et al., 1995).

(vii) Glycoprotein gK: In BHV-1, a gene homologous to HSV-1 gK has been identified and sequenced (Khadr et al., 1996). The BHV-1 gK gene has been located in the unique long region of the genome and codes for a protein of 338 amino acids with a molecular mass of 37.5 kDa. It possesses characteristics of membrane glycoproteins, including a potential cleaveable signal sequence, three transmembrane domains and two potential N-linked glycosylation sites. A 30 kDa primary translation product was shown to be encoded by the BHV-1 gK gene, which is modified by the addition of N-linked oligosaccharides to a 38 kDa product (Khadr et al., 1996). Biological properties of BHV-1 gK are not known, but in HSV-1, gK is an essential glycoprotein involved in cell fusion and several syncytial mutations have been mapped to the gK gene (Debroy et al., 1985; Hutchinson et al., 1992a; MacLean et al., 1991; Pogue-Geile and Spear, 1987; Ramaswamy and Holland, 1992). In addition, HSV-1 gK may also be involved in virus egress (Hutchinson and Johnson, 1995).

2.0 OBJECTIVES

Bovine herpesvirus-1 (BHV-1) is an economically important virus. Furthermore it provides a useful model for studying the interactions between the virus and its natural host. An understanding of these interactions should help us implement control measures and thereby reduce economic losses due to this agent. Since glycoproteins have been implicated as major viral constituents, dictating various virus-cell interactions, characterization of these glycoproteins should establish a basis for defining the role of these glycoproteins in pathogenesis of BHV-1 and immune responses of the host.

The BHV-1 genome codes for a large number of different glycoproteins. The major glycoproteins; gB, gC, and gD have been extensively studied in the past few years and their structure, their role in the pathogenesis of BHV-1, and interactions with the host immune system have been well defined (Tikoo et al., 1995). However, the structure of the minor glycoproteins of BHV-1 and their role in immune responses and virus-cell interactions are still to be defined. The essential minor glycoproteins gH and gL and nonessential minor glycoprotein gM in other herpesviruses characterized to date indicate that they may play important roles in the virus life cycle.

The studies described in this thesis are directed towards the characterization of the minor glycoproteins gL, gH and gM of BHV-1 with the following specific aims:

(i) Sequencing of a 3'-coterminal gene cluster containing UL1, UL2, UL3 and UL3.5 located at the right end of the HindIII L fragment of the BHV-1 genome.

(ii) Transcriptional analysis of the gene cluster mentioned in objective (i).

(iii) Identification and characterization of glycoproteins gL and gH encoded by UL1 and UL22 genes, respectively of BHV-1.

(iv) Construction of infectious vaccinia virus recombinants expressing glycoprotein gL and gH to study the interactions between these glycoproteins and to study the immunogenicity of these glycoproteins.

(v) Identification and characterization of glycoprotein gM encoded by the UL10 gene of BHV-1.

3.0 IDENTIFICATION AND TRANSCRIPTIONAL ANALYSIS OF A 3'-COTERMINAL GENE CLUSTER CONTAINING UL1, UL2, UL3 AND UL3.5 OPEN READING FRAMES OF BOVINE HERPESVIRUS-1 (BHV-1)

3.1 Introduction

Bovine herpesvirus type 1 (BHV-1), a member of the subfamily Alphaherpesvirinae (Roizman et al., 1982), genus Varicellovirus (Brown, 1989), is an important pathogen of cattle which causes severe respiratory infections including shipping fever (Yates, 1982). The BHV-1 genome is a linear double stranded DNA molecule with a length of 135-140 kilobases (Mayfield et al., 1983; Wyler et al., 1989). The genome can be divided into a unique long region (U_L [105 kb]) and a short region which are covalently linked to each other (Mayfield et al., 1983; Wyler et al., 1989). The unique short region (U_S [11kb]) is flanked by two inverted repeats (IR [12 kb each]) which enable the viral genome to exist in two-isomeric structures (Mayfield et al., 1983).

The HSV-1 genome has been shown to contain at least 72 genes (McGeoch et al., 1988), many of which have been characterized at the protein level. The genome of BHV-1 has not been sequenced completely. However, Northern blot analysis has identified 54 to 59 BHV-1 specific transcripts in a productive BHV-1 infection (Seal et al., 1991;Wirth et al., 1989). This compares well with 33 structural proteins and 15 non structural proteins reported to be encoded by the BHV-1 genome (Bolton et al., 1983; Misra et al., 1981). Although the tentative number of BHV-1 specific transcripts and proteins is known, definitive gene assignments are available for only a few proteins (Tikoo et al., 1995).

In this study, we report the DNA sequence, gene arrangement and amino acid sequence of a group of four colinear open reading frames (ORFs) of BHV-1, starting from the right end of the HindIII L fragment of the BHV-1 Cooper isolate (Mayfield et al., 1983), between map units 0.734 and 0.712. Based on positional and sequence similiarities between BHV-1 and other members of alphaherpesviruses (Davison and Scott, 1986;

Dean and Cheung, 1993; McGeoch et al., 1988), we named the identified BHV-1 ORFs UL1, UL2, UL3 and UL3.5. Northern blot analysis helped to map the UL1, UL2, UL3 and UL3.5 transcripts and revealed complex overlapping transcripts having similar transcription termination sequences.

3.2 Materials and Methods

3.2.1 Cells and Virus.

The Cooper isolate of BHV-1 was grown in Madin-Darby bovine kidney (MDBK) cells cultivated in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (Gibco\BRL Burlington, Ontario, Canada). BHV-1 was assayed by plaque titration as described previously (Rouse and Babiuk, 1978).

3.2.2 Cloning and DNA sequencing.

The construction and restriction mapping of a pBR322-based plasmid library, containing fragments of the BHV-1 Cooper strain genome has been described in detail (Mayfield et al., 1983). The HindIII L fragment cloned into pBR322 (pSD72; Mayfield et al., 1983) was digested with appropriate restriction enzymes and 2.2 kb (HindIII-NruI) and 1.8 kb (SacI-SacI) fragments (Fig. 3.1, B) were cloned into pTZ18R and pTZ19R (Pharmacia Biotech., Quebec, Canada). A library of nested deletions was generated using a double stranded nested deletion kit (Pharmacia Biotech., Quebec, Canada). Single stranded copies of cloned inserts were generated using M13KO7 helper phage. Sequencing of single stranded DNA was performed by the dideoxy chain termination method using a T_7 sequencing kit (Pharmacia Biotech., Quebec, Canada). The analog 7-Deaza-2'-deoxy-GTP was substituted for dGTP in sequencing reactions to minimize band compressions resulting from the high G+C content (72%) of BHV-1 DNA (Plummer et al., 1969). Both DNA strands were sequenced for accuracy. Sequencing products were labeled with ^{35}S-dATP (Amersham Oakville, Ontario, Canada) and visualized by autoradiography after electrophoresis. The DNA sequence was analyzed using IBI\Pustell

DNA\Protein Sequence Analysis software (International Biotechnologies Inc., New Haven, Conn.) and PC\Gene software (Intelligenitics).

3.2.3 RNA isolation.

Total cellular RNA was isolated from infected (multiplicity of infection = 10) or mock infected cells at two hour intervals from 2 to 10 hour post infection by the guanidinium method (Chomczynski and Sacchi, 1987). Briefly, BHV-1 infected and normal MDBK cells were washed three times with 0.1M ice cold PBS and lysed with cell lysis buffer (containing equal amounts of water saturated phenol and solution D [4M guanidinium, 50mM EDTA, 25mM citric acid, 0.5% sarcosyl], 0.1M sodium acetate and 0.7% β-mercaptoethanol) pipetted directly on to the cell monolayer. The cell lysate was collected, mixed with chloroform-isoamyl alcohol solution (500 µl\2.5 ml of lysate) and centrifuged at 4000 rpm at 15^0C for 15 min. RNA in the aqueous phase was precipitated with isopropanol and pelleted by centrifugation. The RNA pellet was redissolved in solution D, precipitated with isopropanol, pelleted and finally dissolved in DEPC treated water. Polyadenylated RNA from BHV-1 infected and normal MDBK cells was isolated with a Quick Prep Micro mRNA purification kit (Pharmacia Biotech., Quebec, Canada).

3.2.4 Northern (RNA) blot analysis.

Equal amounts of RNA (20 µg) were run through 1 % agarose gel containing formaldehyde (Sambrook et al., 1989). After electrophoresis, RNA was transferred to Zeta probe nylon membrane (Pharmacia Biotech, Quebec, Canada) with 20X SSC (1X SSC is 0.15 M NaCl with sodium citrate at 0.015 M pH 7.0) by the method of Southern (Southern, 1975). The membrane was baked for 2 hrs at 80^0C under vaccum. The HindIII L fragment and its subclones were radiolabeled with [alpha-^{32}P] dCTP by nick translation (Rigby et al., 1977). Oligonucleotides (27 mer and 30 mer) were end labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Sambrook et al., 1989). The filters were prehybridized for 4 to 6 hrs in a reaction mixture containing 5X SSC, 5X Denhardt's (1X Denhardt's solution is 0.2% each of bovine serum albumin, Ficoll and polyvinyl pyrollidone), 50% formamide, 50mM NaH_2PO_4, 0.1% SDS, 1% glycine solution

[combined pH of solution is 6.5]. Hybridization with nick translated and ^{32}P end labelled oligonucleotide probes were performed at 45^0C in the presence of prehybridization buffer. The blots were washed with 2X SSC containing 0.1% SDS for 15 min at room temperature twice, then at room temperature with 0.5X SSC containing 0.1% SDS for 30 min and finally with 0.1X SSC containing 0.1% SDS at 45^0C for 30 min before exposing to X-Omat AR film (Eastman Kodak, Rochester, New York, USA) at -70^0C.

3.3 Results

3.3.1 Sequence analysis.

We have determined the nucleotide sequence of the right hand portion of the pSD72 (HindIII L fragment) of the BHV-1 Cooper isolate (Mayfield et al., 1983) from 0.734 to 0.712 map units. The sequenced region contains 3113 bp and is G+C rich (71%). The nucleotide sequence is numbered from right to left with respect to the conventional BHV-1 genome map, beginning with the HindIII site at the junction of HindIII L and C fragments (Mayfield et al., 1983). ORF and codon usage analysis indicated the presence of four genes oriented in the same direction. Since the prototypic U_L region of HSV-1 (McGeoch et al., 1988), runs antiparallel to the U_L region of BHV-1 (Mayfield et al., 1983; Tikoo et al., 1995), we analyzed putative translational products of these genes for homology with proteins encoded at the left end of the HSV-1 U_L region (McGeoch et al., 1988). The deduced amino acid sequence of the second ORF revealed the presence of a uracil-DNA glycosylase signature sequence (Caradona and Cheng, 1980) with 68% homology to the HSV-1 UL2 product (uracil-DNA glycosylase; Mullaney et al., 1989), a well conserved gene within herpesviruses. Since amino acid sequence analysis of ORFs 1 and 3 also showed significant homology to the HSV-1 UL1 and UL3 gene products (McGeoch et al., 1988) respectively, we designated these genes as BHV-1 UL1, UL2, UL3 and UL3.5. The location and the predicted amino acid sequences of these ORFs are depicted in Figs 3.1 and 3.2.

Fig. 3.1. Map location of the BHV-1 UL1-UL3.5 gene cluster. (A) Schematic diagram of the BHV-1 (Cooper strain) genome as adapted from Mayfield et.al., 1983. The genome is divided into a unique long (U_L), and a unique short (U_S) segment flanked by inverted repeat regions (R_S). Location of HindIII restriction endonuclease cleavage sites is indicated. (B) Expanded portion of 0.734 to 0.705 map units of HindIII-L fragment. (C) Enlarged portion of HindIII-NruI and SacI-SacI overlapping clones. (D) Location of open reading frames UL1, UL2, UL3 and UL3.5. Arrow indicates direction of transcription. (E) Location of TATA boxes (T), repeats (RR) and polyadenylation site (A_n). (F) Location and orientation of oligonucleotide probes

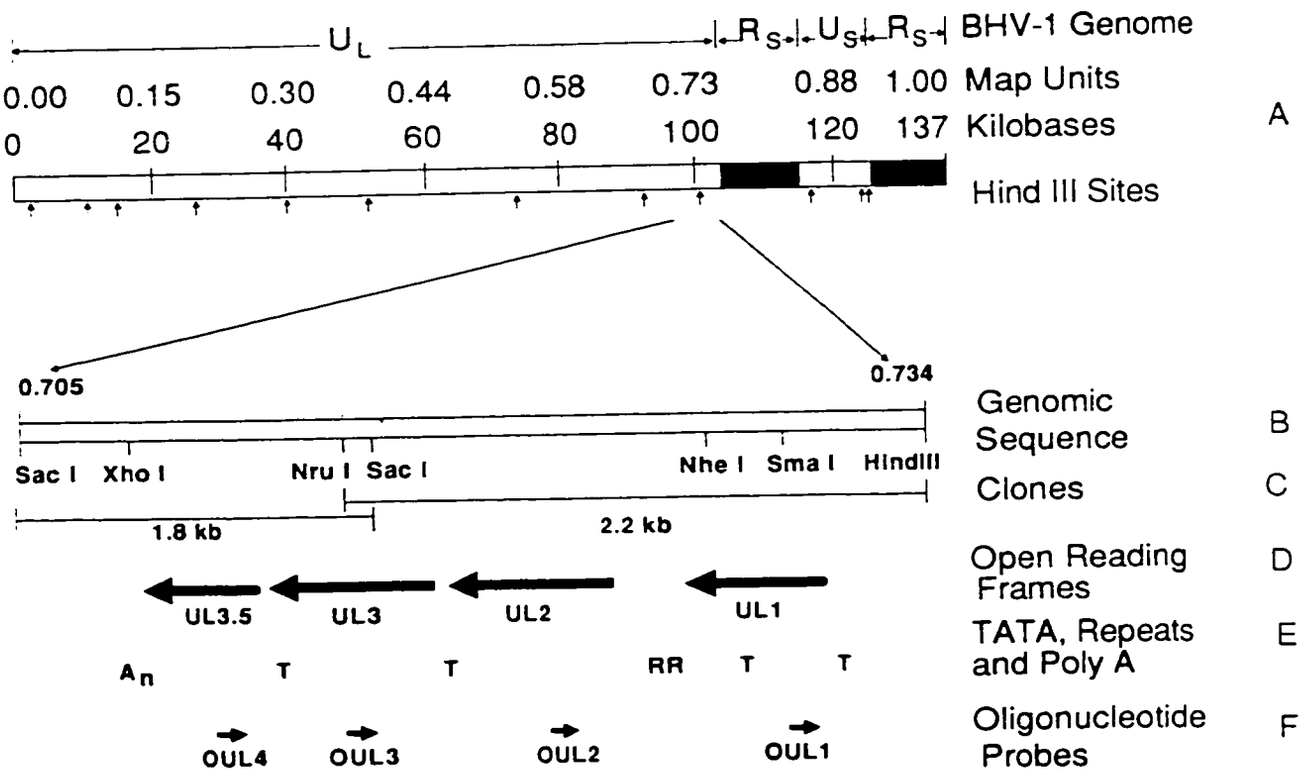
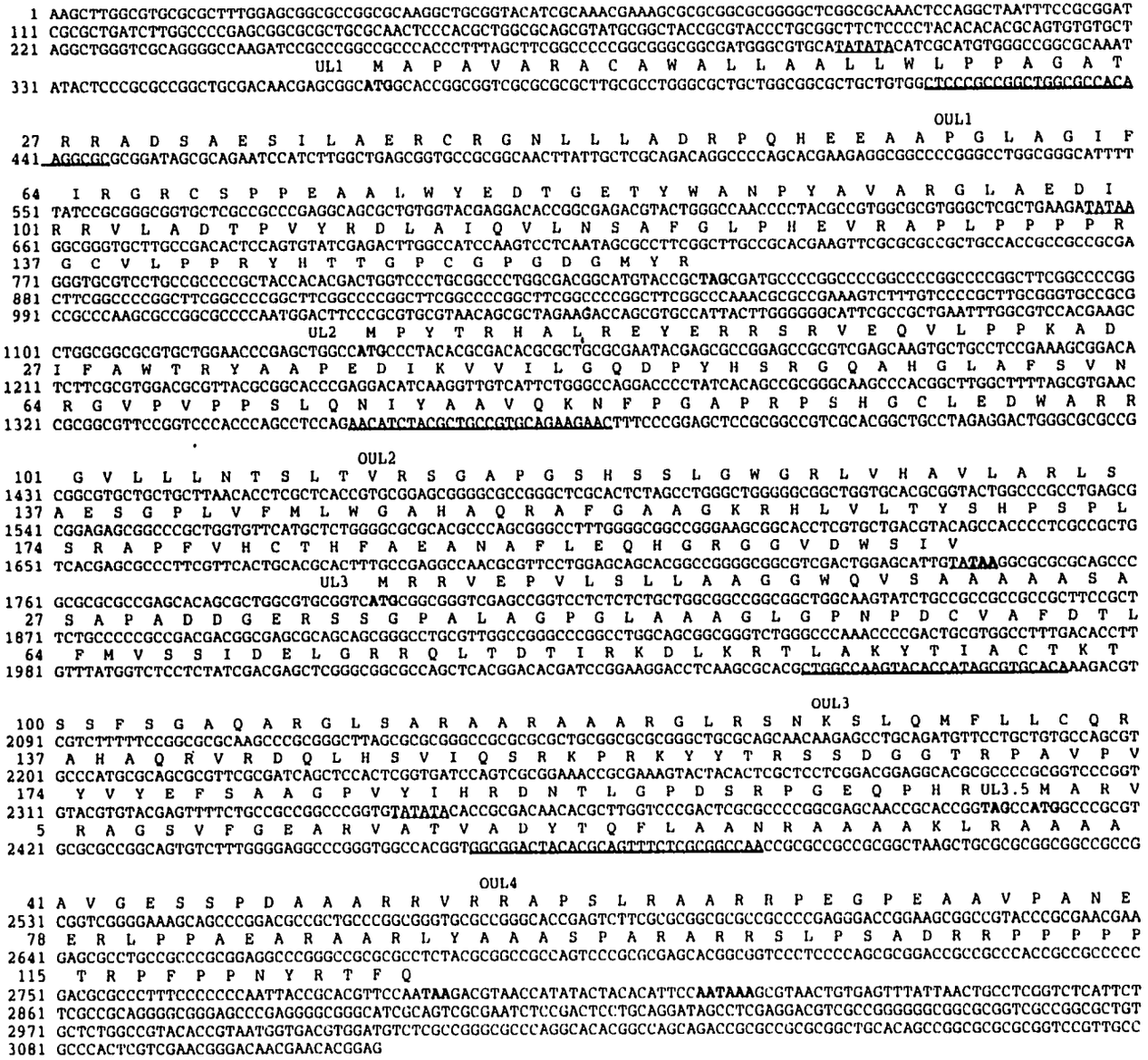

U_L
R_S
U_S
R_S
BHV-1 Genome
0.00 0.15 0.30 0.44 0.58 0.73 0.88 1.00
Map Units
0 20 40 60 80 100 120 137
Kilobases
A
Hind III Sites
0.705
0.734
Genomic Sequence
B
Sac I Xho I Nru I Sac I Nhe I Sma I HindIII
Clones
C
1.8 kb
2.2 kb
Open Reading Frames
D
UL3.5 UL3 UL2 UL1
TATA, Repeats and Poly A
E
An T T RR T T
Oligonucleotide Probes
F
OUL4 OUL3 OUL2 OUL1

Fig. 3.2. DNA sequence and deduced amino acid sequence of the BHV-1 UL1-UL3.5 gene cluster. Nucleotide sequence of the 3113 bp long, right hand portion of the pSD 72 (HindIII L fragment) of BHV-1 Cooper strain from 0.734 to 0.712 map units. Nucleotide sequence is numbered from right to left with respect to the conventional BHV-1 genome map, beginning with HindIII site at the junction of HindIII L and C fragments (Mayfield et.al., 1983). The deduced amino acid sequences of UL1, UL2, UL3 and UL3.5 ORFs are indicated. Location of translation initiation codons, termination codons and polyadenylation signal are indicated by bold face letters. Putative TATA boxes are indicated by a single underline. The 27 or 30 mer oligonucleotide probes used for transcriptional analysis are complimentary to the nucleotide sequences and are indicated by a double underline.

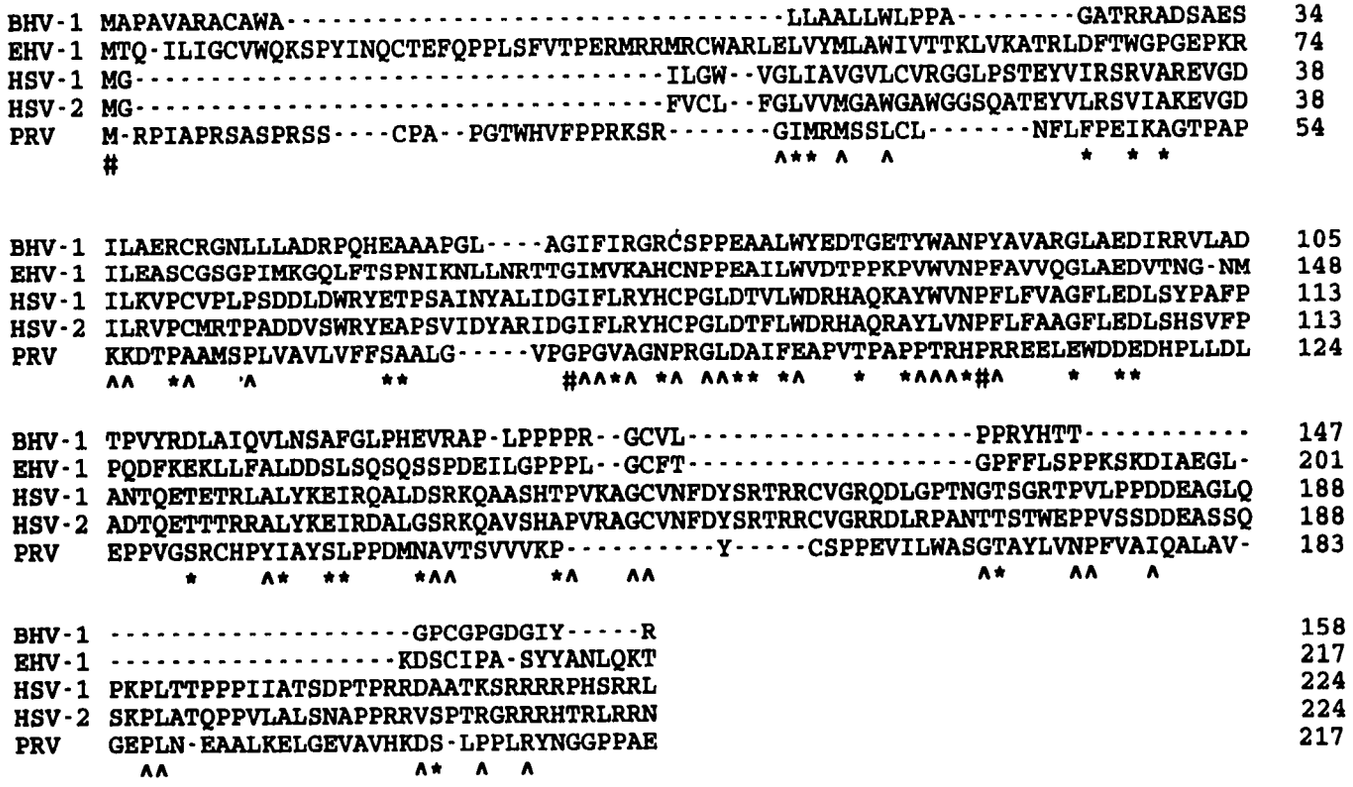

UL1
OUL1
UL2
OUL2
UL3
OUL3
OUL4

3.3.1.1 UL1.

The UL1 ORF starts at nucleotide 363 and ends at nucleotide 836 encoding a protein of 158 amino acids (Fig. 3.2) with a predicted molecular mass of 17 kDa and an isoelectric point of 7.2. It contains an N-terminal signal sequence of 25 amino acids as the length, relative hydrophobicity and consensus cleavage site is characteristic of eukaryotic signal sequences (Fig. 3.2; von Heijne, 1986). Cleavage of the signal sequence would be predicted to occur after the alanine residue at position 25, such that alanine would occupy both positions at -3 and -1, and would conform to the strict requirements for specific amino acids at these key positions (von Heijne, 1986). There is no consensus sequence for the addition of N-linked oligosaccharides (Kornfeld and Kornfeld, 1985). However, there are several serine and threonine residues which could accept O-linked oligosaccharides. Immediately downstream of UL1 is a region of four direct repeats of CCCCGG and six direct repeats of CTTCGGCCCCGG from nucleotide 845 to 940 (Fig. 3.2). The location of the UL1 gene in BHV-1 is equivalent to the genome location of PRV UL1 (Dean and Cheung, 1993), HSV-1 and HSV-2 UL1 (McGeoch et al., 1988, 1991), VZV ORF 60 (Davison and Scott, 1986), EHV-1 ORF 63 (Telford et al., 1992) and Marek's disease virus (MDV) UL1 (Yoshida et al., 1994) genes. Since, gene arrangement appears to be conserved in alphaherpesviruses (Dean and Cheung, 1993) including BHV-1 (Fig. 3.1), the BHV-1 UL1 is expected to encode a positional gL homolog (Forghani et al., 1994; Hutchinson et al., 1992b; Klupp et al., 1994).

