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GENETIC ANALYSES OF THE GENES ENCODING THE MAJOR GLYCO-

PROTEINS OF FELINE HERPESVIRUS TYPE 1

presented by

Stephen Joseph Spatz

has been accepted towards fulfillment
of the requirements for

PhD degree in Microbiology

Major professor

Date 8/10/93

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# GENETIC ANALYSES OF THE GENES ENCODING THE MAJOR GLYCOPROTEINS OF FELINE HERPESVIRUS TYPE 1

BY

Stephen Joseph Spatz

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

GENETIC ANALYSES OF THE GENES ENCODING THE MAJOR GLYCOPROTEINS OF FELINE HERPESVIRUS TYPE 1

By

Stephen Joseph Spatz

The genes encoding six putative glycoproteins of feline herpesvirus type 1 have been identified and their nucleotide sequences determined. Predicted translation products of these genes exhibited significant homology to glycoproteins B, H, G, D, I and E of herpes simplex type 1. The gene encoding glycoprotein B of FHV-1 was located within the unique long region of the genome using an HSV-1 gB hybridization probe. Nucleotide sequencing of the gB gene revealed the presence of an overlapping gene encoding an ICP18.5 homolog. FHV-1 gB polypeptides of 100, 64 and 58 Kd were detected with antisera to gB of HSV-1 in immunoprecipitation and immunoblot assays.

The genomic location of the glycoprotein H gene was determined using a functional assay for a suspected upstream gene which encodes thymidine kinase (Tk). The selectability of the FHV-1 thymidine kinase gene in transfected mouse (Tk-negative) cells under HAT selection allowed for the localization of the Tk/gH gene cluster. Colonies of mouse Tk+ cells only appeared with Tk negative cells transfected with DNA from the SalI A clone and a subfragment (6.6 Kb EcoRI-EcoRI). Nucleotide sequencing of this subfragment from the $U_L$ region has indicated the presence of two ORFs whose predicted

translated polypeptides share similarities with the gene products of the gH and TK genes of HSV-1.

The genes encoding a protein kinase and glycoproteins G, D, E and I have been localized within the unique short region of the FHV-1 genome. Nucleotide sequencing of a 6.2 Kb EcoRI-SalI fragment from this region allowed for the identification of these genes.

The gene products of two of these glycoproteins (gB and gD) genes were characterized by the generation of poxvirus recombinants (vaccinia and raccoon poxviruses) expressing these glycoproteins. High titers of virus neutralizing antibodies were generated in rabbits inoculated with the vaccinia recombinants expressing either FHV-1 gB or gD. Western blot analyses with FHV-1 virions and antisera against the vaccinia recombinants have demonstrated the presence of a 60 Kd (gB) and a 50 Kd (gD) polypeptide.

The identification of the genes encoding these important glycoproteins will form the basis for the assessment of these glycoproteins as potential vaccine antigens.

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Stephen Joseph Spatz

1993

to my family

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# INTRODUCTION

Feline viral rhinotracheitis (FVR) is a major cause of respiratory tract disease in cats and is caused by an alphaherpesvirus, feline herpesvirus type 1 (FHV-1). Several vaccines are currently available against FVR, but there is a need to develop vaccines that are more protective. Current vaccines protect against the development of clinical signs, but fail to protect against reinfection. The goal of research presented in this thesis was to identify the genes encoding major immunogens of feline herpesvirus and to express their gene products in a suitable vaccine vector.

Identification of the genes encoding immunodominant FHV-1 glycoproteins (gB, gH, gG, gD, gI and gE) is described in detail in chapters 2-4. Chapter 2 also contains immunological data on FHV-1 polypeptides which cross react with HSV-1 gB antisera. Nucleotide sequencing information presented in chapters 2-4 formed the basis for expression work described in chapter 5. In chapter 5, generation of poxvirus recombinants containing glycoproteins B and D of FHV-1 are described, along with preliminary immunological data.

# CHAPTER 1

# A REVIEW OF FELINE VIRAL RHINOTRACHEITIS

Stephen J. Spatz and Roger K. Maes

## INTRODUCTION

In 1958, Crandell and Maurer reported the isolation of a viral agent from kittens with acute upper respiratory tract disease. The disease was later designated feline viral rhinotracheitis (FVR) and the new virus referred to as feline rhinotracheitis virus (FRV). Subsequent work by Ditchfield and Grinyer (1965) revealed that FRV had the characteristics of a herpesvirus. Based on its biological properties, FRV is currently classified as a member of the subfamily of Alphaherpesvirinae and commonly referred to as feline herpesvirus-1 (FHV-1).

This virus has a world-wide distribution and serological surveys have shown that 50 - 75% of adult domestic cats have neutralizing antibodies against FHV-1 (Studdert and Martin, 1970; Herbst et al., 1988). Feline herpesvirus-1 and feline calicivirus (FCV) are responsible for about 80 percent of the cases of infectious upper respiratory disease in cats (Kahn and Hoover, 1975). Clinically, it is estimated that up to 45% of all feline respiratory illness is cause by FHV-1 (Studdert, 1978). In a recent study, Harbour et al., (1991) reported on the isolation of FCV and FHV-1 from oropharyngeal swabs collected from 6866 cats from 1980 through 1989. They repeatedly isolated the two viruses at an average ratio of 4.8:1.0 (FCV:FHV-1). For individual years, the ratio varied from 1.3:1.0 to 15.0:1.0. The majority of cats shedding either virus were under 1 year of age (Harbour et al., 1991). Of lesser importance as etiological agents in the induction of

viral respiratory disease in cats are feline Reovirus and Chlamydia psittaci. Feline leukemia virus and feline immunodeficiency virus, via the immunosuppression they induce, can indirectly trigger respiratory disease in cats.

## VIRAL MORPHOLOGY, HOST RANGE AND CROSS-REACTIVITY WITH OTHER HERPESVIRUSES

The morphology of FHV-1 is identical to that of other herpesviruses (Ditchfield and Grinyer, 1965). The capsid is hexagonal and has an average diameter of 108 nm. A membranous envelope surrounds the capsid, giving the complete virion an average diameter of 178 nm. The envelope contains viral glycoprotein antigens that are very important in virus-host cell interactions. Infectivity of FHV-1 is greatly reduced by exposure to lipid solvents (Johnson, 1966) and FHV-1 is more heat labile than the majority of herpesviruses (Povey, 1979). For example, virus stored at -50°C will loose 90% of its infectivity in 5 months.

The *in vivo* host range of FHV-1 is limited to Felidae (Povey, 1979). In vitro replication of this virus is also limited to cells of feline origin. Feline alveolar macrophages, alveolar pneumocytes (Langloss et al., 1978), CD4+ T-lymphoblastoid cells (MYA-1 and FL74 cells), felis catus whole fetus 4 cells (fcwf-4) and Crandell-Reese feline kidney cells are all suspectable and suitable for FHV-1 propagation.

Interestingly, necrotizing alveolar lesions in infected

cats, occasionally observed by Love (1971), may indicate that alveolar macrocytes and pneumocytes are target cells during natural infection (Kawaguchi et al., 1991 and Horimoto et al., 1991). Since FHV-1 can be isolated from feline peripheral blood leukocytes of experimentally infected cats (Tham et al., 1987), Kawaguchi (1991) investigated whether another T-lymphotropic virus, feline immunodefiency virus (FIV), could co-infect T cells in vitro. To accomplish this, MYA-1 cells, a feline T-lymphoblastoid cell line, were dually infected with FHV-1 and FIV. A two color indirect immunofluorescence assay demonstrated that individual cells served as targets for both viruses. Furthermore, it was reported that FHV-1 can transactivate the LTR's of FIV: FHV-1 induced the expression chloramphenicol acetyl transferase (CAT) from a transfected plasmid containing a FIV-LTR directed CAT gene expression cassette (Kawaguchi et al., 1991,1992). This transactivation was likely to occur via the immediate early gene products as observed with other herpesviruses and their species-dependent retroviruses (Yuan et al., 1989). It is not clear whether coinfection of FHV-1 in FIV infected T-lymphocytes is of any physiological significance in vivo, since a large number of T-cells are destroyed as a result of FIV infection. What is significant is that FHV-1 can establish latency in neural tissues. Periodic reactivation of FHV-1 from these tissues may provide infectious virus for suspectable FIV-infected T cells, thus contributing to accelerated clinical symptoms in cats that are dually infected with FIV and FHV-1.