Comparison of the predicted translation product of BHV-1 UL1 with homologs of four alphaherpesviruses showed limited sequence homology (12%; Fig. 3.3). As shown in Fig. 3.3, a highly conserved region extends from amino acid 61 to 88 in BHV-1 UL1. Of the 28 residues in this region, 10 residues are conserved among all four UL1 homologs. BHV-1 UL1 has 27%, 24%, 33%, 29%, 21%, and 15% homology with HSV-1 UL1 (McGeoch et al., 1988), HSV-2 UL1 (McGeoch et al., 1991) EHV-1 ORF 63 (Telford et al., 1992), MDV UL1 (Yoshida et al., 1994), PRV UL1 (Dean and Cheung, 1993) and VZV ORF 60 (Davison and Scott, 1986), respectively.

Fig. 3.3. Amino acid homology of alphaherpesvirus UL1 homologs. Predicted amino acid sequences of UL1 homologs of HSV-1 (McGeoch et al., 1988), HSV-2 (McGeoch et al., 1991), EHV-1 (Telford et al., 1992) and PRV (Dean and Cheung, 1993) were compared with the predicted amino acid sequence of BHV-1 UL1. Gaps (dashes) in the protein sequences have been introduced to yield maximal alignment. Perfectly conserved, well conserved and 3 out of 5 identical residues (consensus sequence) are indicated by #, * and ^ signs, respectively. Alignment and homology searches were performed by using CLUSTAL programme of PC \ GENE software (Intelligenitics).


BHV-1 MAPAVARACAWA-----LLAALLWLPPA--------GATRRADSAES 34
EHV-1 MTQ-ILIGCVWQKSPYINQCTEFQPPLSFVTPERMRRMRCWARLELVYMLAWIVTTKLVKATRLDFTWGPGEPKR 74
HSV-1 MG-----ILGW--VGLIAVGVLCVRGGLPSTEYVIRSRVAREVGD 38
HSV-2 MG-----FVCL--FGLVVMGAWGAWGGSQATEYVLRSVIAKEVGD 38
PRV M-RPIAPRSASPRSS----CPA--PGTWHVFPPRKSR-------GIMRMSSLCL-------NFLFPEIKAGTPAP 54
BHV-1 ILAERCRGNLLLADRPQHEAAAPGL----AGIFIRGRCSPPEAALWYEDTGETYWANPYAVARGLAEDIRRVLAD 105
EHV-1 ILEASCGSGPIMKGQLFTSPNIKNLLNRTTGIMVKAHCNPPEAILWVDTPPKPVWVNPFAVVQGLAEDVTNG-NM 148
HSV-1 ILKVPCVPLPSDDLDWRYETPSAINYALIDGIFLRYHCPGLDTVLWDRHAQKAYWVNPFLFVAGFLEDLSYPAFP 113
HSV-2 ILRVPCMRTPADDVSWRYEAPSVIDYARIDGIFLRYHCPGLDTFLWDRHAQRAYLVNPFLFAAGFLEDLSHSVFP 113
PRV KKDTPAAMSPLVAVLVFFSAALG-----VPGPGVAGNPRGLDAIFEAPVTPAPPTRHPRREELEWDDEDHPLLDL 124
BHV-1 TPVYRDLAIQVLNSAFGLPHEVRAP-LPPPPR--GCVL-----PPRYHTT----- 147
EHV-1 PQDFKEKLLFALDDSLSQSQSSPDEILGPPPL--GCFT-----GPFFLSPPKSKDIAEGL- 201
HSV-1 ANTQETETRLALYKEIRQALDSRKQAASHTPVKAGCVNFDYSRTRRCVGRQDLGPTNGTSGRTPVLPPDDEAGLQ 188
HSV-2 ADTQETTTRRALYKEIRDALGSRKQAVSHAPVRAGCVNFDYSRTRRCVGRRDLRPANTTSTWEPPVSSDDEASSQ 188
PRV EPPVGSRCHPYIAYSLPPDMNAVTSVVVKP-----Y-----CSPPEVILWASGTAYLVNPFVAIQALAV- 183
BHV-1 -----GPCGPGDGIY-----R 158
EHV-1 -----KDSCIPA-SYYANLQKT 217
HSV-1 PKPLTTPPPIIATSDPTPRRDAATKSRRRRPHSRRL 224
HSV-2 SKPLATQPPVLALSNAPPRRVSPTRGRRRHTRLRRN 224
PRV GEPLN-EAALKELGEVAVHKDS-LPPLRYNGGPPAE 217

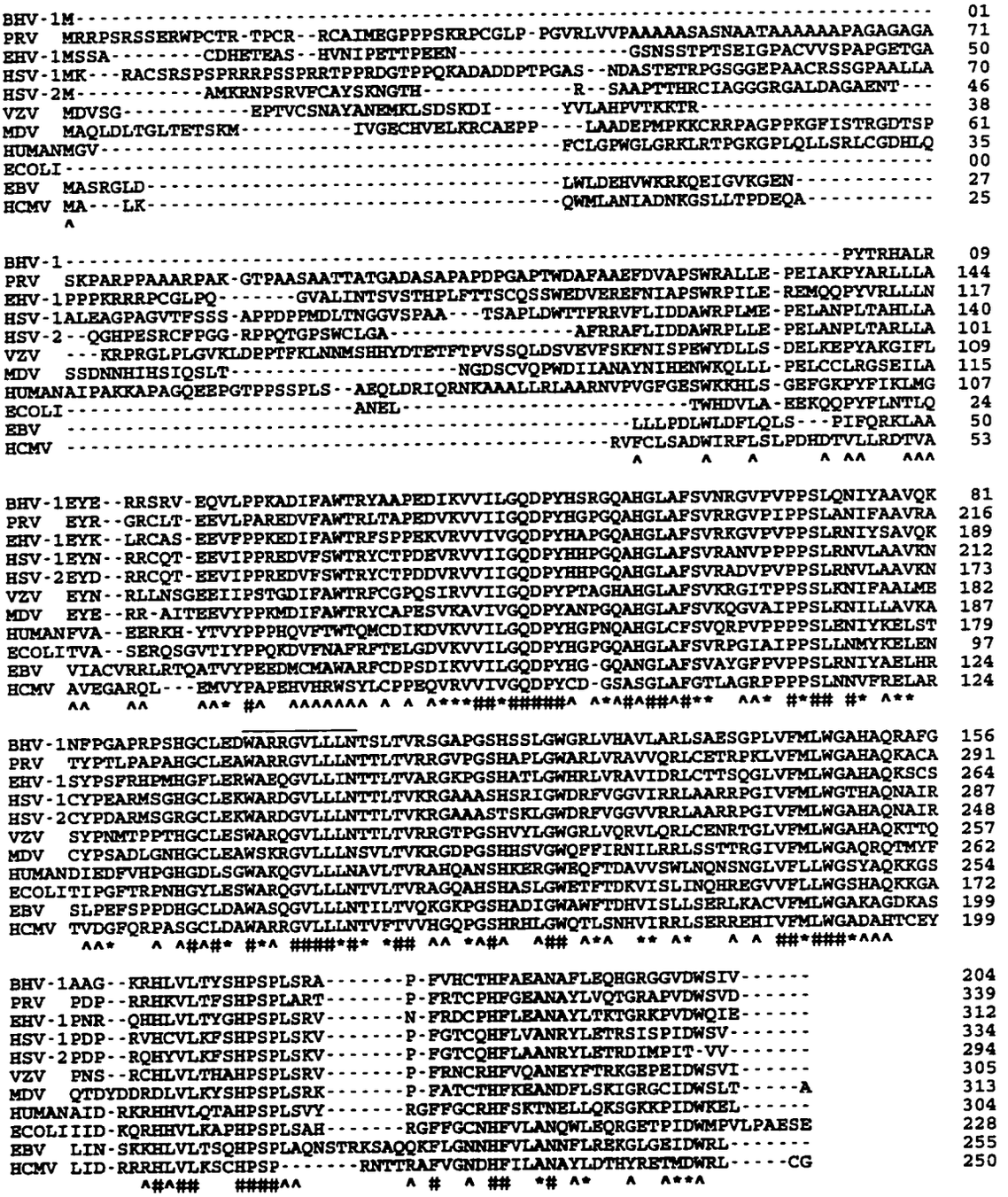
3.3.1.2 UL2.

The BHV-1 UL2 ORF is located between nucleotide 1132 to nucleotide 1743 (Fig. 3.2). No TATA box is found between the termination codon of UL1 and start codon of UL2, however a potential TATA box for UL2 gene is found at nucleotide 656 (5'TATAA3') within the UL1 gene sequence (Fig. 3.1 and 3.2). BHV-1 UL2 is predicted to encode a protein of 204 amino acids with a molecular mass of 22 kDa and an isoelectric point of 10.5. The predicted translation product contains a consensus uracil-DNA glycosylase signature sequence (5'WARRGVLLLN3') (Caradonna and Cheng, 1980; Sancar and Sancar, 1988) from amino acids 97 to 106 (Fig. 3.2). BHV-1 UL2 is 68% homologous with HSV-1 and HSV-2 UL2 (McGeoch et al., 1988, 1991), both of which have been shown to have uracil-DNA glycosylase activity (Mullaney et al., 1989; Worrad and Caradona, 1988).The homology with PRV UL2 (Dean and Cheung, 1993), EHV-1 ORF 61 (Telford et al., 1992), MDV UL2 (Yoshida et al., 1994), VZV ORF 59 (Davison and Scott, 1986), Epstein-Barr virus (EBV) BKRF3 (Baer et al., 1984), human cytomegalovirus (HCMV) UL114 (Chee et al., 1990), human (Olsen et al., 1989) and *Escherichia coli* (Varshney et al., 1988) uracil-DNA glycosylase is 75%, 72%, 69%, 65%, 57%, 55%, 55% and 58%, respectively. The overall homology among uracil-DNA glycosylase sequences is 21% (Fig. 3.4).

3.3.1.3 UL3.

The BHV-1 UL3 ORF is located between nucleotide 1793 to nucleotide 2404 (Fig. 3.2). It encodes a protein of 204 amino acids with a molecular mass of 22 kDa and an isoelectric point of 10.8. A potential TATA box is located upstream of UL3 at nucleotide 1742 (5'TATAA3') just before the stop codon of UL2. The UL3 gene contains two additional in-frame ATG codons at nucleotide 1985 and nucleotide 2180, which if used would yield a protein product of 140 and 75 amino acids, respectively. A potential nuclear localization signal (5'RKPRK3') (Worrad and Caradonna, 1993) is located at the C-terminus of the protein from amino acids 153 to 157 (Fig. 3.2). Comparison of BHV-1

Fig. 3.4. Amino acid homology of BHV-1 UL2 like proteins. Comparison of the predicted amino acid sequences of BHV-1 UL2 with the predicted amino acid sequences of PRV UL2 (Dean and Cheung, 1993), EHV-1 ORF 61 (Telford et al., 1992), HSV-1 UL2 (McGeoch et al., 1988), HSV-2 UL2 (McGeoch et al., 1991), VZV ORF 59 (Davison and Scott, 1986), MDV UL2 (Yoshida et al., 1994), human uracil-DNA glycosylase gene (Olsen et al., 1989), E.coli uracil-DNA glycosylase gene (Varshney et al., 1988), EBV BKRF3 (Baer et al., 1984) and HCMV UL114 (Chee et al., 1990). The uracil-DNA glycosylase signature sequence is overlined. Perfectly conserved, well conserved and consensus residues are indicated by #, * and ^ signs, respectively.



UL3, HSV-1 and HSV-2 UL3 (McGeoch et al., 1988, 1991), MDV UL3 (Yoshida et al., 1994), EHV-1 ORF 60 (Telford et al., 1992), VZV ORF 58 (Davison and Scott, 1986) and PRV UL3 (Dean and Cheung, 1993) amino acid sequences show significant sequence homology (38%; Fig. 3.5). BHV-1 UL3 has 62%, 58%, 56%, 53%, 52% and 62% homology at amino acid level with HSV-1 UL3, HSV-2 UL3, MDV UL3, PRV UL3, VZV ORF 58, and EHV-1 ORF 60, respectively.

3.3.1.4 UL3.5.

The BHV-1 UL3.5 ORF starts at nucleotide 2410 and terminates at nucleotide 2787 (Fig. 3.2). It encodes a protein of 126 amino acid with a predicted molecular mass of 13 kDa and an isoelectric point of 12.2. A potential TATA box (5'TATATA3') is located upstream of UL3.5 at nucleotide 2345 to 2350 within the UL3 ORF (Fig. 3.2). The BHV-1 UL3.5 contains many alanine (28%) and arginine (17%) residues. Comparison of the BHV-1 UL3.5 amino acid sequence with homologs in PRV (Dean and Cheung, 1993) and EHV-1 (Telford et al., 1992) showed limited homology (27%; Fig. 3.6) particularly in the N-terminal region where 15 out of 50 amino acids are conserved.

The sequences upstream and downstream of the ORFs were also analysed for cis-acting elements (Fig. 3.1 and 3.2). Four potential TATA boxes, located at nucleotide 301 (5'TATATA3'), 656 (5'TATAA3'), 1742 (5'TATAA3') and 2345 (5'TATATA3') were found in the UL1 to UL3.5 gene cluster (Fig.3.2). The first TATA box is upstream of the start of UL1, the second is within the UL1 coding sequence, the third overlaps with the stop codon of UL2 and is before the start codon of the UL3 gene and the fourth is within the UL3 nucleotide sequence before the stop codon of UL3 and the start codon of the UL3.5 ORF (Fig. 3.2). The only potential polyadenylation consensus signal (5'AATAAA3') of this gene cluster is located 3' to the UL3.5 ORF at nucleotide 2816 (Fig. 3.1 and 3.2).The putative sequence 5'TTTATT3' at nucleotide 2835 (Fig. 3.2) downstream of the polyadenylation signal of this gene cluster may serve as a potential polyadenylation signal for a UL4 gene (homolog of HSV-1 UL4; McGeoch et al., 1988) transcribed in the opposite direction.

Fig. 3.5. Amino acid homology of BHV-1 UL3 like proteins. Alignment of deduced amino acid sequences of BHV-1 UL3 homologs with those of HSV-1 (McGeoch et al., 1988), HSV-2 (McGeoch et al., 1991), MDV (Yoshida et al., 1994), EHV-1 (Telford et al., 1992), VZV (Davison and Scott, 1986) and PRV (Dean and Cheung, 1993). The nuclear localization signal is overlined. Perfectly conserved amino acids are indicated by # and well conserved amino acids by *. Consensus residues are marked by ^.

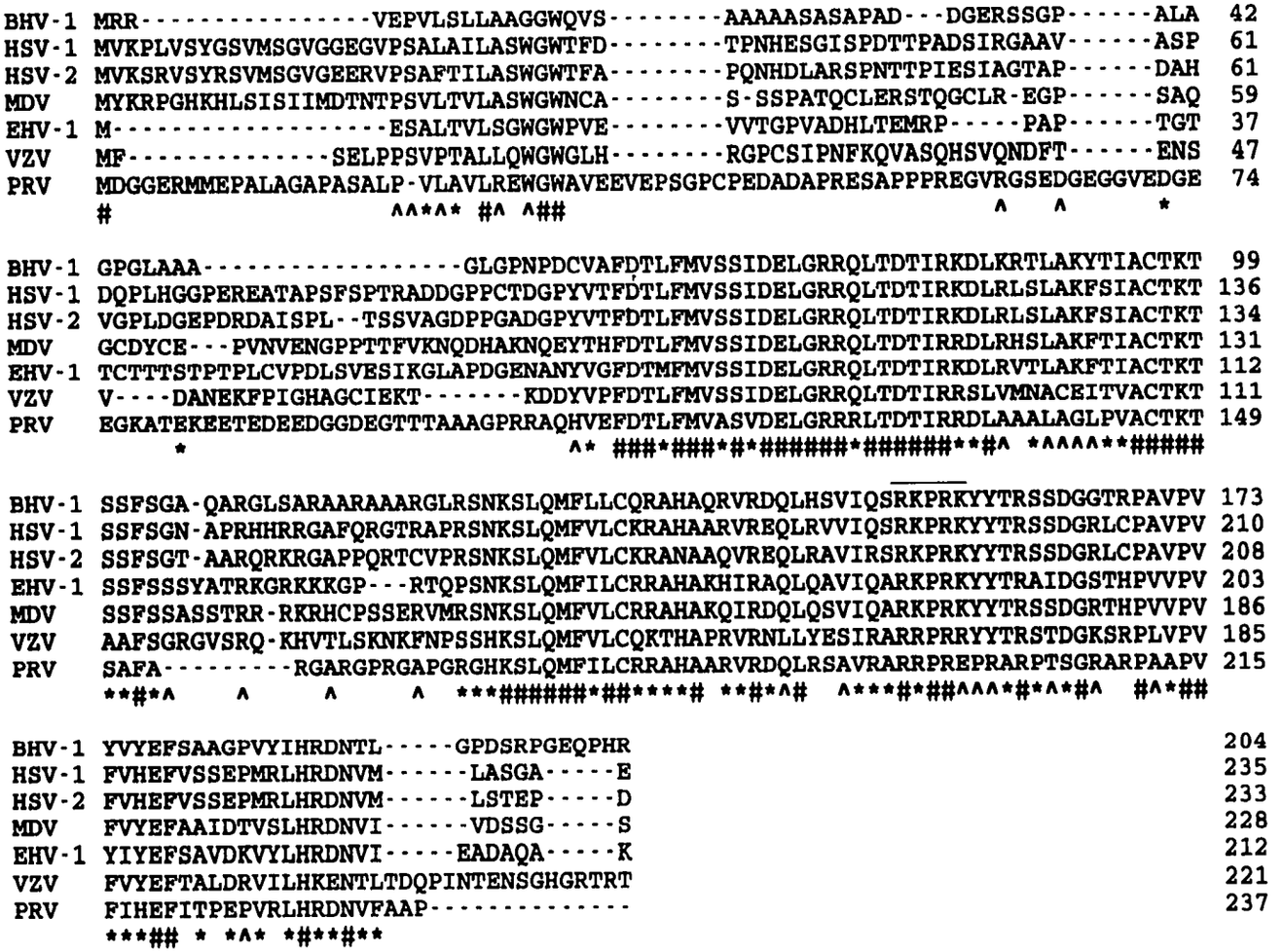
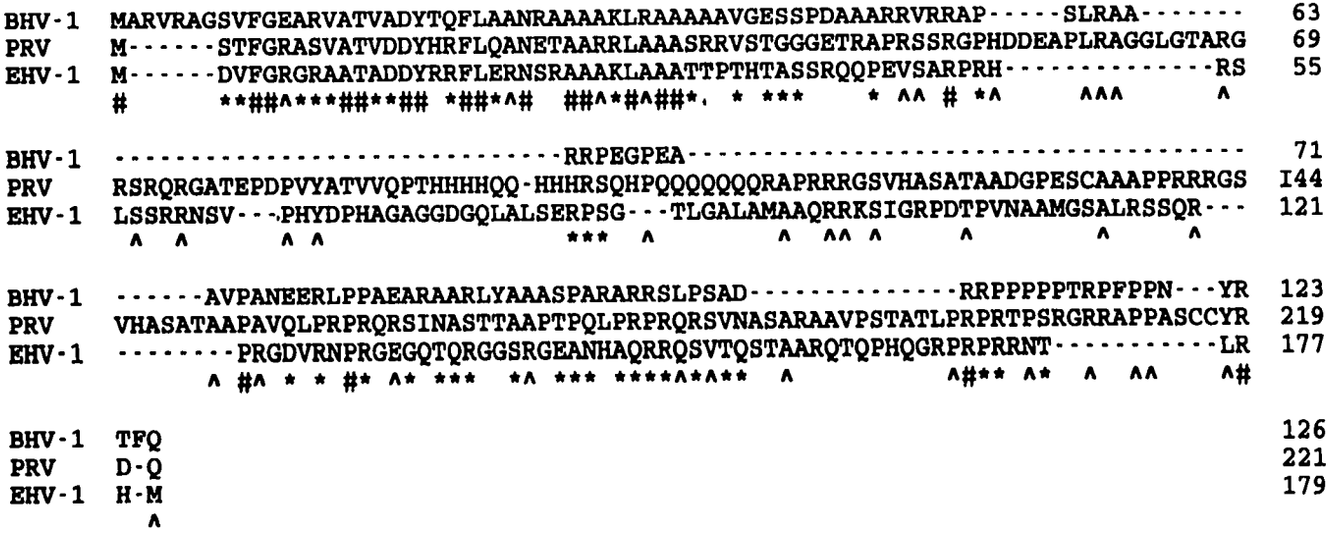