Serological studies, using polyclonal antisera and involving a number of FHV-1 isolates from different parts of the world, have shown that there is only one serotype of FHV-1 (Metianu and Virat, 1974). Differences exist, however, in virulence of clinical isolates. Furthermore, examination of vaccine strains by this approach showed them to be antigenically very similar to field strains of the virus. Serological studies have also indicated that there is no cross-reactivity between FHV-1 and other herpesviruses such as feline cytomegalovirus (FCMV), herpes simplex virus type 1 (HSV-1), pseudorabies (PRV), equine herpes virus type 1 (EHV-1) and bovine herpesvirus type1 (BHV-1) (Fabricant, 1984; Crandell and Weddington, 1967; Johnson and Thomas, 1966; Limcumpao et al., 1990). However, FHV-1 polyvalent antiserum has been reported to neutralize the infectivity of canine herpesvirus (Evermann et al., 1982).

Although feline herpesvirus-1 and canine herpesvirus-1 (CHV-1) have restriction endonuclease patterns that are quite distinct from each other, they are antigenically related. In a study by Xuan et al., (1992) reciprocal cross-neutralization between the two heterogeneous viruses was demonstrated, both with polyvalent serum and with monoclonal antibodies, specific for each virus. It was further reported that both viruses have hemagglutination capability. Antibodies to FHV-1's hemagglutinin (60Kd) can neutralize CHV-1's HA-protein (41Kd) and the reciprocal cross also neutralize the heterologous virus. Species specificity for FHV-1 and

CHV-1 is often regarded as stringent, but FHV-1-like viruses have been isolated from dogs (Rota et al., 1986; Kramer et al., 1991).

Since CHV-1 and FHV-1 seem to be antigenically related and antisera of either FHV-1 or CHV-1 can cross-neutralize virus, it is interesting to speculate whether a vaccine against FHV-1 could protect not only cats against FVR, but also protect dogs challenged with CHV-1.

## GENETIC CHARACTERIZATION OF THE FHV-1 GENOME

The DNA of FHV-1 has been reported to have a density of 1.705 $g/cm^3$ corresponding to 46% G+C (Roizman, 1980). Initially, Herrmann (1984) reported that the FHV-1 genome was remarkably stable with respect to restriction polymorphisms. This was based upon analysis of DNA from 12 isolates and a vaccine strain of FHV-1. However, from the examination of 59 field isolates of FHV-1, Grail et al., (1991) concluded that the FHV-1 genome is not static and that interstrain variants occur spontaneously.

The first restriction map of FHV-1 reported in the literature was that of the C-27 strain of feline herpesvirus-1 by Rota et al. (1986). The map revealed that the C-27 genome is approximately 134 Kb in size and contains a group D genome similar to PRV, VZV, EHV-1 and BHV-1. The longer segment (L) of the genome is composed of 103 Kb of unique DNA ($U_L$) and is adjacent to a 31 Kb short (S) segment. The short segment contains 8 Kb of unique DNA ($U_S$) flanked by inverted repeats

($I_R$'s) of 11 Kb. This genomic structure allows inversion of the unique short relative to the unique long region, thus creating two isomeric arrangements.

Recently, a restriction map of the B927 strain of FHV-1 was published by Grail et al.,(1991). A comparison of the two restriction maps is presented in Figure 1. Although few differences could be demonstrated in the unique short regions of the two genomes, major differences were found in the $U_L$ region. An extra SalI band was mapped between the 6.9 and 16 Kb bands, the SalI bands of 4.7 and 16.5 Kb were reversed and there were differences in the end of the $U_L$ region. Since no hybridization data was presented involving southern blots containing digests of B927 DNA probed with cloned C-27 DNA, or vice versa, little can be said about the absolute validity of the $U_L$ region sequences of either strain.

However, recent nucleic acid sequencing studies involving approximately 13 Kb of C-27 DNA, 6.2 Kb from the $U_S$ region and two 3.0 Kb sections from the $U_L$ region, confirmed the accuracy of the restriction map originally proposed by Rota et al., (1986). Major differences in both the $U_L$ and $U_S$ region of strain B927 could be found when compared to the restriction map generated from the C-27 strain sequencing data.

**Figure 1. Restriction Endonuclease maps of DNA representative of the C-27 and B927 strains of Feline herpesvirus -1.** The 134 Kb genome of FHV-1 is represented as a group D genome with a unique long ($U_L$) region adjacent to a unique short ($U_S$) region. Inverted repeats flank the $U_S$ region. The complete SalI restriction maps of both genomes are presented along with a EcoRI restriction maps of the $U_S$ regions. Sizes of the individual restriction fragments are given in kilobases.

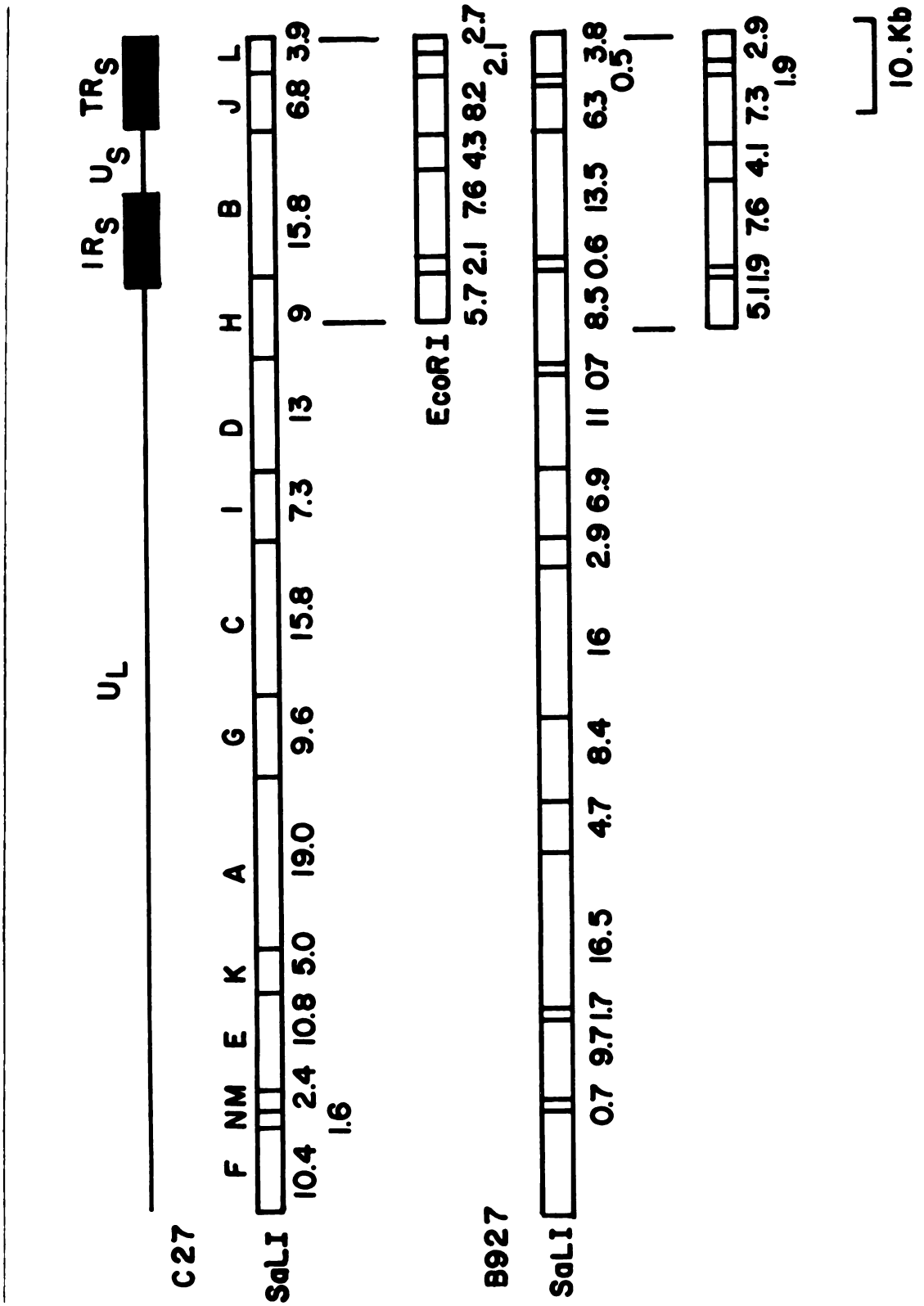
C27
B927
SaLI
EcoRI
SaLI
$U_L$
$IR_S$
$U_S$
$TR_S$
F NM E K A G C I D H B J L
10.4 2.4 10.8 5.0 19.0 9.6 15.8 7.3 13 9 15.8 6.8 3.9
1.6
5.7 2.1 7.8 4.3 8.2 2.1 2.7
0.7 9.7 1.7 16.5 4.7 8.4 16 2.9 6.9 11 0.7 8.5 0.6 13.5 6.3 0.5 3.8
5.1 1.9 7.6 4.1 7.3 1.9 2.9
10.Kb

Figure 1

**GENE MAPPING STUDIES INVOLVING THE GENOME OF FHV-1 (C-27)**

Since it has been established that FHV-1 has a genomic organization similar to that of other alphaherpesviruses, an extensive genetic analysis of the genes encoding the major immunogens has revealed that FHV-1 contains HSV-1 homologs to glycoproteins B, H, D, G, I, and E. The genomic location of three genes encoding the nonstructural proteins, thymidine kinase, serine/threonine protein kinase, and ICP18.5 have also been identified.

The complete nucleotide sequences of the genes encoding gB, gH, gG, gI and gE are presented in the accompanying papers. Predicted translation products of these genes have revealed extensive homology to glycoproteins found in related animal herpesviruses. As shown in Table 1, FHV-1 glycoproteins show more similarity to homologs found in the genomes of EHV-1, PRV, BHV-1 than those found in HSV-1 and 2, human cytomegalovirus (HCMV), Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS). This is also supported phylogenetically. Evolutionary lineage analyses involving gB homologs of alphaherpesviruses (Figure 2) have indicated that FHV-1 evolved along lines giving rise to the Varicelloviruses, VZV, PRV, EHV-1 and BHV-1. This lineage is also supported by the extensive homology at the animo acid level between the other FHV-1 glycoproteins (gH, gD, gG. gI and gE) and homologs of varicelloviruses. Analyses of six glycoproteins of FHV-1 are in agreement with classification of FHV-1 in the genus

Varicellovirus of the subfamily, Alphaherpesvirinae.

Interestingly, many of the FHV-1 genes encoding glycoprotein homologs are colinear with those of other alphaherpesviruses. The genomic organization of unique short regions in FHV-1 is quite similar to that of PRV, with the following gene order (5' > 3') Pk, gX(gG), gp50(gD), gp63(gI) and g1(gE). HSV-1 homologs are indicated with parentheses to eliminate confusion. Relative orientation of the two $U_L$ glycoproteins, B and H, appears to be inverted between FHV-1 and PRV. This inversion in the $U_L$ region of PRV has been reported by Davison and Wilkie (1983) using low-stringency hybridization analysis.

## BIOCHEMICAL AND IMMUNOLOGICAL CHARACTERIZATION OF FHV-1 PROTEINS

Since the genome of FHV-1 is 134,000 base pairs in size, it is estimated that FHV-1 is capable of encoding 50-70 polypeptides. Direct SDS-PAGE analysis of $^{35}S$ methionine or $^{14}C$ glucosamine-labeled FHV-1 virions indicated the presence of at least 17 virion-associated proteins, ranging in molecular weight from >200 Kd to <39 Kd. In a study by Maes et al., (1984), three $^{14}C$ glucosamine-labeled glycoproteins (107-105, 68 and 60 Kd) were detected by immunoprecipitation with goat anti-FHV-1 antiserum (Figure 3). Two glycoproteins with molecular weights of 107 and 76 Kd were detected in infected culture supernatants. Because HSV-1 contains at least 10 glycoproteins, it was expected that FHV-1 should contain more

Table 1. **Homology analyses of the predicted translation products of the genes encoding glycoprotein B, H, D, E, I and G of FHV-1.** Amino acid sequences of six putative glycoproteins of FHV-1 were compared to homologous glycoproteins found in alpha- beta- and gammaherpesviruses using the GAP programs of the University of Wisconsin package (UWGCG) (Devereux et al., 1984). The values presented represent the percentage similarity.

**GLYCOPROTEINS OF FELINE HERPESVIRUS TYPE 1**

| | gB | gH | gD | gE | gI | gG |
|---|---|---|---|---|---|---|
| EHV-1 | 73 | 56 | 49 | 65 | 56 | 57 |
| EHV-4 | 73 | 56 | N/A | N/A | N/A | 59 |
| PRV | 74 | 50 | 50 | 53 | 49 | 56 |
| BHV-1 | 72 | 53 | 54 | N/A | N/A | N/A |
| VZV | 72 | 50 | D/C | 49 | 51 | D/C |
| MDV | 68 | N/A | 47 | 43 | 47 | D/C |
| SA8 | 68 | N/A | N/A | N/A | N/A | N/A |
| BHV-2 | 67 | N/A | N/A | N/A | N/A | N/A |
| ILTV | 61 | N/A | N/A | N/A | N/A | N/A |
| HSV-1 | 64 | 45 | 47 | 47 | 43 | 42 |
| HSV-2 | 66 | N/A | 47 | 43 | 40 | 40 |
| HCMV | 49 | 44 | D/C | D/C | D/C | D/C |
| HHV-6 | 49 | 42 | N/A | N/A | N/A | N/A |
| EBV | 50 | 44 | D/C | D/C | D/C | D/C |
| HVS | 47 | 44 | D/C | D/C | D/C | D/C |

N/A = NOT AVAILABLE

D/C = DOESN'T CONTAIN THIS GLYCOPROTEIN

Table 1

Figure 2. **Evolutionary relatedness of twelve alpha-herpesviruses.** Amino acid sequences of glycoprotein B, the most conserved glycoprotein in all subfamily of herpesviridae, were analyzed for homology and aligned using the GAP and PILEUP programs of the University of Wisconsin (UWGCG) (Devereux et al., 1984). Dendrograms were drawn using the Phylogeny Interference Package (PHYLIP) (Felsenstein, 1985).