BHV-1 MRR----------VEPVLSLLAAGGWQVS--------AAAAASASAPAD---DGERSSGP------ALA 42
HSV-1 MVKPLVSYGSVMSGVGGEGVPSALAILASWGWTFD--------TPNHESGISPDTTPADSIRGAAV------ASP 61
HSV-2 MVKSRVSYRSVMSGVGEERVPSAFTILASWGWTFA--------PQNHDLARSPNTTPIESIAGTAP------DAH 61
MDV MYKRPGHKHLSISIIMDTNTPSVLTVLASWGWNCA--------S-SSPATQCLERSTQGCLR-EGP------SAQ 59
EHV-1 M----------ESALTVLSGWGWPVE--------VVTGPVADHLTEMRP-----PAP------TGT 37
VZV MF----------SELPPSVPTALLQWGWGLH--------RGPCSIPNFKQVASQHSVQNDFT------ENS 47
PRV MDGGERMMEPALAGAPASALP-VLAVLREWGWAVEEVEPSGPCPEDADAPRESAPPPREGVRGSEDGEGGVEDGE 74
BHV-1 GPGLAAA----------GLGPNPDCVAFDTLFMVSSIDELGRRQLTDTIRKDLKRTLAKYTIACTKT 99
HSV-1 DQPLHGGPEREATAPSFSPTRADDGPPCTDGPYVTFDTLFMVSSIDELGRRQLTDTIRKDLRLSLAKFSIACTKT 136
HSV-2 VGPLDGEPDRDAISPL--TSSVAGDPPGADGPYVTFDTLFMVSSIDELGRRQLTDTIRKDLRLSLAKFSIACTKT 134
MDV GCDYCE---PVNVENGPPTTFVKNQDHAKNQEYTHFDTLFMVSSIDELGRRQLTDTIRRDLRHSLAKFTIACTKT 131
EHV-1 TCTTTSTPTPLCVPDLSVESIKGLAPDGENANYVGFDTMFMVSSIDELGRRQLTDTIRKDLRVTLAKFTIACTKT 112
VZV V----DANEKFPIGHAGCIEKT-------KDDYVPFDTLFMVSSIDELGRRQLTDTIRRSLVMNACEITVACTKT 111
PRV EGKATEKEETEDEEDGGDEGTTTAAAGPRRAQHVEFDTLFMVASVDELGRRRLTDTIRRDLAAALAGLPVACTKT 149
BHV-1 SSFSGA-QARGLSARAARAAARGLRSNKSLQMFLLCQRAHAQRVRDQLHSVIQSRKPRKYYTRSSDGGTRPAVPV 173
HSV-1 SSFSGN-APRHHRRGAFQRGTRAPRSNKSLQMFVLCKRAHAARVREQLRVVIQSRKPRKYYTRSSDGRLCPAVPV 210
HSV-2 SSFSGT-AARQRKRGAPPQRTCVPRSNKSLQMFVLCKRANAAQVREQLRAVIRSRKPRKYYTRSSDGRLCPAVPV 208
EHV-1 SSFSSSYATRKGRKKKGP---RTQPSNKSLQMFILCRRAHAKHIRAQLQAVIQARKPRKYYTRAIDGSTHPVVPV 203
MDV SSFSSASSTRR-RKRHCPSSERVMRSNKSLQMFVLCRRAHAKQIRDQLQSVIQARKPRKYYTRSSDGRTHPVVPV 186
VZV AAFSGRGVSRQ-KHVTLSKNKFNPSSHKSLQMFVLCQKTHAPRVRNLLYESIRARRPRRYYTRSTDGKSRPLVPV 185
PRV SAFA---------RGARGPRGAPGRGHKSLQMFILCRRAHAARVRDQLRSAVRARRPREPRARPTSGRARPAAPV 215
BHV-1 YVYEFSAAGPVYIHRDNTL-----GPDSRPGEQPHR 204
HSV-1 FVHEFVSSEPMRLHRDNVM------LASGA-----E 235
HSV-2 FVHEFVSSEPMRLHRDNVM------LSTEP-----D 233
MDV FVYEFAAIDTVSLHRDNVI------VDSSG-----S 228
EHV-1 YIYEFSAVDKVYLHRDNVI-----EADAQA-----K 212
VZV FVYEFTALDRVILHKENTLTDQPINTENSGHGRTRT 221
PRV FIHEFITPEPVRLHRDNVFAAP---------- 237

Fig. 3.6. Amino acid homology of BHV-1 UL3.5 like proteins. Alignment of the predicted amino acid sequence of BHV-1 UL3.5 with the predicted amino acid sequences of PRV UL3.5 (Dean and Cheung, 1993) and EHV-1 ORF 59 (Telford et al., 1992) products. Perfectly conserved, well conserved and consensus residues are marked by #, * and ^ signs, respectively.


BHV-1 MARVRAGSVFGEARVATVADYTQFLAANRAAAAKLRAAAAAVGESSPDAAARRVRRAP-----SLRAA------- 63
PRV M------STFGRASVATVDDYHRFLQANETAARRLAAASRRVSTGGGETRAPRSSRGPHDDEAPLRAGGLGTARG 69
EHV-1 M------DVFGRGRAATADDYRRFLERNSRAAAKLAAATTPTHTASSRQQPEVSARPRH-------------RS 55
BHV-1 ----------RRPEGPEA---------- 71
PRV RSRQRGATEPDPVYATVVQPTHHHHQQ-HHHRSQHPQQQQQQQRAPRRRGSVHASATAADGPESCAAAPPRRRGS I44
EHV-1 LSSRRNSV---PHYDPHAGAGGDGQLALSERPSG---TLGALAMAAQRRKSIGRPDTPVNAAMGSALRSSQR--- 121
BHV-1 ------AVPANEERLPPAEARAARLYAAASPARARRSLPSAD-------------RRPPPPPTRPFPPN---YR 123
PRV VHASATAAPAVQLPRPRQRSINASTTAAPTPQLPRPRQRSVNASARAAVPSTATLPRPRTPSRGRRAPPASCCYR 219
EHV-1 --------PRGDVRNPRGEGQTQRGGSRGEANHAQRRQSVTQSTAARQTQPHQGRPRPRRNT----------LR 177
BHV-1 TFQ 126
PRV D-Q 221
EHV-1 H-M 179

3.3.2 Transcriptional mapping of UL1 to UL3.5 ORFs.

Northern blot analysis was performed to determine the transcripts originating from the UL1-UL3.5 ORFs. A 2.9 kb DNA probe (Fig. 3.1B; Fig. 3.2 nucleotide 2 to nucleotide 2928) was hybridized to total RNA from BHV-1 infected (Fig. 3.7, lane b) or mock infected (Fig. 3.7, lane a) MDBK cells. As shown in Fig. 3.7 (lane b), the DNA probe hybridized to five transcripts of 3.4, 2.4, 1.9, 1.3 and 0.7 kb.

Since sequence analysis identified only one consensus polyadenylation signal downstream of ORF 3.5, we wished to determine if these four transcripts are coterminal. Northern blots were probed with synthetic oligonucleotides derived from ORF 3.5. Oligonucleotide OUL4, complementary to the sequence shown in Fig. 3.2, hybridized to all four transcripts of 2.4, 1.9, 1.3 and 0.7 kb (Fig. 3.8, lane a) while oligonucleotide probes complementary to the opposite strand or derived downstrean of the polyadenylation site did not hybridize to any of the four transcripts (data not shown). This suggests that all four mRNA species are transcribed in the same direction, have some common sequences and terminate at the polyadenylation site at nucleotide 2816 to 2821 (Fig. 3.2). Although the 1.9 kb transcript appeared early, all four transcripts were detected at 6 hr postinfection (Fig. 3.9).

To determine the coding potential of each transcript, Northern blots were probed with oligonucleotides predicted to hybridize to transcripts containing ORFs UL1, UL2, UL3 and UL3.5. The complimentary sequences and location of selected oligonucleotides are indicated in Fig. 3.1 and 3.2 and the hybridization results are shown in Fig. 3.8. Probe OUL1 located within the UL1 ORF hybridized to a single transcript of 2.4 kb (Fig. 3.8, lane b). Probe OUL2 located within the UL2 ORF hybridized to the 2.4 kb transcript and an additional transcript of 1.9 kb (Fig. 3.8, lane c). Probe OUL3 located within the UL3 ORF hybridized to three transcripts of 2.4, 1.9 and 1.3 kb (Fig. 3.8, lane d). As mentioned above, probe OUL4 hybridized to all four transcripts of 2.4, 1.9, 1.3 and 0.7 kb (Fig. 3.8, lane a). Probe OUL0 upstream of UL1 ORF did not hybridize to any of these transcripts, indicating that the 2.4 kb transcript contains sequences for UL1, UL2, UL3 and UL3.5; the 1.9 kb transcript contains sequences for UL2, UL3 and UL3.5; the 1.3 kb transcript

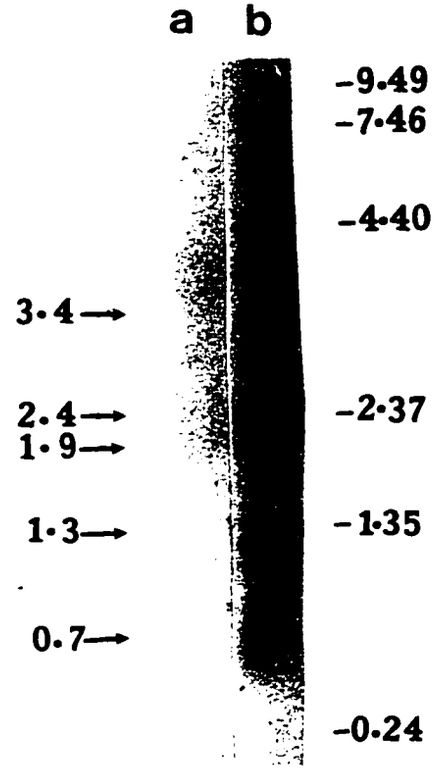


Fig. 3.7. Northern blot analysis of BHV-1 UL1, UL2, UL3 and UL3.5 RNA transcripts. Total RNA isolated from BHV-1 infected (lane b) or mock infected (lane a) MDBK cells at 8 hrs. post infection were hybridized to [^{32}P]dCTP labeled 2.9 kb HindIII-XhoI DNA fragment. The size of transcripts in kilobases is indicated on the left. The molecular sizes of marker RNAs (Gibco-BRL) in kilobases are shown on the right.

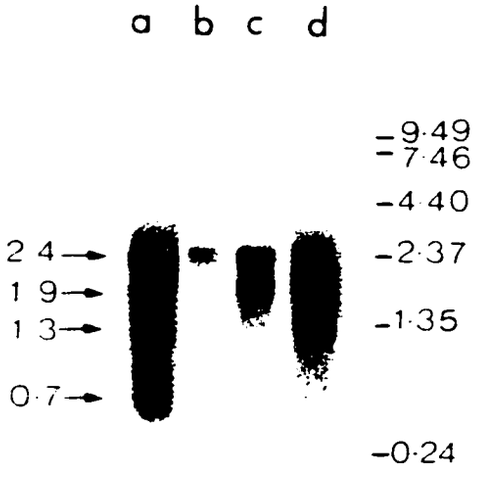


Fig. 3.8. Mapping of UL1 to UL3.5 transcripts by northern blot analysis. Northern blots of total RNA isolated from BHV-1 infected MDBK cells at 8 hrs. post infection were hybridized to 27 or 30 mer γ^{32}P-labelled OUL4 (lane a), OUL1 (lane b), OUL2 (lane c) and OUL3 (lane d) probes. The complimentary sequences and location of probes are shown in Fig.1 and 2. The size of transcripts in kilobases is indicated on the left. The molecular sizes of marker RNAs (Gibco-BRL) in kilobases are shown on the right.

Fig. 3.9. Synthesis of UL1, UL2, UL3 and UL3.5 mRNA. Total RNA isolated from BHV-1 infected MDBK cells at 0 (lane a), 2 (lane b), 4 (lane c), 6 (lane d), 8 (lane e), and 10 (lane f) hr postinfection was hybridized to OUL4 oligonucleotide labeled with $\gamma^{32}P$. The sizes of the transcripts in kilobases are indicated on the left. The molecular sizes of marker RNAs (Gibco-BRL) in kilobases are shown on the right.

contains sequences for UL3 and UL3.5; and the 0.7 kb transcript contains only sequences for UL3.5.

3.4 Discussion

Previous studies reported the sequences of the HSV-1 UL1 to UL3 gene cluster (McGeoch et al., 1988) and its homologs in HSV-2 (McGeoch et al., 1991), VZV (Davison and Scott, 1986), PRV (Dean and Cheung, 1993) and EHV-1 (Telford et al., 1992), and the UL3.5 gene in PRV (Dean and Cheung, 1993) and its homologs in EHV-1 (Telford et al., 1992) and VZV (Davison and Scott, 1986). To search for this gene cluster in BHV-1, we took into consideration that the prototypic orientation of the U_L region in HSV-1 (McGeoch et al., 1988) is inverted relative to the orientation of BHV-1 (Mayfield et al., 1983; Tikoo et al., 1995), PRV (Ben-Porat et al., 1983; Ben-Porat and Kaplan, 1985), EHV-1 (Telford et al., 1992) and VZV (Davison and Scott, 1986). Whereas HSV-1 UL1 to UL3 gene cluster is located to the left end of the prototypic U_L region, the BHV-1 homologs are localized at the right end of the prototypic U_L region. While the gene arrangement in this region of alphaherpesviruses genomes appears to be similiar, our results suggest that this gene cluster may have a different transcriptional strategy.

This region in HSV-1 contains three genes. In contrast, BHV-1 like PRV (Dean and Cheung, 1993), EHV-1 (Telford et al., 1992) and VZV (Davison and Scott, 1986), contains four genes. Upstream of this gene cluster are located homologs of the immediate early ORF BICP0 in BHV-1 (Wirth et al., 1992), ICP0 in HSV-1 (McGeoch et al., 1988), EP0 in PRV (Cheung, 1991), ORF 61 in VZV (Moriuchi et al., 1992) and ORF 63 in EHV-1 (Telford et al., 1992). The UL1 ORF is conserved in genome location but not in sequence. The UL2 and UL3 ORFs are conserved both in genome location and sequence relative to HSV-1 (McGeoch et al., 1988), VZV (Davison and Scott, 1986), EHV-1 (Telford et al., 1992) and PRV (Dean and Cheung, 1993). The UL3.5 ORF is conserved in genome location relative to PRV (Dean and Cheung, 1993), EHV-1 (Telford et al., 1992) and VZV (Davison and Scott, 1986) with limited sequence similiarity, but there is no counterpart in HSV-1 (McGeoch et al., 1988).

The UL1 like ORFs are preceeded by a stretch of direct repeat sequences in HSV-1 (McGeoch et al., 1988), PRV (Dean and Cheung, 1993), EHV-1 (Telford et al., 1992) and VZV (Davison and Scott, 1986). In contrast, in BHV-1, the direct repeats of CCCCGG and CTTCGGCCCCGG are located downstream of UL1 ORF and immediately upstream of UL2 ORF.These repeats may represent part of the vestigial U_L terminal repeat sequences, as has been suggested for PRV (Dean and Cheung, 1993).

The BHV-1 UL1 ORF is collinear with the UL1 gene of HSV-1 (McGeoch et al., 1988) and its homologs in PRV (Dean and Cheung, 1993), VZV (Davison and Scott, 1986), MDV (Yoshida et al., 1994), EHV-1 (Telford et al., 1992), HCMV (Chee et al., 1990) and EBV (Baer et al., 1984), but it does not show significant sequence homology with most of its counterparts. A comparison of the predicted amino acid sequence of BHV-1 UL1 with homologous proteins from other alphaherpesviruses revealed limited amino acid conservation (12% [Fig. 3.3]). The products of HSV-1 UL1 (Hutchinson et al., 1992b), PRV UL1 (Klupp et al., 1994), EBV BKRF2 (Baer et al., 1984), MDV UL1 (Yoshida et al., 1994) and HCMV UL115 (Kaye et al., 1992), human herpesvirus 6 U82 (HHV-6; Liu et al., 1993a, 1993b) and VZV ORF 60 (Forghani et al., 1994) encode a glycoprotein named gL which acts as a chaperone molecule for the correct folding, processing and cell surface expression of their respective gH homologs (Forghani et al., 1994; Hutchinson et al., 1992b; Kaye et al., 1992; Liu et al., 1993a, 1993b; Spaete et al., 1993; Yaswen et al., 1992). Despite the lack of significant sequence homology, preliminary results suggest that BHV-1 UL1 is functionally equivalent to gL, as it is required for the correct folding, processing and cell surface expression of BHV-1 glycoprotein gH (Khattar et al., unpublished data).

BHV-1 UL2, a homolog of the HSV-1 UL2 gene (McGeoch et al., 1988), encodes an uracil-DNA glycosylase enzyme which acts as a DNA repair enzyme by the removal of uracil residues from DNA created from either the deamination of cytosine or the incorporation of dUMP (Caradona and Cheng, 1980; Sancar and Sancar, 1988). The BHV-1 gene encodes a protein of 204 amino acids, which is the shortest of the reported uracil-DNA glycosylase proteins (Fig. 3.4; Baer et al., 1984;, Chee et al., 1990; Davison et al., 1986; Dean and Cheung, 1993; McGeoch et al., 1988, 1991; Mullaney et al.,

1989). Overall these proteins are basic in character and contain a uracil-DNA glycosylase signature sequence (Caradona and Cheng, 1980; Sancar and Sancar, 1988). While uracil-DNA glycosylase has been shown to be dispensable for normal replication of HSV-1 in cultured cells (Mullaney et al., 1989), it is required for efficient viral replication and establishment of latency in the murine nervous system (Pyles et al., 1994). Its role in BHV-1 replication and pathogenesis is presently being investigated.

The BHV-1 UL3 gene encodes a protein of 204 amino acids, which shows a homology of 62% with HSV-1 UL3 (McGeoch et al., 1988). Like HSV-1, BHV-1 UL3 protein contains a hydrophobic N-terminus and a potential nuclear localizing signal (RKPRK) near the C-terminal part of the protein. This nuclear localizing signal is conserved in HSV-1 (McGeoch et al., 1988), HSV-2 (McGeoch et al., 1991; Worrad and Caradona, 1993), MDV (Yoshida et al., 1994), VZV (Davison and Scott, 1986) and EHV-1 (Telford et al., 1992). The function of the products of the UL3 family is not known. While it has been suggested that HSV-1 UL3 may code for a membrane associated protein (McGeoch et al., 1988), the HSV-2 UL3 gene has been shown to encode a nuclear localizing phosphoprotein (Worrad and Caradonna, 1993).

BHV-1 UL3.5 ORF appears to be a positional homolog of PRV UL3.5 (Dean and Cheung, 1993), EHV-1 ORF 59 (Telford et al., 1992) and VZV ORF 57 (Davison and Scott, 1986) proteins as their genome location and orientation of these ORFs is equivalent in these viruses. It has limited homology with PRV UL3 (31%), EHV-1 ORF 59 (20%) and VZV ORF 57 (30%) which is localized particularly in the N-terminal part of the proteins. The amino acid sequence of BHV-1 UL3.5 contains a basic region rich in arginine residues. This region may participate in protein-DNA interactions as has been suggested for the PRV UL3.5 gene product (Dean and Cheung, 1993).

Previous transcriptional analysis indicated six RNAs of 9.0, 5.0, 3.4, 2.8, 1.8, and 1.0 kb mapping to the HindIII L region of BHV-1 (Cooper strain), five of which were classified as late transcripts (Phosphonoacetic acid sensitive) and one as an early transcript (Phosphonoacetic acid resistant) (Seal et al., 1991). We observed five transcripts of 3.4, 2.4, 1.9, 1.3 and 0.7 kb, four of which appear to encode the UL1 to UL3.5 gene cluster. Only one consensus polyadenylation signal was found downstream of the UL3.5 gene,

which suggests that these four transcripts may overlap. Northern blot analysis confirmed that these four transcripts of 2.4 (UL1), 1.9 (UL2), 1.3 (UL3) and 0.7 (UL3.5) kb overlap and are 3'-coterminal. In addition, these results indicated that the larger transcripts contain multiple ORFs of the gene cluster. Since internal initiation of translation (Pelleiter and Sonenberg, 1988) is not common in alphaherpesviruses, it is likely that only the first ORF in each transcript is translated, as UL1, UL2, UL3 and UL3.5 ORFs lie in a different reading frame. A similar situation also occurs in PRV as there is only one polyadenylation signal downstream of the UL3.5 gene (Dean and Cheung, 1993) and the UL1, UL2 UL3 and UL3.5 mRNAs comprise a family of overlapping transcripts that share a 3' terminus (Dean and Cheung, 1993). In contrast, in HSV-1, UL2 and UL3 are followed by separate polyadenylation signals downstream of their termination codons (McGeoch et al., 1988) with several 3'-coterminal mRNAs being described (Worrad and Caradonna, 1988).

In addition, while in PRV (Dean and Cheung, 1993), only UL1 and UL3 of the UL1 to UL3.5 gene cluster is preceeded by a separate A+T rich eukaryotic promoter like element, in BHV-1, HSV-1 (McGeoch et al., 1988) and HSV-2 (McGeoch et al., 1991), each gene of the UL1 to UL3.5 gene cluster, is preceeded by a TATA box. This may suggest that each gene is transcribed from its own TATA box. Alternatively, all four genes may be transcribed from a single TATA box. Since all four transcripts are not of the same kinetic class (Seal et al., 1991), the use of a single promoter for all four genes is unlikely.

In summary, we have identified a 3'-coterminal gene cluster of four genes in the BHV-1 genome named UL1, UL2, UL3 and UL3.5 which are predicted to encode glycoprotein gL, uracil-DNA glycosylase, nuclear targeting protein and a protein of unknown function, respectively.

4.0 IDENTIFICATION AND CHARACTERIZATION OF A BOVINE HERPESVIRUS-1 (BHV-1) GLYCOPROTEIN gL WHICH IS REQUIRED FOR PROPER ANTIGENICITY, PROCESSING AND TRANSPORT OF BHV-1 GLYCOPROTEIN gH.

4.1 Introduction

Bovine herpesvirus-1, an alphaherpesvirus (Roizman et al., 1982), is a predominant cause of respiratory disease, abortion, and genital infections in cattle (Yates, 1982). Like other herpesviruses, BHV-1 consists of a nucleocapsid containing the linear double stranded DNA genome, which is surrounded by an electron-dense zone called the tegument and a lipid bilayer envelope. In this membrane are embedded the virus-encoded glycoproteins. These glycoproteins play an important role in the intial stages of virus-cell interactions and act as major antigenic determinants for the humoral and cellular immune responses of the host. Although ten glycoprotein genes have been localized and sequenced in BHV-1, designated gB, gC, gD, gE, gG, gH, gI and gK gL and gM, only few have been characterized at the protein level (Khadr et al., 1996; Khattar et al., 1995; reviewed by Tikoo et al., 1995; Vlcek et al., 1995), all of which exhibit homology to respective glycoproteins of herpes simplex virus-1. While gC, gE, gG and gI are nonessential for virus replication *in-vitro* (Harrach, unpublished data; Liang et al., 1991; van Engelenburg et al., 1994), gD has been shown to be an essential component of the virion (Fehler et al., 1992).