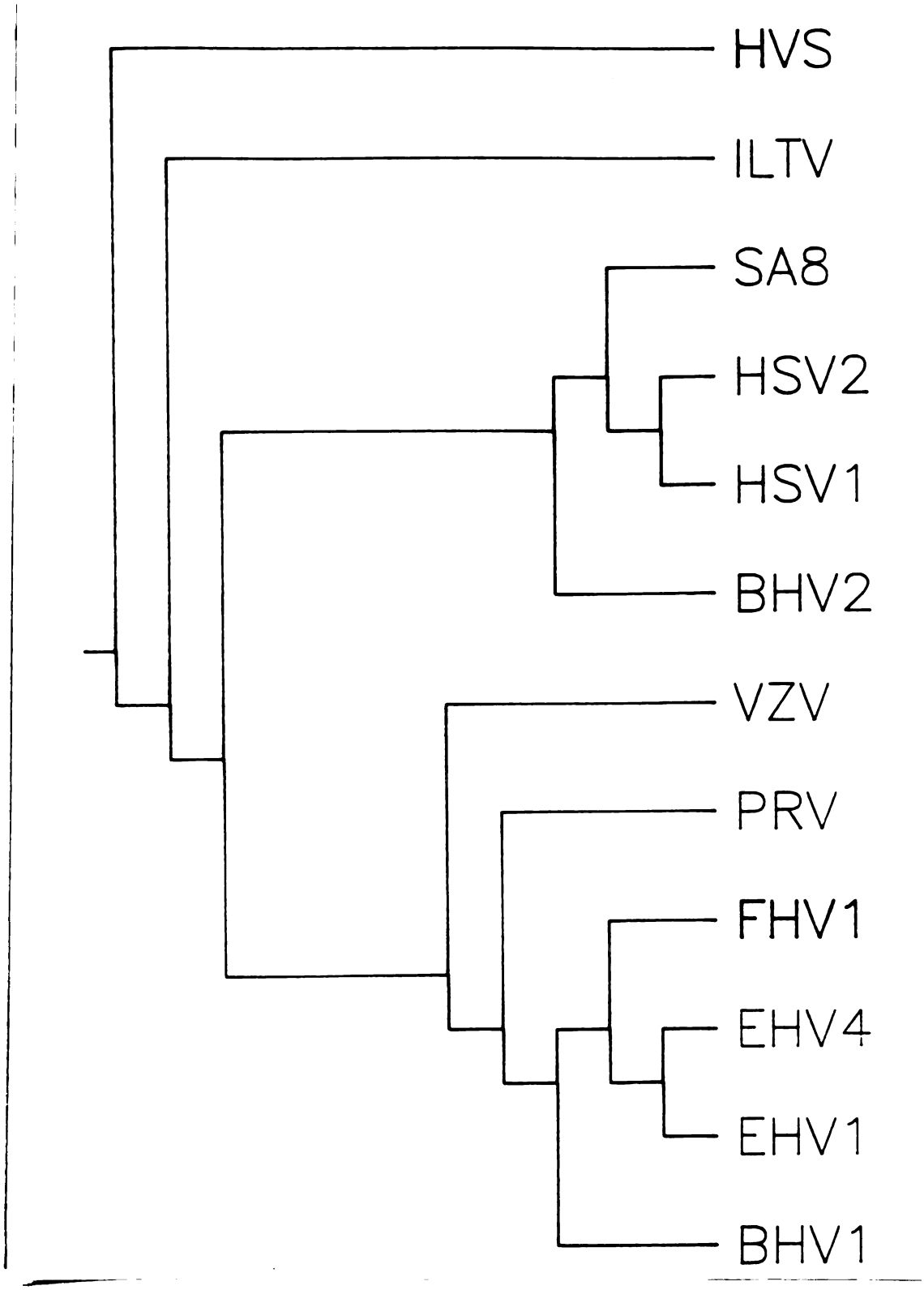


Figure 2

than three glycoproteins. In a 1984 study by Fargeaud and others, six FHV-1 glycoproteins with MW's of 125, 116, 112, 83, 70 and 62, Kd were detected using lectin chromatography. Further protein characterization by Compton (1989) identified five FHV-1 glycoproteins present in cell extracts and purified virions with MW's of 107, 103, 85, 68 and 59 Kd. Two glycoproteins (75 and 107 Kd) were also detected in the culture supernatants with MW's similar to those identified by Maes et al., (1984) although the 85 Kd glycoprotein was not identified in the Maes study, the protein profiles between the two studies are quite similar.

A FHV-1 specific protein of 60 Kd has recently been identified as a hemagglutinin of feline herpesvirus-1 (Horimoto et al., 1989). Although, HA activity is rare in herpesviruses, PRV and BHV-1 have also been reported to contain hemagglutinating activity.

**PATHOGENESIS**

Infections with FHV-1 result from exposure of susceptible cats to virus via the oral, intranasal, or conjunctival routes. From these primary sites of replication, FHV-1 spreads to adjacent sites within the upper respiratory tract, including the trachea and occasionally the bronchi and bronchiolus. Primary interstitial pneumonia resulting from viral replication in the lungs is the exception rather than the rule. Predilection of FHV-1 for the upper respiratory tract can be explained by the fact that this virus replicates

Figure 3. **Analyses of the polypeptides of feline herpesvirus-1** (A) Crandell Reese Feline Kidney (CRFK) cells were infected with FHV-1 (C-27) at a m.o.i. of > 1.0 in the presence of $^{35}$S-Methionine or $^{14}$C-glucosamine. Cytoplasmic extracts were prepared in 1X PBS containing 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS. Lysates prepared from FHV-1 infected cells labeled with either $^{35}$S-methionine (lanes 1 and 2) or $^{14}$C-glucosamine (lane 3) were immunoprecipitated with a goat anti-FHV-1 antisera. The preparation of the goat antisera is described elsewhere (Maes et al., 1984). Immunoprecipitates were dissociated by boiling in a sample containing SDS and electrophoresed through 10% polyacrylamide.

(B) Western blot analysis of virions prepared from FHV-1 infected CRFK cells. Virions were purified through 30% potassium tartrate gradients by the procedure described by Talens and Zee (1976) and dissociated by boiling in SDS sample buffer. Polypeptides were separated through 10% polyacrylamide and electroblotted onto nytran. Blots were blocked with 10% low-fat milk powder in Tris-buffer saline and incubated with a goat anti-FHV-1 antisera for 1 hr. Visualization of reactive polypeptides involved using alkaline phosphatase-labeled rabbit anti-goat conjugates along with the chromogens, BCIP and NBT (Ausubel et al., 1988).

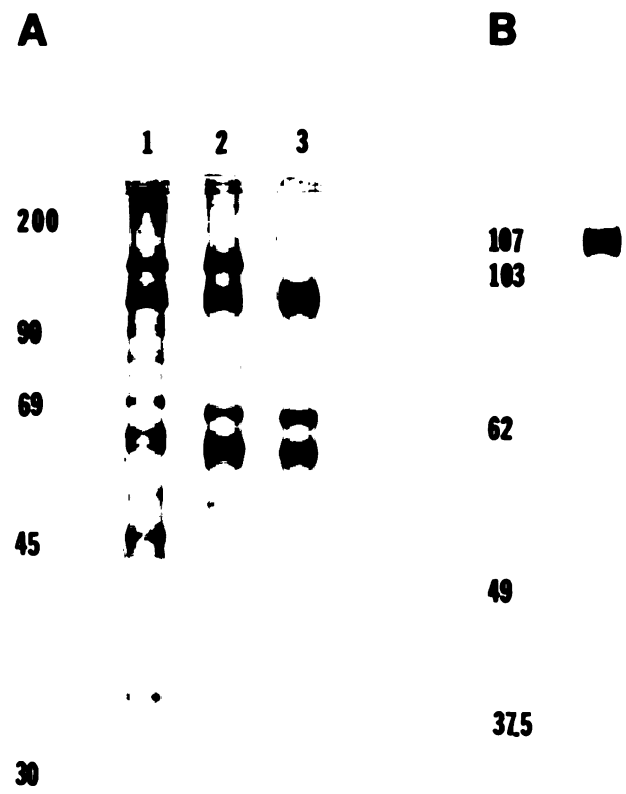


Figure 3

best at temperatures slightly below the normal body temperature of cats.

Viral replication in the upper respiratory epithelium of kittens produces necrosis of the turbinate mucosa and can lead to osteolysis of the turbinates. This results in recurrent rhinitis and possibly nasal deformities. Necrosis of the dorsal tongue surface is a rare feature of FHV-1 infection but is very common in cats infected with feline calicivirus.