Glycoprotein gH, an essential component of the virion, is found in all herpesviruses and constitutes the second most highly conserved group of herpesviral glycoproteins. Homologs of gH have been found to be required for penetration and cell to cell spread (Forrester et al., 1992; Fuller et al., 1989; Gompels and Minson, 1986; Haddad and Hutt-Fletcher, 1989; Peeters et al., 1992). In addition, antibodies against gH homologs possess potent complement-independent neutralizing activity (Buckmaster et

al., 1984; Forrester et al., 1991; Fuller et al., 1989; Miller and Hutt-Fletcher, 1988; Montalvo and Grose, 1986; Gompels and Minson, 1986; Gompels et al., 1991). The expression of gH homologs in the absence of other virus proteins leads to the synthesis of a glycoprotein which is improperly transported and antigenically different from native gH, suggesting that gH homologs must interact with another viral protein in order to attain proper structure and function (Foa-Tomasi et al., 1991; Gompels and Minson, 1989; Gompels et al., 1991; Roberts et al., 1991; Spaete et al., 1993). Recently, an accessory molecule named glycoprotein gL (Hutchinson et al., 1992b; Kaye et al., 1992; Liu et al., 1993; Roop et al., 1993) was identified as the viral protein that forms a complex with gH, which is necessary for attaining proper antigenic conformation and transport of gH.

BHV-1 gII\gp108, a glycoprotein of 108 kDa (van Drunen littel-van den Hurk et al., 1996) is the target of complement independent neutralizing monoclonal antibodies (MAbs) which inhibit penetration but do not prevent attachment of the virus to the cell (Baranowski et al., 1993). The gII\gp108 glycoprotein has been suggested to be involved in viral entry into the cell and in cell to cell spread of the virus (Baranowski et al., 1993). Preliminary evidence suggests that gII\gp108 may be the homolog of glycoprotein H (Baranowski et al., 1995; van Drunen littel-van den Hurk et al., 1996). However, in contrast to other herpesviruses, analysis of purified labelled BHV-1 virions by immunoprecipitation has suggested that gII\gp108 may not be a component of the virion (Baranowski et al., 1993). In addition, no other protein has been found associated with the gII\gp108 glycoptotein (Baranowski et al., 1995). Since we recently described the sequence of the BHV-1 UL1 gene that has the potential to encode the homolog of HSV-1 glycoprotein gL (Khattar et al., 1995), we sought to determine if BHV-1 encodes a functional gL and whether it is neccessary for the correct transport and antigenicity of BHV-1 glycoprotein gH. In this communication, we describe the characterization of gH and gL glycoproteins and show that BHV-1 encodes a functional glycoprotein gL, which forms a complex with gH and is required for proper posttranslational modification and transport of gH.

4.2 Materials and Methods

4.2.1 Reagents and Media.

Cell culture media and fetal bovine serum were purchased from GIBCO/BRL (Burlington, Ontario, Canada). Reagents for DNA manipulations and Protein A sepharose were purchased from Pharmacia (Dorval, Quebec, Canada) and used as recommended by the manufacturer. The enzyme neuraminidase, 5'-bromo-2'-deoxyuridine, biotinylated lectins and other reagents for protein analysis were obtained from Sigma Chemicals Co (St. Louis, MO, USA) and Pierce (Rockford, IL, USA). The enzymes O-glycosidase, PNGase F and Endo H were purchased from Boehringer Mannheim Canada (Laval, Quebec, Canada). The Glycotrack kit was purchased from Oxford Biosystems Ltd. (Rosedale, NY, USA). The reagents for immunofluorescence staining and other immunological assays were purchased from Zymed Laboratories Inc. (San Francisco, CA, USA) and Bio-Rad (Mississauga, Ontario, Canada). Radioisotopically labelled compounds and reagents for fluorography were purchased from ICN (Irvine, CA, USA). Reagents for *in-vitro* transcription and translation including plasmid pSP64polyA were purchased from Promega\Fisher Scientific Ltd (Nepean, Ontario, Canada). RIBI adjuvant was purchased from RIBI Immunochemicals (Hamilton, Ontario, Canada), while VSA3 is a proprietory adjuvant of VIDO\Biostar (Saskatoon, SK, Canada).

4.2.2 Cells and Viruses.

Madin-Darby bovine kidney (MDBK) cells, human thymidine kinase negative (TK^-) cells and BSC-1 cells were grown as monolayers in Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum. $LMTK^-$ cells were grown in Dulbecco's MEM (DMEM) supplemented with 5% fetal bovine serum. Virus stocks of BHV-1 were prepared in MDBK cells (Babiuk and Rouse, 1975). Wild type vaccinia virus (VV) was propagated in BSC-1 cells, while recombinant VVs were cultivated in $LMTK^-$ cells (Mackett et al., 1984).

4.2.3 Antipeptide sera and other antibodies.

Two synthetic peptides BUL1-1 (19 residues) representing amino acid 23 to 41 and BUL1-2 (16 residues) representing amino acid 143 to 158 of UL1 ORF (Khattar et al., 1995) were synthesized onto a hydroxymethyl-phenyl resin using an Applied Biosystems 430 A solid phase peptide synthesizer employing Fmoc chemistry. Rabbits were injected with 0.2 mg each of peptide conjugated to keyhole limpet hemocyanin in Freund's complete adjuvant. Subsequent booster immunizations were given with 0.2 mg peptide conjugated to egg white ovalbumin in RIBI adjuvant at a 4 week interval. MAbs (5C8,1A9 and 3E2) specific for UL22 protein were generated essentially as described previously (van Drunen Littel-van den Hurk et al., 1984b) except that the antigen used for immunizing rabbits was the envelope fraction of BHV-1 depleted of major glycoproteins gB, gC and gD. Rabbit polyclonal serum specific for UL22 was prepared by injecting rabbits twice intramuscularly with 25 μg of affinity purified protein in VSA3.

4.2.4 Plasmid constructions.

The full-length UL1-encoding gene was excised from a plasmid designated pSK2.2 [PTZ18R containing 2.2 kb HindIII-NruI fragment (Khattar et al., 1995)] as a 600 bp NlaIII-NheI fragment, (blunt end repaired by T4 DNA polymerase), and inserted into NcoI (blunt end repaired by T4 DNA polymerase) and SmaI digested pTZ18RgIV (Tikoo et al., 1993a) generating plasmid pSKgL. A 0.6kb BgIII fragment derived from pSKgL was inserted into BamHI digested pSP64polyA generating plasmid pSKiL, and to BglII digested pVVSL (Tikoo et al., 1993a) generating plasmid pSKvL.

The 2.9 kb AvrII fragment containing the UL22 gene (Meyer et al., 1991) was excised from plasmid pSD106 (Mayfield et al., 1983), blunt end repaired with T4 DNA polymerase and ligated to BamHI linkers. After BamHI digestion of the ligation mixture, a 2.8 kb fragment was purified and ligated to BamHI digested pSP64polyA generating plasmid pSKiH; and to BglII digested pVVSL (Tikoo et al., 1993a) generating plasmid pSKvH.

4.2.5 *In-vitro* transcription and translation.

The 0.5-5.0 μg of plasmid DNA (pSKiL or pSKiH) was *in-vitro* transcribed with SP6 polymerase as described by the supplier (Promega). RNA was translated *in-vitro* for 2 hrs at 30^0C using a rabbit reticulocyte lysate containing 40 μCi of [^{35}S]-cysteine. Canine pancreatic microsomal membranes were added to some reaction mixtures to process the *in-vitro* translated proteins. After synthesis, the *in-vitro* translated proteins were analysed on sodium dodecyl sulphate (SDS)-polyacrylamide gels (PAGE) with or without immunoprecipitation.

4.2.6 Radiolabelling of cells and immunoprecipitation.

For immunoprecipitation, LMTK$^-$ cells were infected with VVs at a multiplicity of infection of 5. After 90 min of adsorption, the cells were washed and incubated in cysteine-free DMEM for 3 hrs before labelling with [^{35}S]-cysteine (100 μCi\ml). After 4-8 hrs of labelling, the cells and\or media were harvested. BHV-1 infected MDBK cells were labelled as described previously (van Drunen Littel-van den Hurk et al., 1984). In pulse chase experiments, cells were labelled at 6 hrs postinfection with 100 μCi of [^{35}S]-cysteine for 30 min. Depending on the specific experiment, either the cells were harvested immediately or the label was removed and the cells were incubated for different time periods in DMEM containing an excess of cold cysteine (chase). Proteins were immunoprecipitated from the medium or from infected cells lysed with modified radioimmunoprecipitation assay buffer (RIPA), and analysed by SDS-PAGE as described previously (van Drunen Littel-van den Hurk et al., 1984).

4.2.7 Isolation of recombinant vaccinia viruses.

The desired recombinant VVs were made by homologous recombination as previously described (Mackett et al., 1984), except that the linearized plasmid (pSKvL or pSKvH) DNA was electroporated into the wild-type VV-infected cells by using a Pharmacia Gene Pulser set at 200 V and 500 μF. The recombinant VVs were identified by screening TK$^-$ plaques for the expression of recombinant proteins by immunocytochemistry (Tikoo et al., 1990) before plaque purification and preparing viral

stocks. The recombinant VVs were named SKgL (expressing UL1 protein) and SKgH (expressing UL22 protein).

4.2.8 Western blotting.

Affinity purified protein, potassium tartrate gradient purified BHV-1 and recombinant VV infected LMTK$^-$ cells were suspended in modified lysis buffer and the proteins were separated by SDS-PAGE (van Drunen Littel-van den Hurk et al., 1984). For Western blotting the proteins were subsequently transferred to nitrocellulose membranes and reacted with UL1 or UL22 specific antiserum. Bound antibody was visualised after incubation of membranes with biotinylated secondary antibody using the strepavidin\biotinylated alkaline phosphatase complex catalysed chromogenic reaction of a Bio-rad immunoblot assay kit. For carbohydrate detection, the membranes were treated with periodate followed by biotin-hydrazide (Glycotrack kit). To determine the type of carbohydrate attached, the membranes were incubated with a range of different biotinylated lectins. Finally, the biotinylated compounds were detected with streptavidin-alkaline phosphatase conjugate.

4.2.9 Immunofluorescence.

MDBK or LMTK$^-$ cells were seeded and infected on 4 well Lab-Tek chamber slides. The infected cells were fixed either with 2% paraformaldehyde for 15 min at 4^0C for surface staining or 100% methanol for 15 min at -20^0C for internal staining. The cells were incubated with rabbit antipeptide, rabbit antiprotein or preimmune serum and goat anti-rabbit immunoglobulin G conjugated to FITC and analysed.

4.2.10 Immunization of rabbits and antibody titer determination.

Rabbits were immunized intraperitoneally with 0.2 ml of 10^6 PFU of wild-type VV or recombinant VVs (SKgL or SKgH) per ml at 0 and 21 days post infection. In addition, one group of rabbits was immunized with a 0.2 ml of suspension containing equal PFUs of recombinant VVs SKgL and SKgH. Sera were obtained two weeks after each immunization. BHV-1 specific total antibody responses were measured by enzyme-

linked immunosorbent assay (ELISA)(van Drunen Littel-van den Hurk et al., 1984) with purified BHV-1 as an antigen. Serum neutralization antibody responses were determined as described previously (van Drunen Littel-van den Hurk et al., 1984), using 100 PFU of BHV-1. Titers were expressed as the reciprocals of the highest antibody dilution that caused a 50% reduction in number of plaques relative to the control.

4.3 Results

4.3.1 Production of UL1 protein specific antiserum.

In order to identify and characterize the protein encoded by the UL1 gene, we made antipeptide serum. Analysis of the UL1 gene product by an antigen programme (Hopp and Woods, 1981) of the PC\GENE software package identified two highly immunogenic regions, one site close to the amino terminus of the predicted protein and one at the carboxy-terminal location. Peptide BUL1-1 of 19 amino acids corresponding to residues 23 to 41 and peptide BUL1-2 of 16 amino acids corresponding to residues 143 to 158 of the predicted UL1 primary translation product were synthesized and used for immunization of rabbits. Sera collected after the fourth boost were analysed in detail. As shown below, sera against both peptides showed specific positive reactions. In contrast, none of the sera obtained after immunization of rabbits with an irrelevant peptide recognized any BHV-1 specific proteins (data not shown).

4.3.2 *In-vitro* translation and immunoprecipitation of UL1 and UL22 proteins.

In order to determine the identity of UL1 and UL22 ORFs and to check whether the antibodies against these gene products recognized their respective proteins, two *in-vitro* expression plasmids pSKiL and pSKiH were generated in which the coding sequence of UL1 and UL22, respectively was placed downstream of the SP6 promoter. *In-vitro* translation of pSKiL RNA resulted in the synthesis of a polypeptide of 18 kDa (Fig. 4.1, lane a) The same protein was also recognized by anti BUL1-1 peptide serum (Fig. 4.1, lane b). Similarly, *in-vitro* translation of pSKiH RNA resulted in the synthesis of a polypeptide of 92 kDa (Fig. 4.1, lane d) which was recognized by anti UL22 serum (Fig.

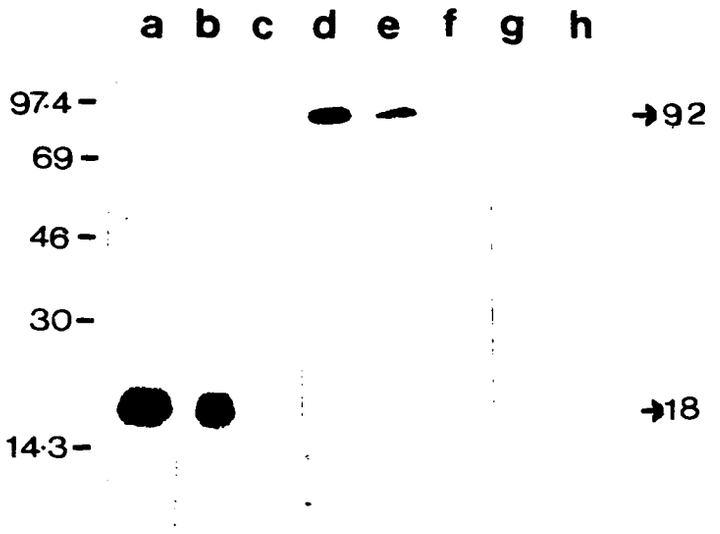


Fig. 4.1. Immunoprecipitation of proteins synthesized by *in-vitro* transcription and translation of pSKiL and pSKiH plasmids. [^{35}S]-cysteine labelled *in-vitro* translated pSkiL products (lane a), immunoprecipitated with anti BUL1-1 peptide serum (lane b), and preimmune serum (lane c); [^{35}S]-cysteine labelled pSkiH products (lane d), immunoprecipitated with anti UL22 serum (lane e) and preimmune serum (lane f); and [^{35}S]-cysteine labelled pSP64polyA products immunoprecipitated with anti BUL1-1 peptide serum (lane g) and anti UL22 serum (lane h) were separated on 12.5% SDS-PAGE under reducing conditions. The mol wt markers in kDa are indicated on the left.

4.1, lane e). However these proteins were not immunoprecipitated with anti UL1 peptide serum (Fig. 4.1, lane g) or anti UL22 serum (Fig. 4.1, lane h) from reactions in which pSP64polyA RNA was translated *in-vitro*. In addition, preimmune serum did not immunoprecipitate any protein from the reactions containing pSKiL (Fig. 4.1, lane c) or pSKiH (Fig. 4.1, lane f) RNAs.

4.3.3 *In-vivo* expression of UL1 and UL22 proteins.

To further characterize the proteins and to confirm the specificity of our antiserum, immunoprecipitation assays were performed. Radiolabelled proteins from mock infected or BHV-1 infected cell lysates were immunoprecipitated and analysed by SDS-PAGE. As compared to preimmune serum (Fig. 4.2A, lane b), both anti BUL1-1 serum (Fig. 4.2A, lane c) and anti BUL1-2 serum (Fig. 4.2A, lane f) recognized two protein species of 16-17 kDa and 108 kDa. Preincubation of anti BUL1-1 serum with BUL1-1 peptide inhibited detection (Fig. 4.2A, lane d), while addition of peptide BUL1-2 had no effect (Fig. 4.2A, lane e). Similarly detection by anti BUL1-2 peptide serum was inhibited by preincubation of serum with peptide BUL1-2 (Fig. 4.2A, lane g) but not with peptide UL1-1 (Fig. 4.2A, lane h). These proteins were not detected in uninfected cells (Fig. 4.2A, lane a). In addition, anti UL22 serum also recognized the two protein species of 108 kDa and 16-17 kDa (Fig. 4.2A, lane i). To determine if the 16-17 kDa and 108 kDa proteins are different and to examine if the anti UL1 or anti UL22 serum cross react with UL22 or UL1 proteins respectively, we constructed recombinant VVs expressing UL1 (SKgL) or UL22 (SKgH) proteins. The anti BUL1-1 peptide serum (Fig. 4.2B, lane c) but not anti UL22 serum (Fig. 4.2B, lane d) recognized a 16-17 kDa protein expressed in pSKgL VV infected cells. The anti UL22 serum (Fig. 4.2B, lane e) but not anti BUL1-1 serum (Fig. 4.2B, lane f) recognized a protein of 100 kDa (precursor of UL22 protein) expressed in pSKgH VV infected cells. These proteins were not detected in $LMTK^-$ cells infected with wild type VV (Fig. 4.2B, lane b) or in uninfected cells (Fig. 4.2B, lane a). These results suggest that UL1 and UL22 protein form a complex in BHV-1 infected cells which is recognized by both anti BUL1-1 and anti UL22 serum.

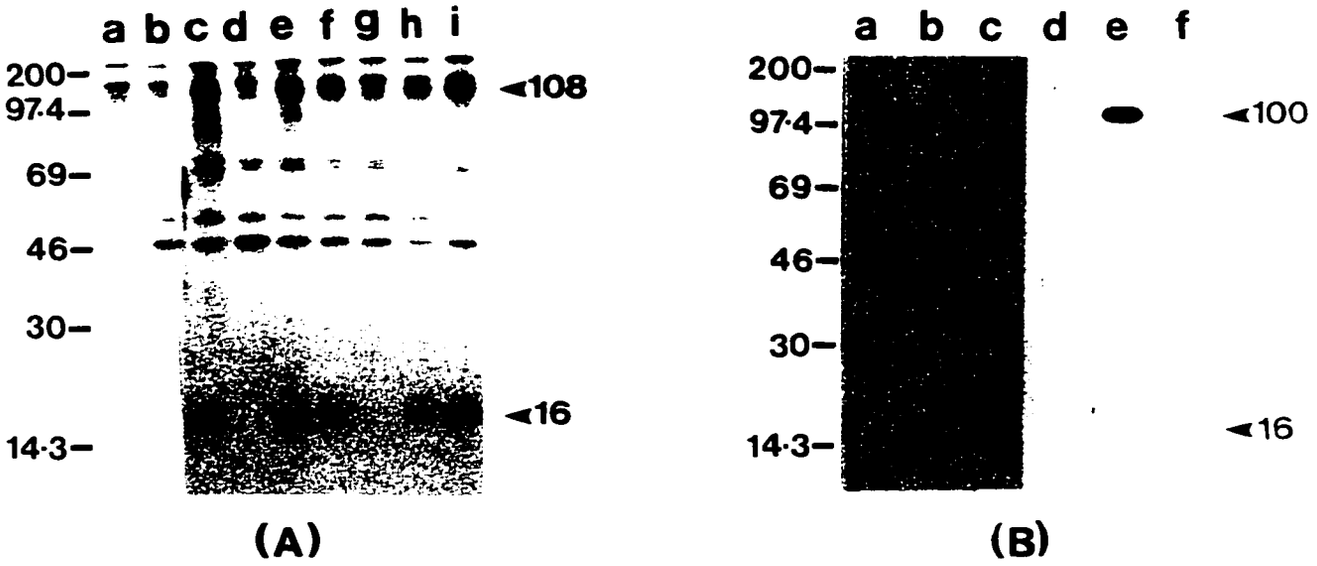


Fig. 4.2. ***In-vivo*** **expression of UL1 and UL22 proteins.** (A) Proteins from lysates of [^{35}S]-cysteine labelled mock infected (lane a) or BHV-1 infected (lanes b-i) MDBK cells were immunoprecipitated with preimmune serum (lane b), anti BUL1-1 peptide serum (lanes a,c), anti BUL1-1 peptide serum preincubated with BUL1-1 peptide (lane d), anti BUL1-1 serum preincubated with BUL1-2 peptide (lane e), anti BUL1-2 peptide serum (lane f), anti BUL1-2 peptide preincubated with BUL1-2 peptide (lane g), anti BUL1-2 peptide preincubated with BUL1-1 peptide (lane h), anti UL22 serum (lane i) and were separated on 15% SDS-PAGE under reducing condition. (B) Proteins from lysates of [^{35}S]-cysteine labelled LMTK$^-$ cells mock infected (lane a) or infected with wild type vaccinia virus (lane b) recombinant VV SKgL (lanes c,d) and recombinant VV SKgH (lane e,f) were immunoprecipitated with anti BUL1-1 peptide serum (lanes a,b,c,f) or anti UL22 serum (d,e) and separated on 12.5% SDS-PAGE under reducing conditions. The mol wt markers in kDa are indicated on the left.

4.3.4 UL1 and UL22 proteins are not associated by disulphide bonds.

To determine if the complex formation between UL1 and UL22 proteins depended on interchain disulphide bonds, the proteins from radiolabelled lysates of BHV-1 infected MDBK cells were immunoprecipitated with anti BUL1-1 (Fig. 4.3, lane a and b) or anti UL22 serum (Fig. 4.3, lane c and d) and analysed by SDS-PAGE under reducing (Fig.4.3, lane a and c) and non reducing (Fig. 4.3, lane b and d) conditions. As seen in Fig. 4.3, both sera clearly recognized two proteins of 108 kDa and 16-17 kDa from BHV-1 infected MDBK cells when the proteins were analysed by either reducing or non-reducing conditions. These results suggest that UL1 and UL22 proteins are not associated by disulphide bonds.

4.3.5 The UL1 and UL22 proteins are glycosylated.

Sequence analysis of UL1 protein homologs of most alphaherpesviruses except pseudorabies virus (PRV), show at least one N-linked glycosylation consensus sequence (Davison and Scott, 1986; Klupp et al., 1994; Kornfeld and Kornfeld, 1985; McGeoch et al., 1988; Telford et al., 1992;). However, PRV and BHV-1 UL1 does not show such a consensus sequence (Khattar et al., 1995; Klupp et al., 1994). *In-vitro* translation of *in-vitro* transcribed UL1 mRNA (Fig. 4.4A, lane a) in the presence of canine microsomal membranes did not show any decrease in the mobility of UL1 protein (Fig. 4.4A, lane b). In addition, treatment of UL1 protein with endo H (Fig. 4.4A, lane d) or PNGase F (Fig. 4.4A, lane c) did not show any increase in the mobility of the protein. Together these results suggest that UL1 protein is not modified by N-linked oligosaccharides. Moreover, an increase in the mobility of UL1 protein translated *in-vitro* in the presence of canine microsomal membranes suggests that the signal sequence is cleaved (Fig. 4.4A, lane b). To determine whether the UL1 protein contained O-linked oligosaccharides we treated the UL1 protein with neuraminidase and O-glycosidase. No increase in the mobility of the protein was observed following such treatment (Fig. 4.4A, lane e). Secondly, attempts to label the protein with [^{3}H]-glucosamine were unsuccessful (data not shown). However periodate oxidation of UL1 protein detected carbohydrates (Fig. 4.4B, lane b). So we tested different lectins with specificities for different carbohydrate groups for their ability

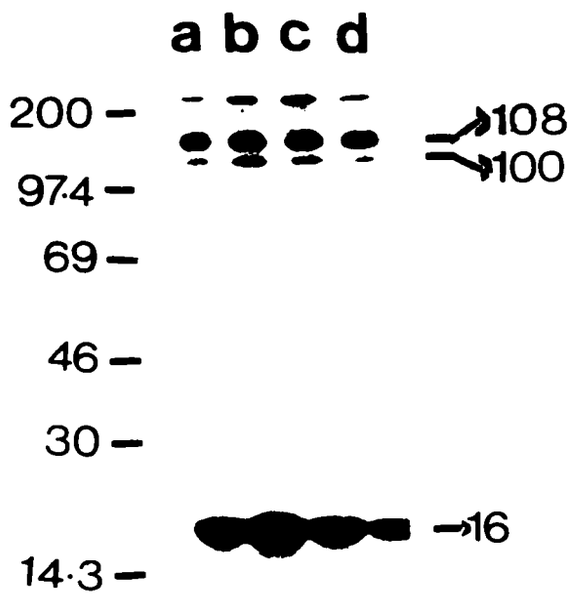
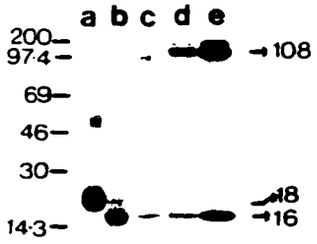
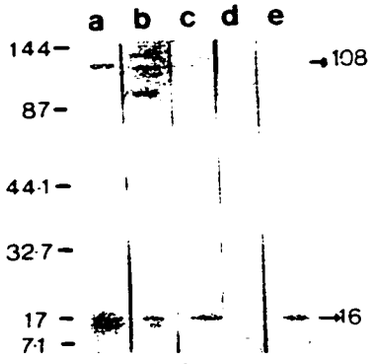


Fig. 4.3. UL1 and UL22 proteins are not associated by disulphide bonds. Proteins from lysates of [^{35}S]-cysteine labelled BHV-1 infected MDBK cells were immunoprecipitated with anti BUL1-1 serum (a,b) or anti UL22 serum (c,d) and separated on 10% SDS-PAGE under reducing (a,c) or non reducing (b,d) conditions. The mol wt markers in kDa are indicated on the left.

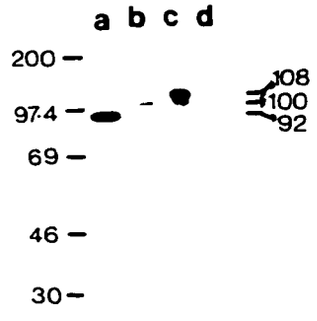
Fig. 4.4. Glycosylation of UL1 and UL22 proteins. (A) Immunoprecipitation of [^{35}S]-cysteine labelled *in-vitro* translated pSKiL RNA products in the absence (lane a) or presence (lane b) of canine microsomal membranes with anti BUL1-1 peptide serum. [^{35}S]-cysteine labelled cytoplasmic extracts from MDBK cells infected with BHV-1 were immunoprecipitated with anti BUL1-1 peptide serum and treated with PNGase F (lane c), endo H (lane d) or neuraminidase and O-glycosidase (lane e). All these proteins were separated on 15% SDS-PAGE. (B) For lectin binding, solublized purified BHV-1 virions (lane a,b) or affinity purified UL1 protein (c,d,e) were separated on 15% SDS-PAGE under reducing condition. The separated proteins were probed in western blots with anti BUL1-1 peptide and anti UL22 serum (lane a), oxidised with periodate and treated with biotin hydrazide (lane b); probed before (lane c) or after digestion with neuraminidase and O-glycosidase (lane d) or PNGase F (lane e) with biotinylated *Dolichus biflorus* lectin. (C) Immunoprecipitation of proteins from [^{35}S]-cysteine labelled *in-vitro* translated pSKiH RNA in the absence (lane a) or presence (lane b) of canine microsomal membranes; or from lysates of [^{3}H]-glucosamine labelled MDBK cells BHV-1 infected (lane c) or mock infected (lane d) by anti UL22 serum. Immunoprecipitated proteins were separated on 10% SDS-PAGE under reducing condition. (D) Proteins from [^{35}S]-cysteine labelled BHV-1 infected MDBK cells were immunoprecipitated with anti UL22 serum. The immunoprecipitated proteins were mock digested (lane a) or treated with PNGase F(lane b), endo H (lane c), neuraminidase (lane d) or neuraminidase and O-glycosidase (lane e) and analysed on 10% SDS-PAGE. The mol wt markers in kDa are indicated on the left.


a b c d e
200
97·4
69
46
30
14·3
108
18
16

(A)


a b c d e
144
87
44·1
32·7
17
7·1
108
16

(B)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97·4
69
46
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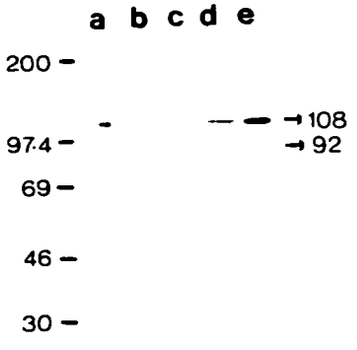
108
100
92

(C)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(D)

to bind to the protein. Only *Dolichus biflorus* lectin, which is specific for N-acetyl galactosamine [(GalNAc)-α-3GalNAc] bound to the UL1 protein (Fig. 4.4B, lane c). Treatment of the protein with neuraminidase and O-glycosidase (Fig. 4.4B, lane d) but not PNGase F (Fig. 4.4B, lane e) caused the loss of *Dolichus biflorus* lectin binding due to removal of sugar moieties. Since these data indicated that O-linked oligosaccharides are attached to the UL1 protein, we named the UL1 translational product BHV-1 glycoprotein gL in accordance with the uniform nomenclature system of alphaherpesvirus glycoproteins as described earlier (Tikoo et al., 1995).

Homologs of the herpesvirus UL22 proteins have been found to be glycosylated (Cranage et al., 1988; Gompels et al., 1989; Klupp et al., 1992). An *in-vitro* translation of *in-vitro* transcribed UL22 mRNA (Fig. 4.4C, lane a) in the presence of canine microsomal membranes showed decrease in mobility of UL22 protein (Fig. 4.4C, lane b). UL22 protein synthesized in BHV-1 infected cells was labelled by [^{3}H]-glucosamine (Fig. 4.4C, lane c). Treatment of UL22 protein (Fig. 4.4D, lane a) with PNGase F (Fig. 4.4D, lane b) but not with endo H (Fig. 4.4D, lane c), neuraminidase (Fig. 4.4D, lane d) or neuraminidase and O-glycosidase (Fig. 4.4D, lane e) showed an increase in the mobility of the protein. None of the lectins specific for O-linked oligosaccharides bound to the UL22 protein (data not shown). Taken together these results suggested that the UL22 protein is modified by N-linked oligosaccharides only and was named glycoprotein gH.

4.3.6 BHV-1 gL and gH are part of BHV-1 virions.

In order to determine whether these two glycoproteins are components of the virion, BHV-1 virions were labelled with [^{35}S]-cysteine and purified by sucrose gradients. Purified labelled virions were solublized, the proteins were immunoprecipitated by anti BUL1-1 serum or anti UL22 serum and analysed by SDS-PAGE. As seen in Fig. 4.5, compared to preimmune serum (lane a) both anti BUL1-1 (lane b) and anti UL22 sera (lane c) specifically recognized 108 kDa and 16-17 kDa virion proteins.

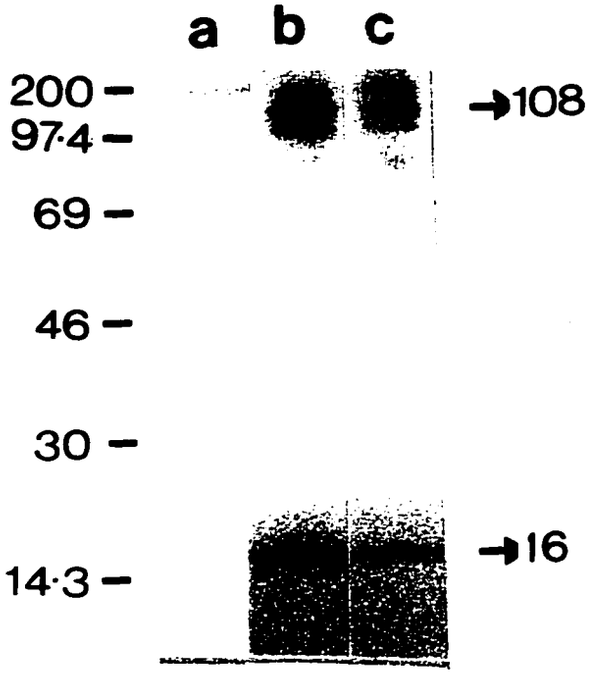


Fig. 4.5. Identification of the BHV-1 gL and gH proteins in purified virions. [^{35}S]-cysteine labelled sucrose gradient purified BHV-1 virions were solubilized and proteins were immunoprecipitated with preimmune serum (lane a), anti BUL1-1 serum (lane b) or anti UL22 serum (lane c) and analysed on 12.5% SDS-PAGE. The mol wt markers in kDa are indicated on the left.

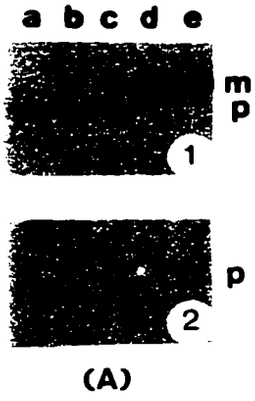
4.3.7 BHV-1 gL is required for proper processing and transport of BHV-1 gH.

Two types of experiments were performed to investigate the processing of recombinant gH protein. i) Pulse chase studies were performed to study the maturation of recombinant gH expressed by SKgH VV. ii) Since endo H sensitivity indicates the retention of protein in the endoplasmic reticulum, endo H enzyme treatment of steady state-labelled proteins was done to investigate whether the recombinant gH was retained in the endoplasmic reticulum (endo H sensitive) or transported to the Golgi apparatus (endo H resistant), where N-linked oligosaccharides are modified and O-linked oligosaccharides are added.

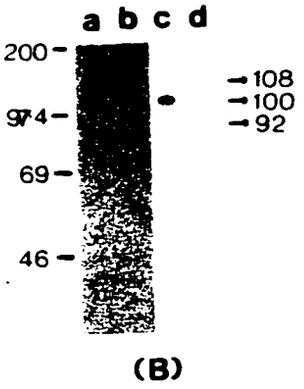
Wild type gH expressed by BHV-1 infected cells was first detected as a 100 kDa precursor (p) which was processed into a 108 kDa mature (m) form of the protein (Fig. 4.6A, panel 1). The mature form of wild type gH (Fig. 4.6B, lane a) was resistant to endo H enzyme treatment (Fig. 4.6B, lane b) indicating that it entered the normal secretory pathway through the Golgi apparatus, where N-linked oligosaccharides on gH were modified to the complex type prior to transport of gH to the cell surface. In contrast, gH protein expressed by recombinant VV SKgH was detected as a precursor (100 kDa) which was never processed into the high-molecular weight mature form (Fig. 4.6A panel 2). In addition, this recombinant protein (Fig. 4.6B, lane c) was sensitive to endo H treatment (Fig. 4.6B, lane d) indicating that this protein contained N-linked oligosaccharides exclusively of the high-mannose type (Kornfeld and Kornfeld, 1985). These findings suggest that recombinant gH was not transported from the endoplasmic reticulum to the Golgi.

To determine if coexpression of gH and gL proteins would help in proper processing and transport of recombinant gH, LMTK$^-$ cells were infected with SKgH or SKgL individually or together. The radiolabelled proteins were immunoprecipitated with anti UL22 serum and analysed by SDS-PAGE. As compared to gH alone (Fig. 4.6C, lane d) coexpression of gL and gH resulted in the synthesis of a 108 kDa protein (mature form) (Fig. 4.6C, lane e) that is indistinguishable from gH synthesized in BHV-1 infected cells (Fig. 4.6C, lane c) and resistant to endo H treatment (Fig. 4.6C, lane f). This suggests that gH and gL form a complex, which assists in the processing and transport of gH.

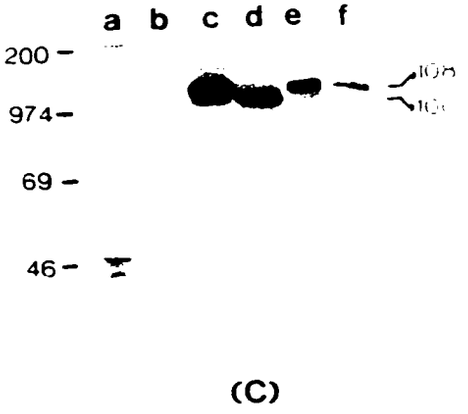
Fig. 4.6. Processing of glycoprotein gH. (A) BHV-1 infected MDBK cells (panel 1) or recombinant VV SKgH infected LMTK$^-$ cells (panel 2) were pulse labelled with [^{35}S]-cysteine for 15 min (lane a) and chased for 15 (lane b), 30 (lane c), 60 (lane d) or 120 (lane e) min in unlabelled medium. Cell extracts were immunoprecipitated with a UL22 MAb pool and analysed by 12.5% SDS-PAGE under reducing conditions. (B) Proteins from [^{35}S]-cysteine labelled lysates of BHV-1 infected MDBK cells (lane a,b) or recombinant VV SKgH infected LMTK$^-$ cells (lane c,d) were immunoprecipitated with anti UL22 serum. Immunoprecipitated proteins were mock digested (lane a,c) or treated with endo-H (lane b,d) and analysed by 7.5% SDS-PAGE under reducing conditions. (C) Proteins from [^{35}S]-cysteine labelled lysates of mock infected (lane a) or BHV-1 infected MDBK cells (lane c); wild type vaccinia virus infected (lane b), recombinant VV SKgH infected (lane d) and recombinant VV SKgH plus recombinant VV SKgL infected (lane e) LMTK$^-$ cells were immunoprecipitated with anti UL22 serum. Immunoprecipitated proteins from recombinant VVs SKgH and SKgL infected LMTK$^-$ cells were treated with endo H (lane f). Proteins were analysed by 7.5% SDS-PAGE under reducing conditions. The mol wt markers in kDa are indicated on the left.



(A)



(B)



(C)

To examine if the gH-gL complex formation was neccessary for the processing and transport of gL, radiolabelled proteins from infected cell lysates were immunoprecipitated with anti BUL1-1 serum . As seen in Fig. 4.7A, the electrophoretic mobility of recombinant gL synthesized in SKgL infected cells (lane d) was similar to authentic gL produced in BHV-1 infected cells (lane c) or gL produced in SKgL and SKgH infected cells (lane b). In addition, recombinant gL produced in SKgL infected LMTK$^-$ cells contained O-linked oligosaccharides (Fig. 4.7B, lane c). This suggests that gH is not required for processing and transport of gL from the endoplasmic reticulum to the Golgi.

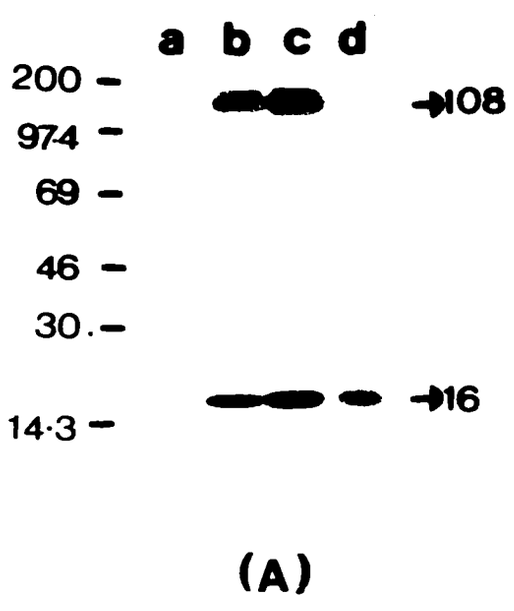
4.3.8 BHV-1 gL is not anchored independently to cell membranes.

Recombinant gH, when expressed alone, was detected intracellularly (Fig. 4.8e) but was not detected on the surface of the infected cells (Fig. 4.8f) or in the culture media (Fig. 4.9, lane b). In contrast, co-expression of gH and gL proteins resulted in the detection of gH on the surface of the infected cells (Fig. 4.8h) but not in the culture media (Fig. 4.9, lane c) confirming that processing and transport of gH is dependent on gL. Similarly, recombinant gL, when expressed alone was detected intracellularly (Fig. 4.8c) but was not detected on the surface of the infected cells (Fig. 4.8d). Co-expression of gL and gH resulted in the detection of gL on the surface of infected cells (Fig. 4.8j). However, gL was immunoprecipitated from the culture media of cells infected with recombinant VV SKgL alone (Fig. 4.9, lane a), but not from cells coinfected with recombinant VVs SKgL and SKgH (Fig. 4.9, lane d). These results suggest that gL is membrane associated as a result of its interaction with gH.

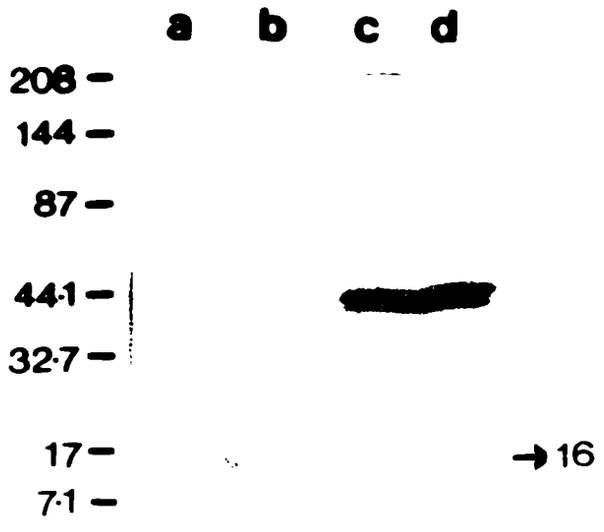
4.3.9 Immunogenicity of recombinant gH and gL proteins.

In order to examine if complex formation between gH and gL was neccessary for the development of neutralizing antibody responses, rabbits were immunized with recombinant VVs SKgH and SKgL individually or in combination. As shown in Table 4.1, ELISA titers were detected in sera from rabbits immunized with gH, gL and gH-

Fig. 4.7. Processing of glycoprotein gL. (A) Proteins from lysates of [^{35}S]-cysteine labelled wild type VV (lane a), recombinant VV SKgL (lane d) and recombinant VV SKgL plus recombinant VV SKgH (lane b) infected LMTK$^-$ cells or BHV-1 infected MDBK cells (lane c) were immunoprecipitated with anti BUL1-1 peptide serum and analysed on 15% SDS-PAGE under reducing conditions. (B) Proteins from lysates of mock infected (lane d), SKgL infected LMTK$^-$ cells (lane b,c) or BHV-1 infected MDBK cells (lane a) were separated on SDS-PAGE under reducing conditions. After western blotting, blots were probed with either anti BUL1-1 serum (lane a) or biotinylated lectin *(Dolichus biflorus)* before (lane c,d) or after neuraminidase and O-glycanase treatment (lane b). The mol wt markers in kDa are indicated on the left.