Viremia following FHV-1 infection is not at all extensive, although the presence of the virus has been demonstrated in mononuclear cells of experimentally infected cats. When cats are viremic, FHV-1 can produce necrosis in the growth regions of the ribs and long bones.

Abortions are not as commonly associated with FHV-1 infections as is the case for other herpesviruses. The low level of viremia can at least be a partial explanation for this. In fact, following experimental infection via the intranasal route transplacental infection could not be demonstrated (Gaskell and Povey, 1982). Abortions in association with FHV-1 infection are, therefore, more a secondary effect due to the debilitating effect of the virus upon pregnant queens than a direct effect of the virus on the developing fetus.

Like human herpes simplex virus, FHV-1 readily replicates in corneal epithelium. Initially this results in the formation of dendritic ulcers (Bistner, 1971). These tend to coalesce in a few days , resulting in large irregularly shaped ulcers. In

more advanced stages, formation of a descemetocele or progression to panophthalmitis, with complete loss of vision are possible (Wasisse, 1990).

Following the acute phase of an FHV-1 infection, clinical signs subside and immunity develops. In the case of herpesviruses this doesn't lead to complete elimination of the virus. Viral genomes persist in certain cell types in a latent form. Latent infections can be reactivated by various natural and artificial stresses. This results in renewed production of infectious virus, which can be spread to susceptible cats. Development of latent infections in sensory neurons and other nervous tissues can be assumed to result from entry of FHV-1 into nerve endings at the site of replication and retrograde axonal transport to sensory ganglia. The biochemical bases of initiation, maintenance and reactivation of latent infections are unclear at this point. It is likely that both viral and cellular factors are involved in these processes.

The presence of latent FHV-1 infections has thus far been demonstrated only in a very preliminary way. Latent FHV-1infections have been reactivated in vivo following the administration of corticosteroids. Ellis (1981) showed that latent FHV-1 could be reactivated from 25.8% of healthy cats by this method. Reactivation can also be induced by so called natural stressors. In a study by Gaskell and Povey (1982), rehousing of cats was sufficient to induce virus shedding in 18% of the carrier cats. Corticosteroid administration to the same group was able to induce reactivation in 64% of latently

infected carriers. Late lactation stress is also an important cause of reactivation of latent virus. Approximately 40% of latently infected queens were found to shed virus at this time. None of the kittens from these shedders showed clinical signs, but 50% were found to be latently infected. Since there is no evidence of *in utero* infection following natural exposure, the most logical explanation of these findings is that the presence of residual passive antibody levels prevented development of clinical signs but not infection.

Research efforts have also been focused on identification of the anatomical site(s) of FHV-1 latency. Gaskell and Povey (1979) were able to isolate FHV-1 from homogenized trigeminal ganglia and olfactory bulbs from carrier cats while they were actively shedding reactivated virus. Ellis (1982) examined a number of tissues, including trigeminal nerve ganglia, from carrier cats by explantation but was unable to show evidence of latent FHV-1 in any of these tissues. However, Gaskell et al. (1985) and Nasisse (1992) have been able to recover FHV-1 from trigeminal ganglia of acutely and chronically infected cats using a tissue fragmentation technique.

The inability to consistently detect latent FHV-1 by tissue explantation is probably more a reflection of the limitations of the method than the actual presence or absence of latency in the tissues examined. We are currently using a very sensitive DNA amplification assay to more definitively determine the tissue tropism of FHV-1 latency.

**CLINICAL SIGNS**

Pedersen (1988) organized the clinical syndromes associated with FHV-1 under 7 headings. These are given here in abbreviated form.

1. "Classical" rhinotracheitis in kittens.

This usually occurs when kittens have lost their passive immunity to FHV-1, between 6 and 12 weeks of age. Clinical signs associated with this form include sneezing and presence of a serous oculonasal discharge which later becomes mucopurulent. Fever is usually low-grade. These clinical signs subside after 1 to 2 weeks. In some cases, fever is high and additional clinical signs seen include pharyngitis, glossitis, tracheitis, depression, open mouth breathing and drooling.

2. Chronic rhinitis and sinusitis

Both of these result from severe upper respiratory infection. Bacteria and Mycoplasma that are part of the normal flora become more invasive in these cases as a result of severe FHV-1 induced mucosal damage.

3. Herpetic ulcers

During the acute stage, corneal ulcers are large, shallow and painful. More chronic ulcers look like small white plaques in the center of the cornea and are less painful. When acute ulcers are secondarily infected or when topical corticosteroid

therapy is used, ulcers tend to deepen, potentially leading to corneal penetration.

4. Recurrent disease

Recurrent infections with FHV-1 result either from reactivation of a latent infection or renewed exposure of cats whose mucosal immunity is sufficiently lowered. Circumstances leading to reactivation are discussed under pathogenesis. Recurrent disease is usually milder in nature and shorter in duration than a primary infection, except in immunosuppressed animals.

5. Abortion

Abortion in pregnant queens can be experimentally induced following intravenous administration of FHV-1. The virus is then recovered from the placenta 1-2 weeks later and is also present in fetal tissues at approximately 3 weeks post-infection. Abortions have been reported after infection via the natural route. In these cases the virus can not be recovered from the placenta, uterus or fetal tissues. The abortion is therefore resulting from a general condition of the pregnant queen, rather than direct infection of fetal tissues.

6. Neonatal disease

Neonatal FHV-1 infections are rather uncommon. Infections are thought to occur during passage through the birth canal or

shortly after birth.

7. CNS signs, skin lesions , glossitis, pancreatitis

FHV-1 has been isolated from the brain of kittens and has also been found to induce CNS signs in naturally and experimentally infected kittens. Other signs infrequently seen with FHV-1 infection include ulcerative glossitis, skin ulcers pancreatitis and pneumonia.

**IMMUNITY**

Immunity induced following natural exposure is protective against clinical disease, but not against reinfection of the upper respiratory tract. A similar situation is seen after vaccination with modified live (MLV) vaccines, given either parenterally or intranasally. A more rapid, and possibly more solid protection, can be an advantage of using the intranasal route of administration. This is illustrated by the work of Slater and York (1976), who attenuated a strain of FHV-1 by serial passage at 25°C in cell culture. Cats intranasally exposed to the virus at the 171$^{th}$ passage level did not develop clinical signs and were clinically protected from challenge with a virulent strain. Later work (Orr et al., 1980) revealed that cats given the same type of vaccine via the intramuscular route did not develop clinical signs but replicated challenge virus. A proportion of the cats were shown to be latent carriers. These studies suggest that local immunity elicited

by antigenic stimulation of mucosal surfaces may have an important influence on protection against reinfection, and therefore against the frequency and intensity of latent infections.

The nature of immune responses against FHV-1 at mucosal surfaces was further examined by Cocker (1984). It was found that cats vaccinated by the intranasal route were specifically resistant to FHV-1 challenge by 6 days post-vaccination. Analysis of sera and nasal secretions at this point revealed the presence of only low levels of neutralizing antibodies and interferon. Lymphocytes from blood and tonsil showed no proliferative response to FHV-1 antigens. The authors concluded that a "local cytotoxic cell" response in the tonsil, an important primary replication site of FHV-1, was responsible for the observed protection against reinfection.

Wardley (1976) investigated components of the immune response which could play a role in the prevention of establishment of latent infections. They found that spread of FHV-1 within the body of a susceptible cat during an acute infection is kept under control by antibody-complement mediated lysis and antibody-directed cellular cytotoxicity (ADCC), involving both lymphocytes and macrophages. The authors postulated that defects in the immune response needed to control viral dissemination, may contribute to establishment of latent infections and also to more severe recrudescent disease. A similar study by Goddard and Gaskell

(1984) attempted to evaluate immune functions in cats during reactivation of latent FHV-1 infections. Rehousing stress, a natural stressor, was used to induce reactivation. No specific suppression of specific or non-specific immunity was associated with viral reactivation and subsequent shedding. It has been noted in the study of other herpesvirus infections that levels of effector functions may be more important than memory functions but this remains to be examined in FHV-1 infections.