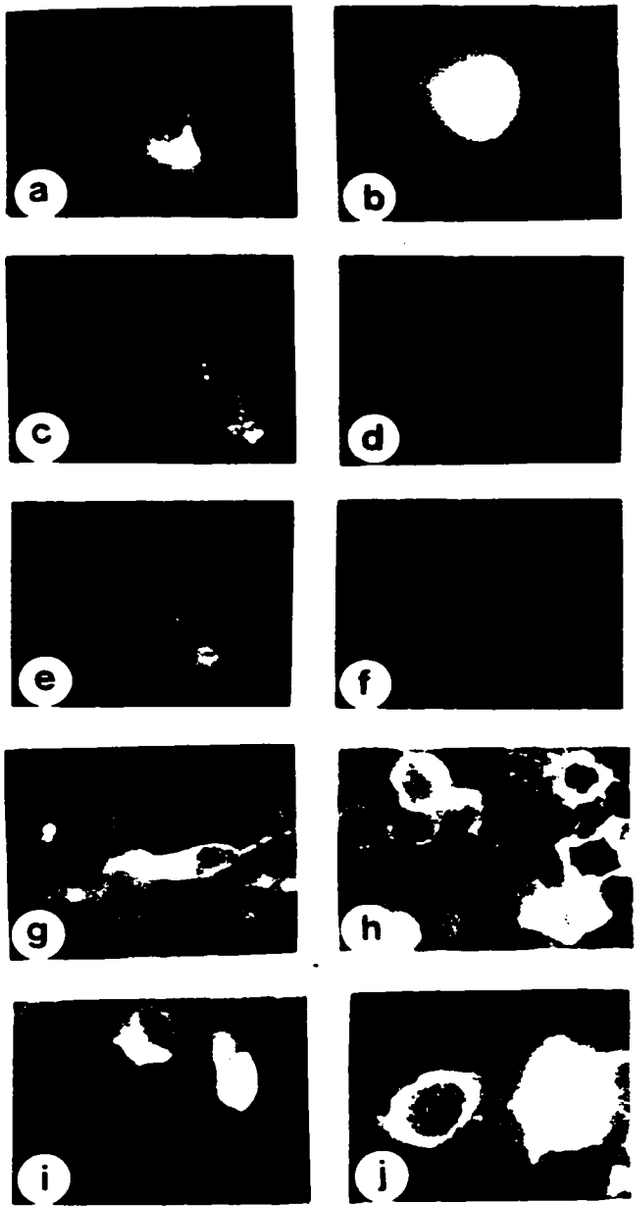


(A)



(B)

Fig. 4.8. Analysis of distribution of gH and gL glycoproteins. MDBK cells infected with BHV-1 (a,b), or LMTK$^-$ cells infected with recombinant VV SKgL (c,d), recombinant VV SKgh (e,f) or recombinant VVs SKgH plus SKgL (g,h,i,j) were fixed either with 2% paraformaldehyde to detect protein on the surface of the cells (b,d,f,h,j) or permeabilized with methanol to detect protein in the cells (a,c,e,g,i). The fixed cells were treated with either anti UL22 serum (a,b,e-h) or anti BUL1-1 peptide serum (c,d,i,j) followed by fluorescein-conjugated goat anti-rabbit IgG. Mock infected BHV-1 or LMTK$^-$ cells after fixing with paraformaldehyde or permeabilized with methanol, looked similar to those in panel d.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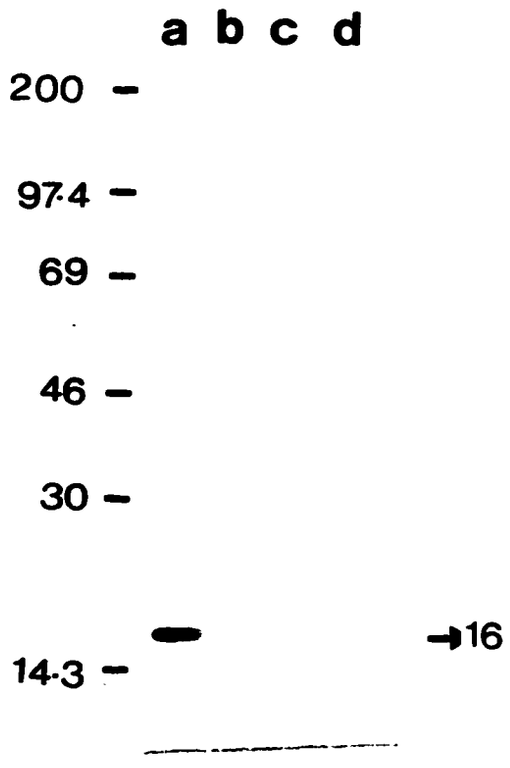


Fig. 4.9. Secretion of glycoprotein gH and gL. [^{35}S]-cysteine labelled culture medium from LMTK$^-$ cells infected with recombinant SKgL (lane a), recombinant SKgH (lane b) or recombinant SKgL plus SKgH (lane c,d) were immunoprecipitated with either anti BUL1.-1 peptide serum (lane a,d), anti UL22 serum (lane b,c) or with anti BUL1-1 peptide serum (lane a,d), and proteins were analysed by 15% SDS-PAGE under reducing conditions. The mol wt markers in kDa are shown on the left.

Table 4.1 Serologic Response of Rabbits Immunized with Recombinant SKgL or SKgH[a]

Antigen	ELISA antibody titers[b]	Virus neutralizing titre[c]
Wild-type VV	<10	<2
Recombinant gL	160	<2
Recombinant gH	640	<2
Recombinant gH plus gL	2560	16

[a] Rabbits were immunized intraperitonially with 0.2ml of 10^6 pfu per milliliter at 0 and 21 days and sera were obtained 2 weeks after the second immunization.

[b] ELISA titers versus 0.1μg of purified BHV-1 per well (van Drunen Littel-van den Hurk et al., 1984).

[c] Virus neutralizing antibody titers versus 100 pfu of BHV-1 in the absence of complement (van Drunen Littel-van den Hurk et al., 1984).

gL.However, complement independent serum neutralizing antibody responses could only be detected in rabbits immunized with gH-gL.

4.4 DISCUSSION

Several studies have shown that a novel glycoprotein designated gL is encoded by members of all herpesvirus subgroups including alpha, beta and gamma herpesviruses and required for the correct processing and transport of glycoprotein H (Forgani et al., 1994; Hutchinson et al., 1992b; Kaye et al., 1992; Liu et al., 1993; Spaete et al., 1993; Yaswen et al., 1993). This glycoprotein complex (gH-gL) has been proposed to be essential for virus penetration and cell to cell spread. Although these two glycoproteins are structural components of all herpesviruses examined so far, the interaction between them differs amongst the different herpesviruses (Duus et al., 1995) suggesting that this complex may be involved in virus specific interactions and thus, may determine the tropism of the virus. In this report we showed that BHV-1 also encodes functional gL and gH glycoproteins, which are structural components of the virion and form a complex, which appears to be

required for the proper processing, transport and antigenicity of gH and anchoring of gL on the cell surface.

The BHV-1 UL1 gene has been suggested to encode a protein of 158 amino acids, which is predicted to contain a N-terminal signal sequence with no potential site(s) for the addition of N-linked oligosaccharides (Khattar et al., 1995). Two different antipeptide sera immunoprecipitated a protein of 16-17 kDa from BHV-1 infected cells and purified virions, which we designated glycoprotein gL based on following observations i) the BHV-1 UL1 gene is a positional homolog of other herpesvirus glycoptotein gL, ii) the N-terminal hydrophobic region of the BHV-1 UL1 protein acts as a signal sequence as it is cleaved, iii) the BHV-1 UL1 protein is glycosylated, iv) the BHV-1 UL1 protein is functionally equivalent to glycoprotein gL of other herpesviruses. BHV-1 gL does not need any other viral protein for its posttranslational processing, since cells infected with recombinant VV SKgL secrete gL, which is indistinguishable from gL produced in BHV-1 infected cells or in cells transfected with gL (data not shown). In contrast, a recent report suggests that HSV-1 gL produced in transfected cells (Dubin and Jiang, 1995) is processed differently than gL produced in recombinant VV infected cells (Hutchinson et al., 1992b).

However, BHV-1 gL does need a glycoprotein for its association with the plasma membrane. Three lines of evidence suggest that glycoprotein gH is required for the anchoring of gL to the plasma membrane. i) There is no other hydrophobic domain in gL that might serve as putative transmembrane anchor except the N-terminal signal sequence, which is cleaved in the mature form, ii) gL is secreted from cells infected with recombinant VV SKgL but not from cells coinfected with recombinant VV SKgL and SKgH, iii) cell surface expression of gL results from complex formation with gH, which has a well defined carboxy-terminal hydrophobic transmembrane anchor domain. A similar observation was made earlier for human cytomegalovirus (HCMV) gL (Spaete et al., 1993) and herpes simplex virus-1 (HSV-1) gL (Dubin and Jiang, 1995).

Previous studies have demonstrated that BHV-1 gII and gp108 are identical proteins using anti-gH peptide sera (van Drunen Littel-van den Hurk et al., 1996). Using cross-immunoprecipitation with monoclonal antibodies to BHV-1 gp108 and anti UL22

peptide antiserum, it was suggested recently that gp108 is the product of the UL22 (gH) ORF (Baranowski et al., 1995). Our results involving the expression of the UL22 gene *in-vitro* and *in-vivo* confirm and extend the previous observations and provide further evidence that gp108, gII and the translation product of UL22, glycoprotein gH are identical proteins and contain only N-linked oligosaccharides.

BHV-1 glycoproteins gB, gC and gD expressed by recombinant VVs are transported and processed similar to the authentic glycoproteins produced in BHV-1 infected cells (Tikoo et al., 1993a, 1993b; van Drunen Littel-van den Hurk et al., 1989). However, the characteristics of gH expressed by recombinant VV SKgH differed from those of authentic gH produced in BHV-1 infected cells. The recombinant gH was slightly smaller, possessed endo H sensitive glycans and was not transported to the cell surface. This is unlikely to be due to some anomaly in the vaccinia virus expression system since similar results were found with BHV-1 gH expressed by transfected MDBK cells (unpublished results). This defect in intracellular transport appears to be a conserved feature of herpesvirus gH homologs including HSV-1 (Gompels and Minson, 1989). The proper processing and transport of recombinant gH could be restored by coexpression of gH with gL. Further evidence that gH and gL form a complex comes from the observation that both glycoproteins were immunoprecipitated from BHV-1 infected cells with either anti-gH or anti-gL serum. This gH-gL interaction appears to be noncovalent since proteins with the same molecular weight were detected by PAGE analysis under reducing and nonreducing conditions. Although similar gH-gL complex formation has been observed in other herpesviruses, the nature of the interactions differ. For example the HSV-1 gH is associated noncovalently with gL (Hutchinson et al., 1992b; Kaye et al., 1992) and CMV gH is associated with gL by disulphide linkages (Spaete et al., 1993).

Like other herpesvirus gH proteins, BHV-1 gH has been suggested to be involved in virus penetration, cell to cell spread and induction of neutralizing antibodies (Baranowski et al., 1993). Using an affinity purified gH:gL complex, we demonstrated that preincubation of cells with the complex prevented virus penetration but did not prevent virus attachment (van Drunen Littel-van den Hurk et al., 1996). In addition, neutralizing antibodies could be detected in sera of rabbits immunized with both

recombinant VVs SKgL and SKgH but not when the rabbits were immunized with individual recombinant VV. These results clearly suggested that formation of the gH-gL complex is neccessary for the biological and immunological functions of gH. However, it is not clear what the contibution of gL is to these biological and immunological functions. It is possible that a functional domain comprised of parts of both gH and gL is produced as a result of their interaction. Alternatively, gL may be helping gH in attaining the biologically active configuration and thus may not be directly involved in these functions.

5.0 THE UL10 GENE OF BOVINE HERPESVIRUS TYPE-1 (BHV-1) ENCODES GLYCOPROTEIN gM, A COMPONENT OF THE VIRION ENVELOPE AND INFECTED CELL MEMBRANES

5.1 Introduction

Bovine herpesvirus-1 (BHV-1), a member of the subfamily alphaherpesvirinae, is a predominant cause of respiratory diseases, abortion and genital infections in cattle (Yates, 1982). To-date, 10 glycoprotein genes of BHV-1 have been identified and designated as gB, gC, gD, gE, gG, gH, gI, gK, gL and gM, but only a few have been characterized at the protein level (Baranowskiet al., 1996; Khadr et al., 1996; Khattar et al., 1995, 1996; Tikoo et al., 1995; Vlcek et al., 1995). Although all BHV-1 glycoproteins described to date exhibit some homology to respective glycoproteins of other alphaherpesviruses, yet detailed analysis has demonstrated the existence of structural and functional differences among different homologs (Fehler et al., 1992; Ligas & Johnson, 1988; Mettenleiter & Spear, 1994; Peeters et al., 1992; Rauh et al., 1991). While gB, gC and gD are the major glycoproteins, which are present in large amounts in the virion envelope and the plasma membrane of infected cells (reviewed by Tikoo et al., 1995), gH, gE, gI and gG have been identified as minor glycoproteins (reviewed by Baranowski et al., 1996).

Recently, glycoprotein gM encoded by the UL10 gene of HSV-1 (McGeoch et al., 1988) has been analyzed in detail (Baines & Roizman, 1991; Baines & Roizman, 1993; MacLean et al., 1993). It is one of the nonessential glycoproteins, which is conserved in all the herpesvirus subfamilies. The herpesvirus gM homologs are characterized by the presence of eight stretches of hydrophobic membrane spanning domains typical of class III integral membrane glycoproteins (Baines & Roizman, 1993; Dijkstra et al., 1996; Lehner et al., 1989; MacLean et al., 1991; Pilling et al., 1994). Glycoprotein gM has been shown to represent a major structural component of the

virion in EHV-1 (Pilling et al., 1994) and PRV (Dijkstra et al., 1996) and a minor virion component in HSV-1 (Baines & Roizman, 1993; MacLean et al., 1993). Enzymatic deglycosylation studies in HSV-1 (Baines & Roizman, 1993; MacLean et al., 1993), PRV (Dijkstra et al., 1996) and EHV-1(Osterrieder et al., 1997) have shown that gM is modified by the addition of only N-linked glycans. Although gM homologs are dispensable for replication in cell culture, they appear to play a modulatory role in membrane fusion during initial stages of virus-cell interactions in HSV-1, EHV-1 and PRV (Baines & Roizman, 1991; Davis-Poynter et al., 1994; Dijkstra et al., 1996; MacLean et al., 1993; Osterrieder et al., 1996).

Recently, a homolog of the HSV-1 UL10 gene has been sequenced in BHV-1 (Vlcek et al., 1995). Sequence analysis of BHV-1 UL10 reveals an ORF of 411 amino acids, which contains eight well conserved transmembrane hydrophobic domains and a potential N-linked glycosylation site located at asparagine 57 amino acid residue. In this communication, we demonstrate that BHV-1 UL10 gene encodes a glycoprotein gM, which contains both N-linked and O-linked oligosaccharides. In addition, gM is a major structural component of the virion and forms disulphide linked dimers.

5.2 Materials and Methods

5.2.1 Reagents and media.

Cell culture media and fetal bovine serum were purchased from GIBCO/BRL, Burlington, Ontario, Canada. Reagents for DNA manipulations and Protein A sepharose were purchased from Pharmacia, Dorval, Quebec, Canada and used as recommended by the manufacturer. The enzyme neuraminidase was obtained from Sigma Chemicals Co, St. Louis, Mo and Pierce, Rockford, IL, USA. The enzymes O-glycosidase, PNGase F and endo H were purchased from Boehringer Mannheim Canada, Laval, Quebec, Canada. The reagents for immunofluorescence staining and other immunological assays were purchased from Zymed Laboratories Inc. San Francisco, California USA and Bio-Rad Mississauga, Ontario, Canada. Radioisotopically labelled compounds and reagents for fluorography were purchased from ICN, Irvine, California. Reagents for *in-vitro*

transcription and translation including plasmid pSP64polyA were purchased from Promega\Fisher, Scientific Ltd. Nepean, Ontario, Canada. Freund's incomplete and complete adjuvants were purchased from Difco Laboratories, Detroit, Michigan, USA. Anti-GST goat antibodies for Western blotting was purchased from Pharmacia, Dorval, Quebec, Canada.

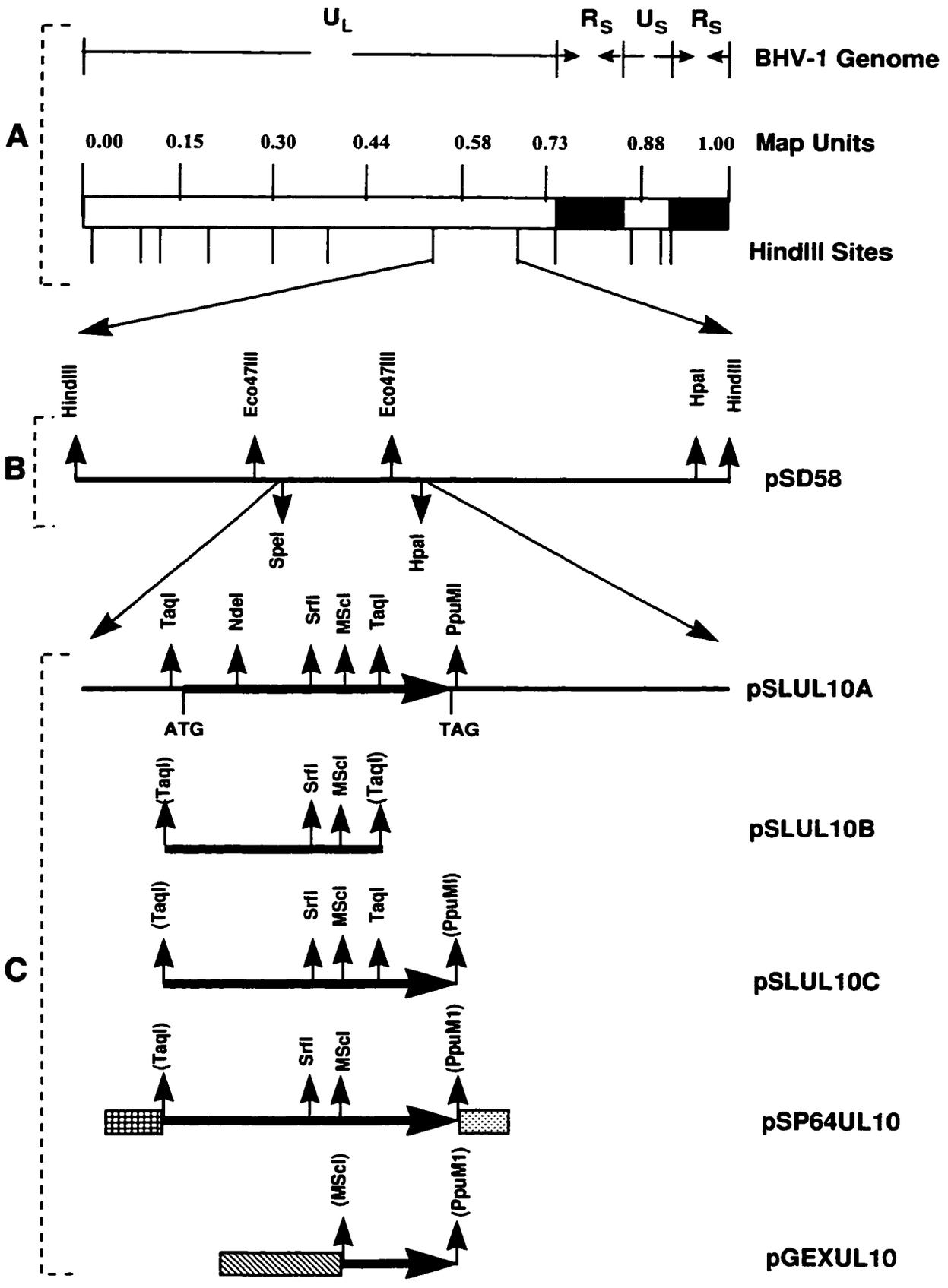
5.2.2 Cells and Viruses.

Madin-Darby bovine kidney (MDBK) cells were grown as monolayers in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum. Virus stocks of BHV-1 were prepared in MDBK cells (Babiuk & Rouse, 1975).

5.2.3 Plasmid constructions.

The construction and restriction mapping of a pBR322-based plasmid library, containing fragments of the BHV-1 Cooper strain genome have been described in detail (Mayfield et al., 1983). As shown in Fig. 5.1, the HindIII B fragment cloned into pBR322 (pSD58; Mayfield et al., 1983) was digested with appropriate enzymes and a 3.9 kb (SpeI-HpaI) fragment was cloned into SpeI-Eco47III digested pSL301 (Invitrogen Corporation, San Diego, California, USA), generating a plasmid pSLUL10A. A 1.1 kb TaqI fragment isolated from plasmid pSLUL10A was inserted into HpaI digested pSL301, generating a plasmid pSLUL10B. A 0.4 kb fragment was isolated from plasmid pSLUL10A after digestion with PpuMI (blunt end repaired with T4 DNA polymerase) and SrfI enzymes. Plasmid pSLUL10C [containing full length UL10-encoding gene (1.3 kb)] was constructed by ligating a 0.4 kb PpuMI-SrfI fragment to SrfI-Eco47III digested plasmid pSLUL10B. The full length UL10 gene was excised from the plasmid pSLUL10C after digesting the plasmid with SnaBI and EcoRV enzymes, and inserted into HincII digested pSP64polyA, generating plasmid pSP64UL10. A plasmid pGEXUL10 was constructed by ligating a 265 bp MscI-EcoRV fragment (containing the carboxy-terminal 80 amino acids of UL10 gene) isolated from plasmid pSLUL10C into XmaI digested (blunt end repaired) pGEX-2T vector.

Fig. 5.1. Map location of the BHV-1 UL10 gene and UL10 plasmids. (A) Schematic diagram of the BHV-1 (Cooper strain) genome as adapted from Mayfield et al., 1983. The genome is divided into a unique long (U_L) and unique short segment (U_S) flanked by inverted repeat regions (R_S). Location of HindIII restriction endonuclease cleavage sites are indicated. (B) Expanded portion of HindIII B fragment (pSD58) (C) Plasmid pSLUL10A contains a 3.9 kb SpeI-HpaI fragment derived from pSD58. UL10 ORF is shown as an arrow in this plasmid. Plasmid pSLUL10B contains a 1.1 kb TaqI-TaqI fragment (indicated by ▬) derived from plasmid pSLUL10A. Plasmid pSLUL10C contains the 1.3 kb full length UL10-encoding gene (TaqI-PpuMI) (indicated by →). Plasmid pSP64polyA used for invitro trancription and translation contains the UL10 open reading frame (indicated by →) flanked by a SP6 promoter (indicated by ▦) and polyA (indicated by▭). Plasmid pGEXUL10 used for generation of a fusion protein contains the C-terminal 80 amino acids of the UL10 gene (indicated by →) fused to glutathione S-transferase gene (indicated by▨). Filled arrows indicate direction of transcription. Relevent restriction endonuclease cleavage sites are indicated. Brackets around some restriction endonuclease cleavage sites indicate that these sites were lost when those fragments were cloned into appropriate vectors.


A
U_L
R_S U_S R_S
BHV-1 Genome
0.00 0.15 0.30 0.44 0.58 0.73 0.88 1.00
Map Units
HindIII Sites
B
HindIII
Eco47III
Eco47III
HpaI
HindIII
pSD58
SpeI
HpaI
TaqI
NdeI
SrfI
MscI
TaqI
PpuMI
pSLUL10A
ATG
TAG
C
(TaqI)
SrfI
MscI
(TaqI)
pSLUL10B
(TaqI)
SrfI
MscI
TaqI
(PpuMI)
pSLUL10C
(TaqI)
SrfI
MscI
(PpuM1)
pSP64UL10
(MscI)
(PpuM1)
pGEXUL10

5.2.4 Generation of anti-UL10 serum.

Plasmid pGEXUL10, containing the C-terminal 80 amino acids of the UL10 protein fused to the gene encoding GST protein, was introduced into *E.coli.* and the fusion protein was expressed following induction with 0.1 mM isopropyl β-D-thiogalactoside (IPTG). GST-UL10 fusion protein was purified by excising the 38 kDa protein band from sodium dodecyl sulphate (SDS)-polyacrylamide gels (PAGE) and eluting from the gel. Two rabbits were immunised subcutaneously with approximately 300 μg of the fusion protein in Freund's complete adjuvant. The rabbits were boosted with 100 μg of the fusion protein in Freund's incomplete adjuvant at 4, 8, and 12 weeks after the primary injections. Ten days after the last boost, the rabbits were bled out and antisera were prepared. Production and characterization of BHV-1 gD and gB specific monoclonal antibodies has been described (Hughes et al., 1988; van Drunen Littel-van den Hurk et al., 1984).

5.2.5 *In-vitro* transcription and translation.

One to five μg of plasmid DNA (pSP64UL10) was *in-vitro* transcribed using SP6 polymerase promoter as described by the supplier. RNA was translated *in-vitro* for 2 hr at 30°C using rabbit reticulocyte lysate containing 40 μCi of [^{35}S] cysteine. Canine pancreatic microsomal membranes were added to some reaction mixtures to process the *in-vitro* translated proteins. After synthesis, the *in-vitro* translated proteins were analyzed on SDS-PAGE with or without immunoprecipitation.