It has been well established that the major immunogens of herpesviruses are the envelope-bound glycoproteins. These glycoproteins are the major targets for both humoral and cell-mediated immunity in the infected host. Natural exposure to FHV-1 results in parallel development of neutralizing antibodies and an antibody response to the major viral glycoproteins. Burgener and Maes (1988) have reported that, by twelve days postinfection, cats which were synchronously infected with the C-27 strain of FHV-1, had virus neutralizing (VN)-antibodies. Moveover, only antisera collected at 12 days P.I. reacted with $^{14}$C-glucosamine-labelled glycoproteins of FHV-1 in immuoprecipitation assays. The concurrent development of virus neutralizing antibodies and glycoprotein specific immunity indicates that FHV-1 glycoproteins, like other viral glycoproteins, are important in the induction of protective immunity.

## HERPESVIRUS GLYCOPROTEINS: IMMUNITY AND PATHOBIOLOGY

Because feline herpesvirus type 1 has been classified as an alphaherpesvirus and contains many glycoprotein homologs to those of the prototype herpesvirus, HSV-1 and other herpesviruses, a brief description of the immunity to glycoproteins of herpesviruses is presented.

Over the last ten years, a large amount of information has accumulated concerning immunity induced by the glycoproteins of alphaherpesviruses HSV-1, PRV, EHV-1, MDV and BHV-1 and other herpesviruses (i.e. EBV, HCMV, HVS). It has been established that these glycoproteins can be classified as either essential or nonessential for replication of the virus. Because of their biological role in virion absorption and eggression from infected cells, viral glycoproteins are generally conserved throughout related subfamilies. Based on extensive work with HSV-1 and the animal herpesviruses, it has been determined that glycoproteins B, D and C are major immunogens, eliciting high titers of virus neutralizing (VN) antibodies and providing protective immunity in vaccinated animals against lethal challenge. So far, HSV-1 is the best model for comparison of the immune response induced by various glycoproteins of a specific herpesvirus (Blacklaw et al. 1990). Individual HSV-1 glycoproteins (gB, gD, gH, gI, gE and gG) expressed in vaccinia virus were evaluated for their ability to (1) elicit neutralizing antibody titers, (2) increase the rate of HSV-1 clearance and (3) protect against

lethal challenge and latency. Vaccinia recombinants expressing gB and gD were reported to be superior in eliciting high titers of VN-antibodies and full protection from establishment of latency.

Glycoprotein D has been reported to be essential for virus entry into cells (Fuller and Spear, 1985; Spear et al., 1989; Johnson et al., 1990). Although genes encoding gD homologs are generally conserved throughout herpesvirinae, VZV and the distantly related herpesvirus, channel catfish herpesvirus do not contain gD homologs (Davison and Scott, 1986; Davison 1992). Early studies with monospecific gD antisera or monoclonal antibodies have indicated that gD plays a role in virus penetration and cell fusion (Noble et al., 1983). In one study by Johnson et al., (1988) UV-inactivated (gD+) virions were reported to block entry of WT-HSV-1 or HSV-2 into cells, whereas UV-inactivated virions which are phenotypically gD- were unable to block WT-HSV-1 or HSV-2 entry. Furthermore, mutant (gD-) virions were shown to be able to adsorb to cellular membranes but could not penetrate into cells. These competition experiments and the fact that cell lines expressing high amounts of gD were resistant to infection, lead to a model that herpesviruses initially bind to the cell membrane probably through interaction with glycoprotein C and cellular heparin sulfate moieties on the cell surface. After this initial attachment to cells, the gD receptor is sequestered in gD-expressing cells (Petrovskis et al., 1988; Campadelli-Fiume et al., 1988).

Biologically, another important glycoprotein of herpesviruses is glycoprotein B. The genes encoding homologs to this essential and highly conserved glycoprotein have been mapped within the genomes of 16 herpesviruses. Like gD, glycoprotein B has been reported to be important in penetration of virus capsids into host cells by fusing the viral envelope with cell membranes. Temperature-sensitive viruses with mutations in the gB gene, when propagated at the nonpermissive temperature attach to cells but fail to penetrate, unless a fusogenic agent such as polyethylene glycol is added to the cells (Haffey and Spear, 1980; Little et al., 1981; Sarmiento et al., 1979; Navarro et al., 1992). Likewise, engineered virions lacking gB fail to penetrate susceptible cells (Cai et al, 1988). Interestingly, many syncytial phenotypes in mutant viruses have been attributed to amino acid changes in gB, further supporting gB's role in cell fusion and cell-to-cell spread. These mutant viruses have also been reported to display a slower rate of entry into cells (Bzik et al., 1984).

Besides their biological significance, gB and gD are the major immunodominant polypeptides of herpesviruses, capable of inducing protective immunity. Of all the HSV-1 glycoproteins, only antibodies to glycoprotein D and B can crossreact with the two types of simplex viruses (Marchioli et al., 1987). It has also been demonstrated that gD of HSV-1 induces the most potent monoclonal antibodies with the highest affinity for HSV-1 virions (Para et al., 1985; Iglesias et al., 1990).

Furthermore, anti-gD monoclonal antibodies have been routinely generated from animals immunized with crude virion preps of HSV-1.

There is good evidence that glycoprotein B is as important an immunogen as gD. In HCMV seropositive individuals, for example, 40-70% of total virus-neutralizing activity in serum has been reported to be directed against gB (Britt et al., 1990). Such a preferential reactivity of human sera for a single virion component is unique, due to the fact that herpesviruses contain many glycoproteins. However, the bias for the tremendous response against gB may include; (1) the abundance of gB in the virion, (2) its expression on the surface of infected cells and (3) its numerous epitopes, due to its size. In addition, monoclonal antibodies against gB of HSV-1 can passively protect animals against acute virus-induced neurological illness and death when administered i.p. two hours prior to footpad challenge.

Both glycoproteins D and B of HSV-1, PRV and EHV-1 have been reported to protect mice from lethal challenge (Long et al., 1984). In one study, mice immunized with gD, affinity-purified from cells infected with either HSV-1 or HSV-2, were protected from a lethal intraperitoneal (i.p) challenge by virus of either serotype (Eisenberg et al., 1985). Similarly, gp50 of pseudorabies virus, a gD homolog in the porcine herpesvirus, has been reported to elicit VN-antibodies (Eloit et al., 1990) and when expressed in adenovirus (Wachsman et al., 1989), vaccinia virus or Chinese hamster ovary cells,

protect immunized mice or rabbits from virulent challenge with PRV. In addition, a recombinant gp50 protects pigs, the natural host, from lethal challenge (Marchioli et al., 1987; Reviere et al., 1992). Likewise, protection of mice immunized with recombinant adenoviruses expressing glycoprotein B of HSV-1 has also been demonstrated. Unlike for gD, correct glycosylation of gB appears to be essential for optimal immunogenicity. Mice immunized with recombinant gB isolated from mammalian cells produced significantly higher titers of virus-neutralizing antibodies, when compared to animals immunized with recombinant gB isolated from procaryotes. An enhanced level of protection from lethal challenge was also demonstrated in vaccinates receiving the glycosylated (eukaryotic) recombinant polypeptide. In a study by van Drunen littel-van den Hurk et al., (1990), deglycoslyation of gI(gB) of BHV-1 resulted in a significant decrease in production of serum neutralizing antibodies, due to modifications of three distinct carbohydrate containing continuous epitopes. Likewise, nonglycosylated HCMV gB produced in recombinant prokaryotic systems has been reported to be less immunogenic than the glycosylated protein produced in eukaryotes (Britt et al., 1990). In contrast, nonglycosylated forms of glycoprotein D, for example gIV of BHV-1, stimulate neutralizing antibodies at levels similar to those elicited by glycosylated forms. This comes as no surprise, since the nucleotide sequence of gp50 (gD) of PRV lacks potential N-linked glycosylation sites (Petrovskis et al., 1986). Recently, gD of HSV-1 has been

expressed at high levels in baculoviruses. Although the recombinant protein was slightly smaller than gD in HSV-1 infected Vero cells, due to differences in glycosylation patterns of the two cell lines, the expressed protein was present on membranes of SF9 cells and reacted with gD specific antibodies. Vaccination with the expressed protein resulted in production of neutralizing antibodies to HSV-1 and complete protection against lethal HSV-1 challenge (Ghiasi et al., 1991).

Because of these results, gD and gB of HSV-1 are the prime candidates for subunit vaccines. The genes encoding gD and gB of various herpesviruses have been expressed in both prokaryotic and mammalian cells. Studies on mammalian cells expressing native and truncated gD polypeptides, along with synthetic peptides and V8 protease digestion products have enabled researchers to map its immunologically important continuous and discontinuous epitopes. Synthetic peptides representing one continuous epitope (amino acids 9-21) of gD(HSV-1) conjugated to ovalbumin or BSA were reported to elicit high titers of antipeptide neutralizing antibodies in mice after immunization with adjuvants. Resistance to lethal challenge was also demonstrated in synthetic peptide-immunized mice (Eisenberg et al., 1985).

From the above, it is clear that humoral immunity to gD and gB appears to be a significant contributor to virus clearance. However, this type of immunity is primarily important during initial infection. Overall, cell-mediated

immunity (CMI) appears to be more important. Not only is CMI essential in the acute phase of a herpesvirus infection but it is also involved in virus clearance following reactivation or reinfection. The importance of CMI in resistance to HSV-1 is apparent by the fact that 80-90% of immunosuppressed patients have a high incidence of recurrence (Bernstein et al., 1991). Supporting the role of cell-mediated immunity are numerous reports of adoptive transfer experiments, conferring resistance to lethal HSV challenge. In a study by Rooney et al., (1988), vaccinia recombinants containing the gD(HSV-1) gene under control of an early vaccinia promoter were reported to elicit a better T-cell response than recombinants in which gD expression is controlled by a late vaccinia promoter. Both recombinant viruses produced potent neutralizing antibodies and protected immunized mice from lethal HSV-1 challenge and latency establishment by challenge virus for at least 6 weeks after immunization (Rooney et al., 1988; Wachsman et al., 1989; Wachsman et al., 1989). However, reimmunization with recombinants containing the early vaccinia promoter/gD construct resulted in a significant increase in neutralizing antibody titers lasting over 1 year. Vaccinia recombinants containing the late vaccinia promoter/gD gene fusion failed to protect from cutaneous disease following administration of a high dose of HSV-1. Protection against cutaneous lesions is associated with the induction of HSV-1 specific T-cell responses. Furthermore, proliferation of lymph node cells in response to HSV-1 antigens was demonstrated only in mice

immunized with the Vac(early promoter)/gD- and not Vac(late promoter)/gD-constructs. It appears that temporal expression of glycoprotein genes in antigen presenting cells is important in the induction of immunity to herpes viral disease (Wachsman et al., 1992).

Additional evidence for the role of these glycoproteins in cell-mediated immunity response comes from studies involving immunized mice transplanted with cells expressing herpesvirus glycoproteins. Nakagama et al., (1991) reported significant differences in lymphocyte infiltration and antigen clearance in syngeneic unimmunized mice transplanted with (HSV-1) gD-transfected BALB/3T3 cells, as compared to mice immunized with HSV-1. In the later case, transfected cells elicited massive lymphocyte infiltration of mainly THY1+ and CD8+ lymphocytes along with a small number of CD5+, CD4+, and B-lymphocytes in the HSV-1 immunized mice. In contrast, in unimmunized mice, little evidence of cellular infiltration could be detected and transplanted cells could be detected for as long as 7 days. In immunized animals however, the transplanted cells were mostly destroyed by day 4, despite the presence of anti-HSV-1 antibodies at the time of transplantation. Likewise, cells from the spleen and lymph nodes of gB-immunized mice have been reported to protect syngeneic mice against lethal challenge.

It is generally believed that reactivation of latent herpesvirus occurs more frequently than episodes of recurrent disease. Administration of gD or gB to latently infected

animals reduces the frequency of reactivation, the severity of recurrent disease and the duration of shedding (Bernstein et al., 1991). In guinea pigs latently infected with HSV-2, the adoptive transfer of clones expressing either glycoprotein D or B significantly reduced the number and severity of subsequent symptomatic recurrent infections with a concomitant reduction in cervicovaginal HSV-2 shedding. In this study the author concluded that the reduction in clinical disease was the result of lymphokine activated cellular immunity in which transfer of HSV-1 gD or gB into latently infected animals resulted in production of other cytokines by HSV-1 sensitized T-cells. This could further increase critical responses, such as natural killer cells, needed for clearance of reactivated virus. Further evidence for involvement of lymphokine activity in CMI elicited by herpesvirus glycoproteins was provided by Zarling et al., (1986). Administration of gD or gB, expressed in mammalian cells to HSV-1 seropositive individuals stimulated proliferation of their peripheral blood lymphocytes and interleukin-2 production by these cells. Interestingly, IL-2 can also significantly enhance cellular and humoral immunity in cows when included in either a gD subunit or modified live viral (MLV) vaccine (Reddy et al., 1989; Hughes et al., 1991). Likewise, high antibody responses and cell mediated immunity to HSV-1 were recently reported in mice immunized with a recombinant expressing a glycoprotein D/Interleukin-2 fusion protein (Hinuma et al., 1991).

Although other glycoproteins (gC, gH, gI and gE) of alphaherpesviruses are undoubtedly important for induction of humoral and cell-mediated immunity in infected animals, glycoprotein B is the major immunodominant protein found in members of all subfamilies of Herpesviridae. Glycoprotein D is also a major immunogen, conserved in most viruses of the subfamily Alphaherpesvirinae. The finding that animals can be protected against lethal and latent herpesvirus infections by immunization with either gD or gB, suggest that subunits vaccines containing these glycoproteins will be at least as protective as currently available inactivated vaccines and likely safer than modified live viral (MLV) vaccines.

**PREVENTION**

Because of the prevalence and clinical implications of FHV-1, various vaccines against feline viral rhinotracheitis have been developed and licensed. These include inactivated, modified live (MLV) and subunit vaccines against FHV-1. In a number of these vaccines, FHV-1 is combined with calicivirus and panleukopenia virus in the form of a trivalent vaccine. In other instances rabies virus and chlamydia psittaci are also included. Most recently, two divalent vaccines (generation II) against feline viral rhinotracheitis and feline leukemia have been engineered by Cole and others (1991). One vaccine contained the (FeLV) genes encoding the envelope *env* and *gag*. The other contained the *gag* and protease genes, both inserted

into the thymidine kinase gene of FHV-1. Cats vaccinated with various combinations of these recombinant viruses were fully protected against FeLV challenge (Wardley et al., 1992).

One of the most successful attenuated strains of FHV-1 was developed by serial passage of FHV-1 in Crandell-Reese feline kidney cells at 32°C. Other MLV vaccines against FVR have been generated using classical tissue culture passage and are commonly referred to as generation I vaccines. These vaccines generally protect cats against clinical symptoms when naturally exposed to the virus, but do not protect against challenge with a virulent laboratory strain. Although the duration of clinical signs is lessened in most MLV-vaccinated cats that are challenged with virulent strains, the route of administration of MLV vaccines appears to be an important determinant in eliciting protective immunity. MLV vaccines are generally administered by the natural route of infection (intranasally), thereby inducing a more rapid (48-96 hours) and more solid local immune response such as secretory IgA. However, parenteral administration of MLV-vaccines is often preferred in catteries or multiple cat-households due to the fact that they do not evoke sneezing or other postvaccinal signs.