5.2.6 Radiolabelling and immunoprecipitation.

BHV-1 infected MDBK cells were labelled by [^{35}S]cysteine or [^{3}H]glucosamine as described previously (van Drunen Little-van den Hurk et al., 1984). [^{35}S]cysteine labelled virons were purified from BHV-1 infected MDBK cells by sucrose gradient centrifugation as described previously (Liang et al., 1991). Proteins were immunoprecipitated from the infected cell lysate or virons using anti-UL10 serum as described previously (van Drunen Littel-van den Hurk et al., 1984). After immunoprecipitation, the protein A-Sepharose beads were resuspended in 2XSDS-

PAGE sample buffer with or without 2% 2-mercaptoethanol. The samples were either heated at 37°C for 30 min, 56°C for 5 min or boiled for 2 min and separated by SDS-PAGE.

5.2.7 Enzyme digestions.

The immunoprecipitated proteins were eluted from protein A-Sepharose in 20µl of 0.5% SDS by heating the samples at 56°C for 5 min. The digestions of protein with endo H (to remove high mannose forms of N-linked sugars), PNGase F (to remove high mannose and complex forms of N-linked sugars), neuraminidase (to remove sialic acid), and O-glycosidase(to remove O-linked oligosaccharides) were done as described previously (Tikoo et al., 1993a, 1993b).

5.2.8 Western blotting.

UL10-GST fusion proteins from uninduced and induced bacterial cell lysates were suspended in modified lysis buffer and the proteins were separated by 12.5%SDS-PAGE (van Drunen Littel-van den Hurk et al., 1984). For Western blotting the proteins were subsequently transferred to nitrocellulose membranes and reacted with anti-GST antibodies. Bound antibody on the membranes was reacted with horseradish peroxidase conjugated secondary antibody and visualized after developing with hydrogen peroxide and chloronapthol of Bio-Rad immunoblot assay kit.

5.2.9 Immunofluorescence staining.

MDBK cells were seeded and infected in four-well Lab-Tek chamber slides. The infected cells were fixed with either 2% paraformaldehyde for 15 min at 4°C for surface staining or 100% methanol for 15 min at -20°C for internal staining. The cells were incubated with anti-UL10 serum and followed by goat anti-rabbit immunoglobulin G conjugated to FITC and analyzed with the aid of a fluorescent microscope.

5.3 Results

5.3.1 Production of UL10-GST fusion protein for immunization of rabbits.

Computer analysis of the deduced BHV-1 UL10 protein predicted a potential antigenic site close to the carboxy terminus (last 80 amino acids) (Fig 5.2). A plasmid designated pGEXUL10 was generated by fusing the last 80 amino acids of the UL10 ORF to the gene encoding GST. The junction of the sequences encoding GST and UL10 was sequenced to ensure that the two coding domains were in frame (data not shown). Plasmid pGEXUL10 was transformed into *E.coli* strain DH5α and after induction by IPTG, the lysate was analysed by 12.5% SDS-PAGE using Coomassie blue staining (Fig 5.3A) and by Western blotting using anti-GST antibodies (Fig. 5.3B). As compared to uninduced (Fig. 5.3A, lane a and Fig. 5.3B, lane b), the induced UL10-GST fusion protein migrated at the expected position of 38 kDa (Fig. 5.3A, lane b and Fig. 5.3B, lane a). The fusion protein was purified by excising the 38 kDa band from a SDS-PAGE gel and subsequently eluting from the gel. The purified fusion protein was checked for purity (Fig. 5.3A, lane c) and used to immunize the rabbits.

5.3.2 *In-vitro* translation and immunoprecipitation of UL10 protein.

In order to determine the identity of the UL10 ORF and to check whether antibodies against this gene product recognized the UL10 protein, an *in-vitro* expression plasmid pSP64UL10 was generated in which the coding sequence of UL10 was placed downstream of the SP6 promoter. *In-vitro* translation of pSP64UL10 RNA resulted in the synthesis of a polypeptide of 36-37 kDa (Fig. 5.4A, lane a) The same protein was also recognized by anti-UL10 serum (Fig. 5.4A, lane b). However this protein was not immunoprecipitated with anti-UL10 serum when pSP64polyA RNA was translated *in-vitro* (Fig. 5.4A, lane C). In addition, preimmune serum did not immunoprecipitate any protein from the reaction containing pSP64UL10 RNA (Fig. 5.4A, lane d). These results suggest that serum against GST-UL10 fusion protein specifically recognises UL10 protein.

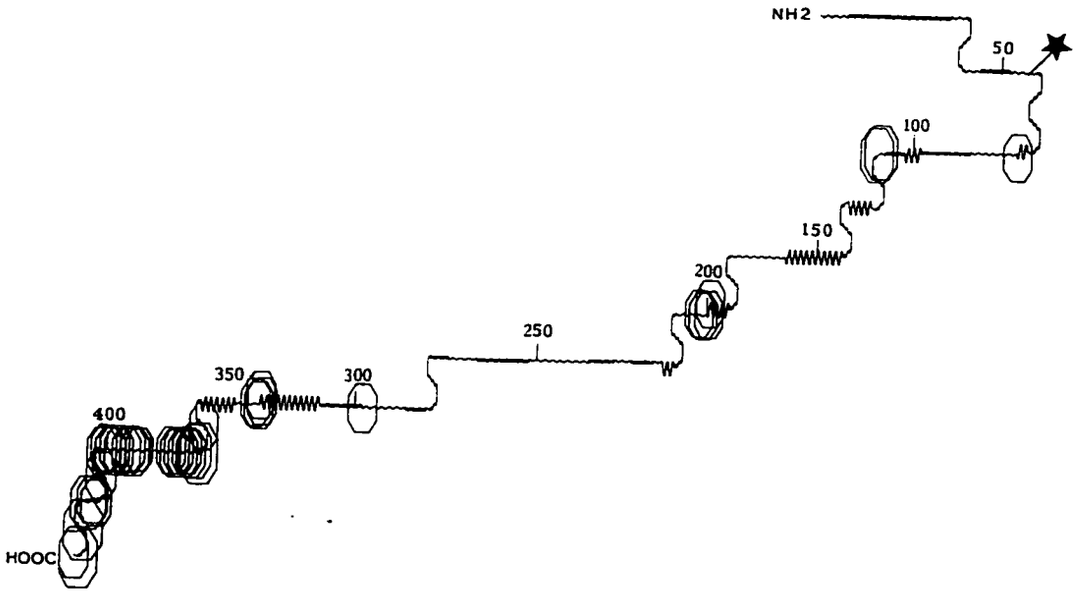


Fig. 5.2. Prediction of secondary structure and antigenicity of UL10 protein. Secondary structure and antigenicity were predicted from the deduced amino acid sequence of BHV-1 UL10 open reading frame (Vlcek et al., 1995) by the method of Chou and Fasman, 1978, and Jameson and Wolf, 1988, respectively. Octagons indicate local antigenic indices higher than 1.2. Amino acid residues from 332-411 present at the C-terminal end of the UL10 open reading frame (represented by cluster of octagons) were used to generate anti-UL10 serum. Potential N-linked glycosylation site (star) is also indicated.

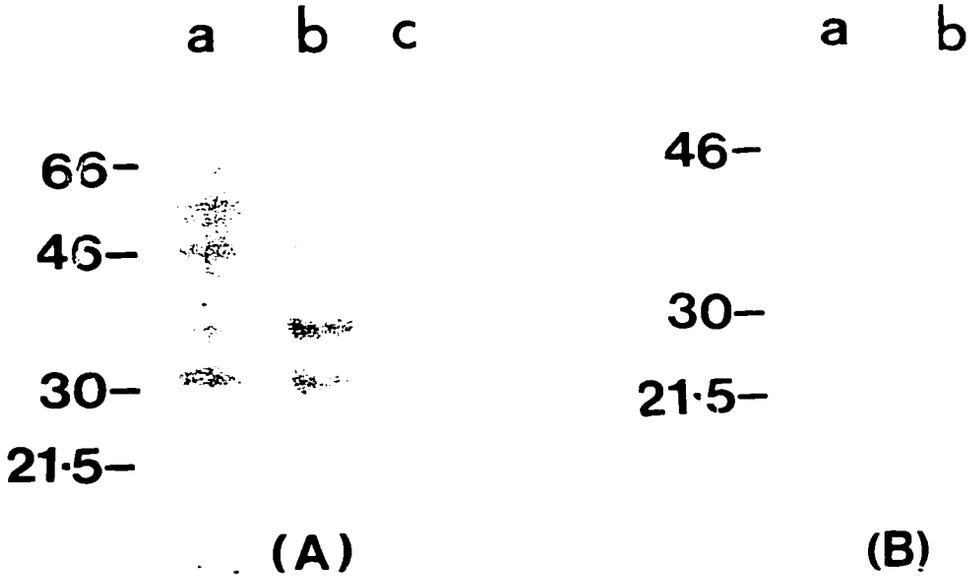


Fig. 5.3. Production of UL10-GST fusion protein. (A) The UL10-GST fusion protein from lysates of uninduced (lane a) and induced (lane b) bacterial cultures was separated by 12.5% SDS-PAGE under reducing conditions and stained with Coomasssie brilliant blue. The fusion protein was purified, separated on SDS-PAGE under reducing conditions and stained with Coomassie brilliant blue (lane c). (B) Proteins from lysates of uninduced (lane b) and induced (lane a) bacterial cultures containing pGEXUL10 plasmid were separated by 12.5% SDS-PAGE under reducing conditions, and separated proteins were probed in western blots with anti-GST antibodies. The mol wt markers in kDa are indicated on the left.

5.3.3 *In-vivo* expression of UL10 protein.

To further characterize the proteins and to confirm the specificity of our antiserum, immunoprecipitation assays were performed. Radiolabelled proteins from mock infected or BHV-1 infected cell lysates were immunoprecipitated and analysed by SDS-PAGE. Anti-UL10 serum recognized a protein of 43-44 kDa in BHV-1 infected (Fig. 5.4B, lane b) as compared to mock infected MDBK cells (Fig. 5.4B, lane a). Since the UL10 protein aggregated when heated to 100°C (Fig. 5.4B, lane b), even in the presence of SDS, BHV-1 infected MDBK cell lysates were mixed with an equal volume of electrophoresis sample buffer and incubated at 37°C for 30 min (Fig. 5.4B, lane c) or 56°C for 5 min (Fig. 5.4B, lane d). These results suggest that anti-UL10 serum recognizes a protein of 43-44 kDa in BHV-1 infected MDBK cells. In addition, these results also demonstrate the sensitivity of UL10 protein to boiling and its conversion to aggregates, which are partially entering the separating gel.

5.3.4 The UL10 protein is glycosylated.

Homologs of the herpesvirus UL10 proteins have been shown to be glycosylated (Baines & Roizman, 1993; Dijkstra et al., 1996; MacLean et al., 1993; Osterrieder et al., 1997). Sequence analysis of the UL10 ORF of BHV-1 shows one potential site for the attachment of N-linked oligosacchrides (Vlcek et al., 1995). Analysis of *in-vitro* translated UL10 mRNA (Fig. 5.5A, lane a) in the presence of canine microsomal membranes (Fig. 5.5A, lane b) showed a decrease in the mobility of UL10 protein. Treatment of *in-vitro* translated and processed UL10 protein with endo H showed an increase in the mobility of the protein (Fig. 5.5A, lane c). Further support for the glycosylation of the UL10 protein was obtained by labelling BHV-1 infected MDBK cells with [^{3}H] glucosamine (Fig. 5.5A, lane d). In addition, treatment of the UL10 protein (Fig. 5.5B, lane a) with PNGase F (Fig. 5.5B, lane c), neuraminidase (Fig. 5.5B, lane d), or neuraminidase and O-glycosidase (Fig. 5.5B, lane e) but not with endo H (Fig. 5.5B, lane b), showed an increase in the mobility of the protein. As a control, BHV-1 glycoprotein gD (Fig. 5.5B, lane f), which contains both N-linked and O-linked glycans was treated with PNGase F (Fig. 5.5B, lane g), neuraminidase (Fig. 5.5B, lane h) or neuraminidase

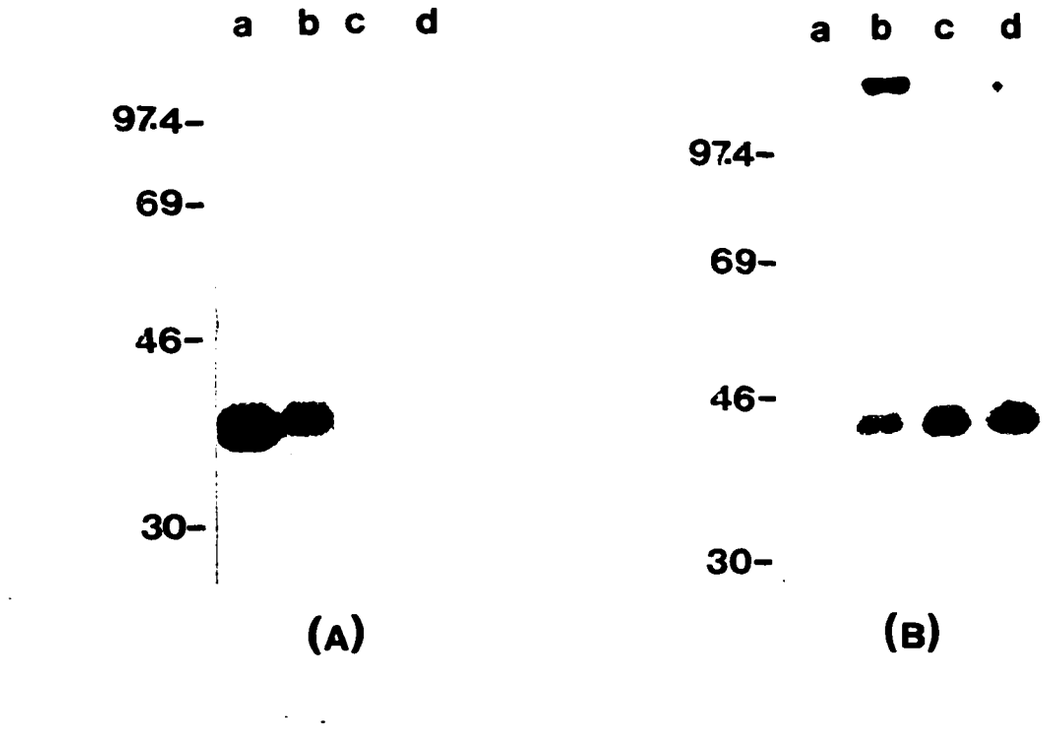


Fig. 5.4. Immunoprecipitation of proteins. (A) *In-vitro* expression of UL10 protein. [^{35}S] cysteine-labelled *in-vitro* translated pSP64UL10 products (lane a), immunoprecipitated with anti-UL10 serum (lane b) and preimmune serum (lane d); and [^{35}S] cysteine-labelled pSP64polyA products, immunoprecipitated with anti-UL10 serum (lane c) were separated on 12.5% SDS-PAGE under reducing conditions. The mol wt markers in kDa are indicated on the left. (B) *In-vivo* expression of UL10 protein. Proteins from lysates of [^{35}S] cysteine-labelled mock infected (lane a) or BHV-1 infected (lanes b to d) MDBK cells were immunoprecipitated with anti-UL10 serum. Immunoprecipitated samples were incubated at 100°C for 2 min (lane a, b), 37°C for 30 min (lane c) or 56°C for 5 min (lane d) in electrophoresis sample buffer and analyzed by 12.5% SDS-PAGE under reducing conditions. The mol wt markers in kDa are indicated on the left.

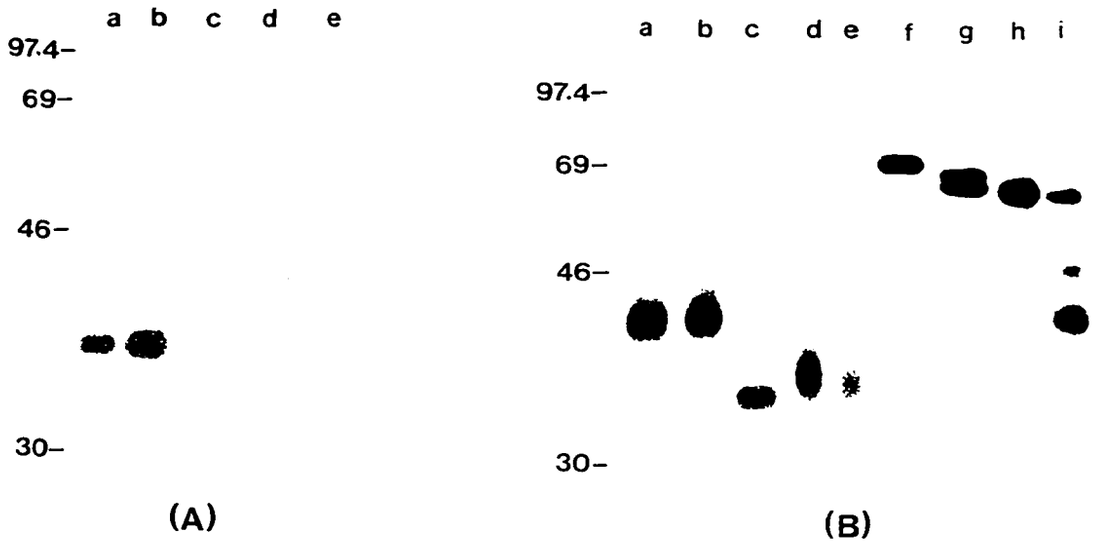


Fig 5.5. Glycosylation of UL10 protein. (A) Immunoprecipitation of proteins from [^{35}S] cysteine-labelled *in-vitro* translated pSP64UL10 RNA in the absence (lane a) or presence (lane b) of canine microsomal membranes or from lysates of [^{3}H] glucosamine-labelled mock infected (lane e) or BHV-1 infected (lane d) MDBK cells by anti-UL10 serum. Immunoprecipitated proteins were separated by 12.5% SDS-PAGE under reducing conditions. (B) Proteins from [^{35}S] cysteine-labeled BHV-1 infected MDBK cells were immunoprecipitated with anti-UL10 serum (lane a to e) or gD specific monoclonal antibodies (lane f to i). The immunoprecipitated proteins were mock digested (lane a, f) or treated with endo H (lane b), PNGase F (lane c, g),neuraminidase (lane d, h), neuraminidase and O-glycosidase (lane e, i) and analyzed on 12.5% SDS-PAGE. The mol wt markers are indicated on the left.

and O-glycosidase (Fig. 5.5B, lane i). These results suggest that the UL10 protein is a glycoprotein, which is modified by attachment of both N-linked as well as O-linked oligosaccharides and hence was named glycoprotein gM, in accordance with the uniform nomenclature system of alphaherpesvirus glycoproteins described earlier (Tikoo et al., 1995).

5.3.5 Glycoprotein gM is a component of the BHV-1 virions.

In order to determine if BHV-1 gM is a component of the virions, BHV-1 virions were labelled with [^{35}S]-cysteine and purified by sucrose gradients. Purified labelled virions were solublized, the proteins were immunoprecipitated by anti-UL10 serum and analysed by SDS-PAGE under reducing conditions. As seen in Fig. 5.6, compared to preimmune serum (lane a), anti-UL10 serum specifically recognized a 43-44 kDa virion protein (lane b). In addition, a band of 88-90 kDa is also visible (lane b). As a control, glycoprotein gD was immunoprecipitated from [^{35}S]-cysteine labelled purified solublized virions with gD specific monoclonal antibodies and analysed by SDS-PAGE (Fig. 5.6, lane c). These results suggest that BHV-1 gM is a structural component of BHV-1 virions.

5.3.6 Glycoprotein gM forms disulphide linked dimers.

To determine if the UL10 protein exists as disulphide linked dimeric or multimeric forms, the proteins from radiolabelled lysates of purified virions (Fig. 5.7, lane a,b,e,f) or BHV-1 infected MDBK cells (Fig. 5.7, lane c,d,g,h) were immunoprecipitated with anti-UL10 serum (Fig. 5.7 lane a,b,c,d) or gB specific monoclonal antibodies (Fig. 5.7, lane e,f,g,h), and analysed by SDS-PAGE under reducing (Fig. 5.7, lane a,c,e,g) and non reducing (Fig. 5.7, lane b,d,f,h) conditions. As seen, anti-UL10 serum recognized a protein of 43-44 kDa (Fig. 5.7, lane a,c) and a protein of 88-90 kDa (Fig. 5.7, lane b,d) from purified virions or BHV-1 infected MDBK cells. As a control BHV-1 glycoprotein gB (Fig. 5.7, lane e,f,g,h) was analysed by SDS-PAGE under reducing (Fig. 5.7, lane e,g) and non-reducing (Fig. 5.7, lane f,h) conditions. These results suggest that UL10 protein forms disulphide linked dimers.

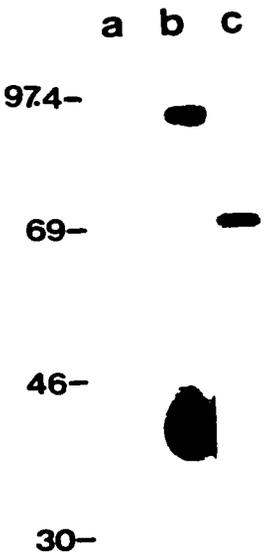


Fig. 5.6. Identification of BHV-1 gM in purified virions. [^{35}S] cysteine-labelled sucrose gradient purified BHV-1 virions were solublized and proteins were immunoprecipitated with preimmune serum (lane a), anti-UL10 serum (lane b) or gD specific monoclonal antibodies (lane c) and analyzed on 12.5% SDS-PAGE. The mol wt markers are indicated on the left.

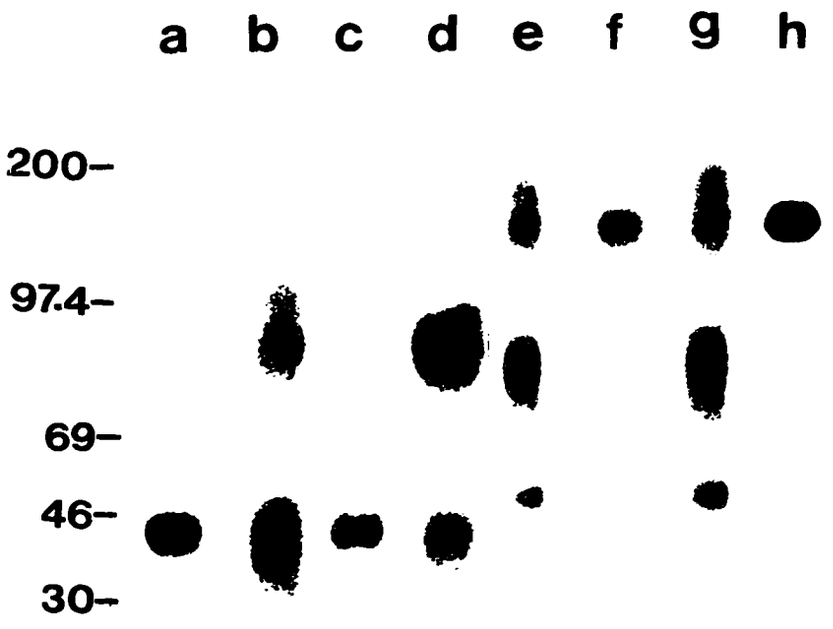


Fig. 5.7. Glycoprotein gM forms disulphide linked dimers. Proteins from lysates of [^{35}S] cysteine-labelled purified virions (lane a, b, e, f) or BHV-1 infected MDBK cells (lane c,d,g,h) were immunoprecipitated with anti-UL10 serum (lane a,b,c,d) or gB specific monoclonal antibodies (lane e,f,g,h) and separated on 12.5% SDS-PAGE under reducing (lane a,c,e,g) or nonreducing conditions (lane b,d,f,h). The mol wt markers in kDa are indicated on the left.

5.3.7 BHV-1 gM is present inside the cytoplasm and on the surface of infected cells.

In order to determine the intracellular distribution of BHV-1 gM during the course of BHV-1 infection, MDBK cells were infected with BHV-1 at a low multiplicity of infection. After 12 h of incubation at 37°C, gM was detected by indirect immunofluorescence inside the cytoplasm (Fig. 5.8, panel a) and on the surface of infected cells (Fig. 5.8, panel c). These results suggest that at least a portion of carboxy-terminal 80 amino acids against which the rabbit polyclonal antibody is directed is exposed on the surface of infected cells.

5.4 Discussion

Our goal in this and previous studies (Khattar et al, 1995, Khattar et al., 1996) has been to precisely dissect the structure and function of minor glycoproteins of BHV-1 both with respect to immune response to BHV-1 and virus-host cell interactions. Genes homologous to the HSV-1 UL10 gene (McGeoch et al., 1988) have been identified in the genomes of all the herpesvirus subfamilies studied to date, including alpha, beta and gamma herpesviruses (Albrecht et al., 1992; Baer et al., 1984; Chee et al., 1990; Davison and Scott, 1986; Dijkstra et al., 1996; Lawrence et al., 1995; Lehner et al., 1989; Telford et al., 1992; Vlcek et al., 1995). In addition, analysis of amino acid sequences of gM homologs has shown that, while the presence of number of transmembrane domaims among gM homologs is highly conserved, they do not share overall hydrophobicity and extensive primary amino acid sequence homology. This suggests that gM homologs may have structural differences and thus may not necessarily perform identical functions in different herpesviruses. In this report, we show that BHV-1 UL10 encodes glycoprotein gM, which a) contains both N-linked and O-linked oligosaccharrides , b) forms disulphide linked dimers and c) is a major component of the virion.

The BHV-1 UL10 gene is predicted to encode a protein of 411 amino acids (Vlcek et al., 1995). It has features characteristic of a typical type III integral membrane protein,

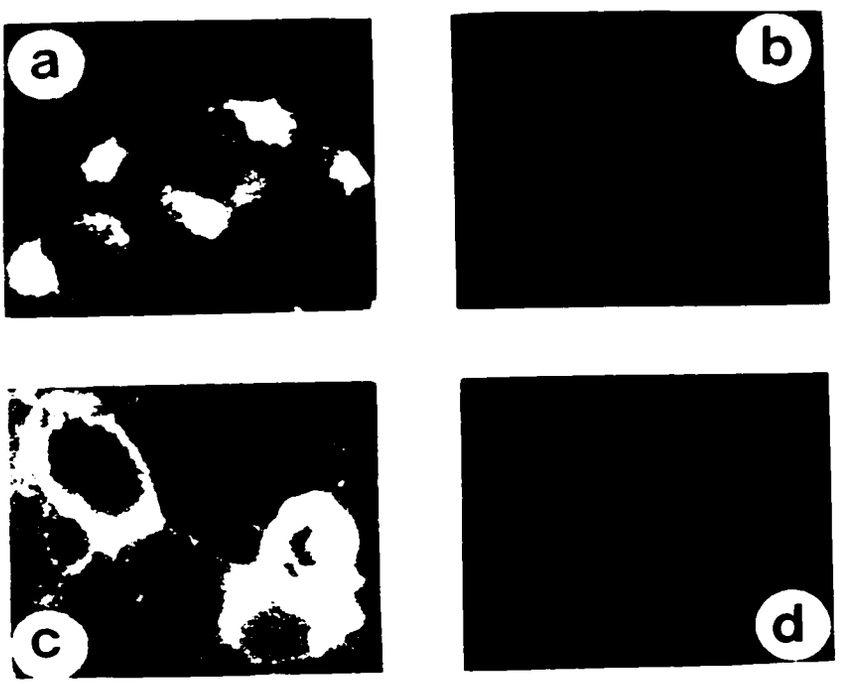


Fig. 5.8. Analysis of cellular distribution of glycoprotein gM. BHV-1 (a, c) or mock (b, d) infected MDBK cells were either permeabilized with methanol (a, b) to detect protein inside the cells or fixed with 2% paraformaldehyde (c, d) to detect protein on the surface of the cells. The fixed cells were treated with anti-UL10 serum followed by flourescein-conjugated goat anti-rabbit IgG.

with eight stretches of hydrophobic amino acids, suitable to span the lipid bilayer, which are conserved in all the other known corresponding herpesvirus open reading frames. The BHV-1 UL10 protein contains one potential site for the addition of N-linked oligosaccharides, which is conserved in all the UL10 homologs. These features would suggest that the UL10 product is a glycosylated membrane protein. Antiserum raised against the UL10-GST fusion protein of BHV-1 immunoprecipitated a protein of 43-44 kDa and its dimeric form of 88-90 kDa from BHV-1 infected cells and purified virions, which we named glycoprotein gM based on following observations: (i) the BHV-1 UL10 gene is a positional homolog of other herpesvirus glycoprotein gM, (ii) the BHV-1 UL10 open reading frame contains a strongly hydrophobic region at the N-terminus, which might function as a signal sequence, with signal peptidase cleavage probably occurring between amino acid 36 and 37 (von Heijne, 1986), (iii) BHV-1 UL10 protein is transported to cell surface and (iv) the BHV-1 UL10 protein is glycosylated.

Enzymatic deglycosylation with PNGase F led to the decrease in apparent molecular weight of the BHV-1 glycoprotein gM, which indicated that BHV-1 gM, like its homologs in other herpesviruses (Baines & Roizman, 1993; Dijkstra et al., 1996; Oesterrieder et al., 1997), contains N-linked oligosaccharides. However, in contrast to other gM homologs, enzymatic deglycosylation with neuraminidase and O-glycosidase also led to the decrease in apparent molecular weight of the BHV-1 gM suggesting that BHV-1 gM also contains O-linked oligosaccharides. Wheather O-glycosylation has a role in the function of BHV-1 gM remains to be determined.

Like glycoprotein gK (Khadr et al., 1996), BHV-1 glycoprotein gM from infected cells, differs from other virus encoded glycoproteins (Tikoo et al., 1995) in that it is hydrophobic and aggregates when boiled in buffers containing SDS. However, boiling of immunoprecipitated sample did not substantially alter the appearance of BHV-1 gM in SDS-PAGE gel compared with samples incubated at 56^0C or 37^0C suggesting that BHV-1 gM is not as hydrophobic as HSV-1 gM (Baines & Roizman, 1993).

Glycoprotein gM is dispensible for virus replication in vitro, and has been shown to play a modulatory role in cell to cell fusion and spread of virus in cell culture (Baines & Roizman, 1991; MacLean et al., 1993; Dijkstra et al., 1996; Osterrieder et al.,

1996). In light of the observations that gM homologs are highly conserved among different herpesviruses, and the highest similarity between gM homologs lies within the transmembrane regions, it is not unreasonable to expect that they may play an important role in the function of the protein. In this regard, the identification and characterization of BHV-1 glycoprotein gM establishes a basis for further elucidation of gM function(s).

6.0 GENERAL DISCUSSION AND CONCLUSION

When I began this project, the major glycoproteins gB, gC and gD encoded by the BHV-1 genome had already been identified and characterized with respect to their role in pathogenesis of BHV-1 and their interactions with the host immune system were extensively studied. However, there were no published reports regarding the structure and function of BHV-1 minor glycoproteins gL, gH and gM. Therefore the primary objective of my studies was to identify and characterize the minor glycoproteins gL, gH and gM. In this thesis, several issues, starting from sequencing and transcriptional analysis of the right end of HindIII L fragment of BHV-1 genome, in vitro transcription and translation, in vivo expression and post translational modifications of BHV-1 glycoproteins gL, gH and gM, were addressed. In addition, folding, complex formation, intracellular transport, cell surface expression, secretion and immunogenecity of glycoproteins gL and gH were studied using recombinant vaccinia viruses.

In this study, I identified and sequenced 3113 nucleotides located at the right end of the HindIII L fragment of the bovine herpesvirus-1 (BHV-1) genome from map units 0.712 to 0.734. (Fig. 3.1, 3.2). Analysis of the sequence identified four open reading frames (ORFs) which are designated UL1, UL2, UL3 and UL3.5 (Fig. 3.1, 3.2) based on their homology with proteins of herpes simplex virus-1 (HSV-1), pseudorabies virus (PRV), equine herpes virus-1 (EHV-1) and variella-zoster virus (VZV). The UL1 ORF of 158 amino acids exhibits limited homology with UL1 (glycoprotein gL) of HSV-1 (27%) and PRV (21%) (Fig. 3.3). The UL2 ORF of 204 amino acids showed significant homology to UL2 (uracil-DNA glycosylase) of HSV-1 (68%) and PRV (75%) (Fig. 3.4). The UL3 ORF of 204 amino acids showed significant homology to UL3 (nuclear phosphoprotein) of HSV-1 (62%) and PRV (53%) (Fig. 3.5). The UL3.5 ORF of 126 amino acids showed limited homology to the UL3.5 ORF of PRV (31%) (Fig. 3.6). The homolog of this gene is absent in HSV-1. These comparisons of sequences between different herpesviruses helped us to reveal conserved genes between different members of the herpesvirus family and thus demonstrated their evolutionary relationship. Further, with the availability of the nucleotide sequence of UL1, UL2, UL3 and UL3.5, it became

possible to study these proteins/glycoprotein at the molecular level. We started a detailed analysis of the structural, functional, and antigenic properties of these proteins.

Nucleotide sequence analysis of UL1, UL2, UL3, and UL3.5 genes revealed potential TATA boxes located upstream of each ORF (Fig. 3.1, 3.2). However, putative polyadenylation signal AATAAA was only detected downstream of the UL3.5 ORF (Fig. 3.1, 3.2), indicating that the preceding genes might be transcribed into 3'-coterminal mRNAs. This assumption was confirmed by Northern blot analysis of total RNAs from BHV-1 infected MDBK cells with gene-specific oligonucleotide hybridisation probes. Northern blot analysis revealed four transcripts of 2.4, 1.9, 1.3 and 0.7 kb, which are transcribed in the same direction and are 3' co-terminal. (Fig. 3.8). These mRNAs appear to yield proteins encoded by UL1 (2.4kb), UL2 (1.9kb), UL3 (1.3 kb) and UL3.5 (0.7kb) ORFs. The strategy of using a common poly A by these 3'-coterminal transcripts should help to conserve space in the large BHV-1 genome.

DNA sequence analysis of the bovine herpesvirus-1 (BHV-1) genome revealed the presence of an open reading frame named UL1 which exibited limited homology to glycoprotein gL of herpes simplex virus-1. To identify the BHV-1 UL1 protein, rabbit antisera were prepared against two synthetic peptides that were predicted by computer analysis to encompass antigenic epitopes. Sera against both peptides immunoprecipitated a 16-17 kDa protein from *in-vitro* translated and *in-vitro* transcribed UL1mRNA (Fig. 4.1), BHV-1 infected MDBK cells (Fig. 4.2) and purified virions (Fig. 4.5). Enzymatic deglycosylation and lectin binding assays confirmed that the BHV-1 UL1 protein contained only O-linked oligosaccharides (Fig. 4.4) and was named glycoprotein gL. Sera against UL22 protein immunoprecipitated a protein of 92 kDa from in vitro translated in vitro transcribed UL22 mRNA (Fig. 4.1), which was processed to a 100 kDa protein in the presence of canine microsomal membranes (Fig. 4.4). However, anti-UL22 serum immunoprecipitated a protein of 108 kDa from BHV-1 infected MDBK cells and purified virions (Fig. 4.2, 4.5). Pulse chase studies with BHV-1 infected MDBK cells confirmed that the 100 kDa protein is a precursor of the 108 kDa mature form of the UL22 protein (Fig. 4.6), which was derived from the 92 kDa primary translation product. Enzymatic

deglycosylation studies have shown that the UL22 protein is modified by only N-linked oligosaccharides (Fig. 4.4) and was named glycoprotein gH.

Previously it has been shown that BHV-1 glycoproteins expressed by recombinant vaccinia viruses are transported and processed similar to the authentic glycoproteins produced in BHV-1 infected cells (Tikoo et al., 1993a, b; van Drunen Littel-van den Hurk et al., 1989). In order to study the processing and transport of glycoprotein gL and gH, I constructed infectious vaccinia virus recombinants expressing glycoprotein gL and gH. Glycoprotein gL expressed by recombinant vaccinia virus was properly processed and secreted into the medium (Fig. 4.7, 4.9). In contrast, glycoprotein gH expressed by recombinant vaccinia virus was slightly smaller in size, possessed endo-H sensitive glycans, and was not transported to the cell surface. This is unlikely to be due to some anomaly in the vaccinia virus expression system since similar results were found with the BHV-1 gH expressed in transfected MDBK cells. This defect in intracellular transport appears to be a common feature of herpesvirus gH homologs, including HSV-1 (Gompels and Minson, 1989). However coexpression of gH with gL by recombinant vaccinia viruses resulted in proper processing and transport of gH to the cell. These results suggest that complex formation between gH and gL is neccessary for the proper processing and transport of gH (Fig. 4.6, 4.8, 4.9) but not gL. (Fig. 4.7). By analyzing BHV-1 infected MDBK cells, using SDS-PAGE under reducing and nonreducing conditions, it was shown that gL associates with gH by noncovalent interactions (Fig. 4.3).

As sequence analysis revealed the presence of a single hydrophobic N-terminal signal sequence in the gL gene which was shown to be cleaved, it was presumed that BHV-1 gL might require interaction with another glycoprotein for its association with the plasma membrane. Using a vaccinia virus recombinant expression system, I showed that gL is secreted from cells infected with recombinant vaccinia virus expressing gL but not from cells coinfected with recombinant vaccinia viruses expressing gL and gH (Fig. 4.9). These results strongly support the contention that gL is anchored to the membrane by association with gH.

Immuninization of the rabbits with recombinant vaccinia virus expressing gH and gL individually or in combination, resulted in production of complement-independent

neutralizing antibodies only in rabbits immunized with both vaccinia virus gH and gL recombinants but not with individual recombinants (Table 4.1). Preincubation of MDBK cells with an affinity purified gH-gL complex prevented virus penetration but did not prevent virus attachment (van Drunen Littel-van den Hurk et al., 1996). These results clearly indicate that complex formation between gH and gL is necessary for biological and immunological functions.

The next step in my thesis project was to identify and characterize the protein encoded by the BHV-1 homolog of the HSV-1 UL10 gene. The BHV-1 homolog of HSV-1 UL10 had been sequenced previously (Vlcek et al., 1995). Sequence analysis of BHV-1 UL10 revealed eight transmembrane hydrophobic conserved domains typical of a class III integral membrane glycoprotein and an N-linked glycosylation site. Antiserum raised against a UL10-GST fusion protein immunoprecipitated a protein of 43-44 kDa from BHV-1 infected MDBK cells and purified virions (Fig. 5.4A, 5.6). Enzymatic deglycosylation studies confirmed that the BHV-1 UL10 protein contained N-linked and O-linked oligosaccharides and was therefore named glycoprotein gM (Fig. 5.5B). Compared to BHV-1, gM homologs in HSV-1 (Baines and Roizman, 1993; MacLean et al., 1993), PRV (Dijkstra et al., 1996) and EHV-1 (Osterrieder et al., 1997) have been shown to be modified by the addition of N-linked oligosaccharides. Although the role of these O-linked glycans present in BHV-1 gM in addition to N-linked glycans is not known, they may be necessary for the maintenance of conformation of this glycoprotein in BHV-1.

The number of transmembrane domains and overall hydrophobicity among gM molecules identified to date are highly conserved (Dijkstra et al., 1996; McGeoch et al., 1988; Telford et al., 1992; Vlcek et al., 1995). Like other gM homologs (Baines and Roizman, 1993; Dijkstra, et al., 1996; Osterrieder et al., 1997), BHV-1 gM has been found to be hydrophobic in nature as it aggregates into large complexes upon heating in the presence of SDS (Fig. 5.4B). This observation combined with the analysis of the secondary structure of BHV-1 gM (Fig. 5.2) suggests that gM might form membrane ion channels and function in transmembrane transport events.

The identification and characterization of these minor glycoproteins has provided important information regarding the structure of these glycoproteins and established a basis for further elucidation of their functions. However, this study is just the start, rather than a conclusion, of the investigation into the structure and function of these glycoproteins. It has created many unanswered questions which are now the subject of further investigations. They include the following:

(i) Glycoprotein gL of BHV-1 has been shown to form a complex with another glycoprotein gH, and is required for proper antigenicity, processing and transport of gH.. Previously, in HHV-6, it has been shown that a 230 amino acid domain present at the N-terminus of gH is required for interaction with gL (Anderson et al., 1996). In BHV-1, further studies are needed to investigate the domains present on gH and gL that are required for their interactions.

(ii) The results presented in this thesis clearly suggest that in BHV-1, formation of the gH-gL complex is necessary for the biological and immunological functions of gH. However, the contribution of gL to these biological and immunological functions is not clear. It is presumed that a functional domain comprised of parts of both gH and gL may be produced as a result of their interaction or gL may help gH in attaining functional conformation. Further studies are needed to prove or refute these alternatives.

(iii) It is well documented that both fusion of the virion envelope and the plasma membrane and fusion of an infected cell with its neighbour are mediated by virus encoded glycoproteins. Some of these glycoproteins are involved in both forms of fusion and others are required only for cell to cell spread. This implies that although the two processes are in some ways analogous, there are likely to be differences in the precise molecular interactions involved. In HSV-1, it has been shown that glycoproteins gB, gD and the gH-gL complex are essential for both types of fusion, since they are essential for virus entry (Cai et al., 1988; Desai et al., 1988; Forrester et al., 1992; Ligas and Johnson, 1988; Roop et al., 1993) and deletion of these glycoprotein genes on a syncytial genetic background abolishes cell-cell fusion (Cai et al., 1988; Davis-Poynter et al., 1994; Ligas and Johnson, 1988). In contrast glycoproteins gE, gI and gM are not required for entry but are required for cell-cell fusion, at least in certain syncytial strains (Davis-Poynter et al.,

1994). In BHV-1, gB and gD are involved in cell-cell fusion (Fitzpatrick et al., 1988, 1990; Li et al., 1997; Tikoo et al., 1990) and the gH-gL complex is involved in penetration (van Drunen Littel-van den Hurk et al., 1996). Presently, glycoprotein gB in BHV-1 is suspected to be the main fusion protein and the other glycoproteins modulate this fusion protein in order to form the conformation required for fusion (Li et al., 1997). Although preliminary data suggest the involvement of BHV-1 glycoproteins gB, gD and gH-gL in fusion/penetration, it is not yet confirmed how these molecules either individually or in combination mediate these fusion/penetration events. In addition, the role of BHV-1 minor glycoproteins gM, gE or gI in these events is also not clear. The characterization of BHV-1 glycoproteins gH, gL and gM forms the basis for determining the role of these glycoproteins in these processes. The role of the essential BHV-1 glycoprotein gH or gL in these processes may be determined by constructing a cell line stably expressing BHV-1 gH or gL and a gH or gL negative BHV-1 mutant. Similarly the role of non-essental BHV-1 glycoprotein gM in these processes, may be determined by constructing a gM negative BHV-1 mutant.

(iv) Previously, the vaccine potential of a mutant HSV-1, with a deletion in glycoprotein gH, was studied (MacLean et al., 1994; Speck et al., 1996). The virus requires a gH-expressing cell line for multicycle growth and can complete an abortive single replication cycle in noncomplementing cells. Such viruses are designated DISC (disabled infectious single cycle) viruses. DISC viruses have been proposed as vaccines or vaccine delivery vehicles (MacLean et al., 1994; Speck et al., 1996). Similarly, DISC viruses may be generated in BHV-1 using the above mentioned approach and the vaccine potential of these viruses can be evaluated in cattle.

(v) By immunizing rabbits with vaccinia virus recombinants expressing BHV-1 gH and gL or with an affinity purified BHV-1 gH-gL complex (van Drunen Littel-van den Hurk et al., 1996), it has been shown that the gH-gL complex is immunogenic. I have also shown that the BHV-1 gH-gL complex is attached to the plasma membrane, using the transmembrane anchor domain of gH. It should be possible to construct a truncated version of the gH-gL complex by terminating the protein at an amino acid immediately upstream of the transmembrane anchor. Like the truncated BHV-1 gD (Kowalski et al., 1993) and

gB (Li et al., 1996) derivatives, the truncated BHV-1 gH-gL derivatives could be expressed in MDBK cells under the control of a heat-inducible, bovine heat-shock protein (hsp) 70A gene promoter. Using this expression system, truncated gH-gL should be produced at high level in the media. The immunogenic potential of this secreted and truncated version of gH-gL complex may be studied and possibly used as a subunit vaccine.

(vi) We could also express the genes of different BHV-1 gH derivatives containing specific truncations of the transmembrane anchor region in MDBK cells under the control of the bovine hsp70A gene promoter. Using this approach we could study the role of the transmembrane anchor of BHV-1 gH in membrane fusion and define the fusion domains of BHV-1 gH as has been done previously for BHV-1 gB (Li et al., 1997)

(vii) In recent years, adenovirus vectors have been used for the expression of foreign genes in mammalian cells. A great advantage of adenoviral delivery system is that adenoviruses naturally target mucosal tissues. Indeed, mucosal adminstration of recombinant human adenoviruses have been shown to induce immunity to the foreign genes from a wide variety of heterologous viruses (reviewed by Imler, 1995). Using human or bovine adenovirus as a vector, recombinants expressing BHV-1 gH-gL together or gM glycoproteins can be constructed. Mucosal expression of these glycoproteins could help to determine the potential role of these glycoproteins in the induction of the protective immune response in cattle.

(viii) Analysis of the structure of BHV-1 glycoprotein gM indicated a role of this glycoprotein in transmembrane transport events. In this context it would be interesting to extend the present study by analyzing the putative ion channel function of this glycoprotein. The ion channel function of BHV-1 gM could be assessed by expression of BHV-1 gM into *Xenopus laevis* oocytes and voltage clamp analysis as has been done recently for EHV-1 gM (Osterrieder et al., 1997)

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