The greatest shortcoming of the available MLV vaccines against FVR, regardless of the route of administration, is that they do not replicate well in cats. The most common FHV-1 vaccine, a ts-mutant of FHV-1 which replicates in the upper

respiratory tract epithelium rather than the lungs, induces a rather weak local immune response in vaccinates. Also, MLV vaccines against FVR may lead to persistent infections in vaccinates, if the dosage is large enough to allow adequate replication and seeding of suspectable ganglia.

Inactivated and subunit vaccines against FVR have also been developed (Benoit-Jeanin, 1983; Limcumpao et al., 1991) and are inherently safer than MLV vaccines. However, in order to be sufficiently immunogenic, these vaccines must contain large amounts of antigen and at least two doses have to be given in order to elicit a solid immune response. To obtain immunogens in usable amounts for inactivated vaccines, the inactivation process has to be gentle enough to not destroy immunogenic components of the virus. Also, inactivated virus and subunit vaccines must be combined with an adjuvant that maximizes the immune response without causing side effects. Recently, animals immunized with a protein construct containing HSV-1 gD fused to IL-2 have been reported to be protected against lethal challenge.

Because of their safety, these vaccines should be given to colostrum-deprived neonates or pregnant, debilitated, or immunosuppressed animals. Although it has been reported that intranasal administration of MLV vaccines to pregnant cats did not produce ill effects, this practice is generally not recommended (Pearson et al., 1986).

## CONCLUSION

From this review it is apparent that infections with FHV-1, especially in young kittens, can be fairly severe and that latently infected carriers are an important link in perpetuation of the virus. Vaccination with currently available vaccines is protective against clinical disease, but not against reinfection and latency. There is a need, therefore, to develop vaccines and vaccination strategies that offer a more comprehensive protection against different clinical forms of this important viral disease of cats.

Chapter 2

# Immunological Characterization of the Feline Herpesvirus-1 Glycoprotein B and Determination of its Nucleotide Sequence.

Stephen J. Spatz

**ABSTRACT**

Feline herpesvirus 1 (FHV-1) is an important viral pathogen of cats. Like other alphaherpesviruses, FHV-1 contains a herpes simplex 1 (HSV-1) glycoprotein B (gB) homolog. In this study, monospecific antisera to HSV-1 gB reacted with three FHV-1 proteins (100, 64 and 58 Kd) present in virion lysates using immunoprecipitation and immunoblot analyses. Reduced stringency hybridization experiments using a HSV-1 gB probe localized the FHV-1 gB gene to a 9.6 Kb SalI fragment in the unique long region of the genome. Northern analyses further localized the entire coding region within a 3.3 Kb SacI fragment. This fragment was sequenced and analyzed for open reading frames. The predicted amino acid sequence of the 2,829 b.p. ORF was shown to have a high degree of homology with gB analogs of HSV-1, EHV-1, BHV-1, EHV-4, and especially PRV. Two unique characteristics of glycoprotein B of FHV-1 were the unusually long signal sequence of 73 amino acids and two proteolytic cleavage sites, RTRRS and RSRRS. An evolutionary tree, based on gB homologs from 12 alphaherpesviruses suggests that feline herpesvirus-1 evolved along similar lines as members of the genus Varicellovirus.

## INTRODUCTION

Feline herpesvirus (FHV-1), a member of the genus alphaherpes virinae, is one of the most important causes of viral upper respiratory diseases in cats (Povey, 1979; Maes et al., 1984). Glycoproteins, present in the envelope of herpesviruses play an important role in induction of humoral, cell-mediated and nonspecific host defense mechanisms (Pereira et al., 1989; Eberle et al., 1985; Blacklaw et al., 1987; Hanke et al., 1991). The genome of herpes simplex virus-type 1 codes for at least 10 antigenically distinct glycoproteins: gB, gC, gD, gE, gG, gH, gI, gJ, gK and gL (Spear, 1984; Hutchinson et al., 1992). These glycoproteins have been well characterized and are fairly conserved among related herpesviruses. Glycoprotein B homologs have been mapped within the genomes of 14 herpesviruses: herpes simplex virus-1, herpes simplex virus-2, varicella-zoster virus, Epstein-Barr virus, human cytomegalovirus, equine herpesvirus-1, equine herpesvirus-4, bovine herpesvirus-1, bovine herpesvirus-2, pseudorabies virus, Marek's disease virus, herpesvirus saimiri, infectious laryngotracheitis virus and simian agent type 8 virus (Bzik et al., 1984; Pellett et al., 1985), (Bzik et al., 1986; Zwaagstra et al., 1987; Stuve et al., 1986), (Keller et al., 1986), (Pellett et al., 1985; Gong et al., 1987), (Cranage et al., 1986; Mach et al., 1986), (Whalley et al., 1989), (Riggio et al., 1989), (Whitbeck et al., 1988; Misra et al., 1988; Lawrence et al., 1986), (Hammerschmidt

et al., 1988), (Robbins et al., 1987), (Ross et al., 1989), (Albrecht et al., 1990), (Griffin, 1991) and (Borcher, et al., 1991). This conservation is not surprising since gB, as well as glycoproteins D, H, K and L, have been shown to be essential for production of enveloped viruses (Spear, 1984; Hutchinson et al., 1992; MacLean et al., 1991)

HSV-1 gB and also the gB homolog of Pseudorabies virus (gII) have been shown to form a dimeric protein on the surface of virions and infected cells. (Claesson-Welsh et al., 1986; Whealy et al., 1990). Furthermore, glycoprotein B has been implicated in the penetration of the host cell membrane and also in cell-to-cell spread of virus by fusion (Cai et al., 1988; Highlander et al., 1988; DeLuca et al., 1982).

The aim of this work was to immunologically define the existence of an FHV-1 gB homolog, to map its genomic location and to define its nucleotide sequence. To accomplish this radiolabeled plasmids containing the HSV-1 gB gene were used to probe southern blots of cloned fragment of FHV-1 DNA. The coding region of the FHV-1 gB homolog was localized within a 3.3 Kb SacI fragment in the unique long region. Two different rabbit antisera to HSV-1 gB reacted strongly with a 64 and 58 Kd and more faintly with a 100 Kd FHV-1 protein from virion lysates, in immunoprecipitation and western blot analyses. In this paper we present the nucleotide sequence of FHV-1 gB, immunoblot and immunoprecipitation analyses of FHV-1 polypeptides crossreacting with anti-HSV-1 gB antisera and an evolutionary lineage of 12 gB homologs of alphaherpesviruses.

## MATERIALS AND METHODS

### Bacterial strains and vectors

*Escherichia coli* JM101 and JM109 were grown in LB medium and used to propagate pBluescript-KS and M13 mp18 and mp19.

### Viruses, cells, and medium

FHV-1 strain (C-27) was obtained from the American Type Culture Collection. Crandell Reese Feline Kidney (CRFK) cells were grown in Dulbecco's modified Eagle Medium, containing 100 Units/ml of Penicillin, 100 ug/ml of Streptomycin and 10% heat-inactivated fetal bovine serum. The CRFK cells were infected with plaque-purified virions as described previously (Maes et al., 1984).

### *In-vitro* labelling and Immunoprecipitation of FHV-1 Infected Cells

Radiolabelling with $^{14}C$ glucosamine and immunoprecipitation were performed as previously described (Maes et al., 1984). Briefly, cytoplasmic extracts were prepared in 1X PBS containing 1.0% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS (PLB). Virion lysates were prepared from virions purified through 30% potassium tartrate cushions. Monospecific rabbit anti-gB (HSV-1) sera (gB1 and R69) were obtained from Drs. N. Balachandran (anti-gB1) and R. Eisenberg (R69). Immunoprecipitins were boiled in 20 ul of 1.25% SDS Sample buffer containing 1.0 ul of 2-mercaptoethanol and electrophoresed through 10.0% polyacrylamide.

**Western Blot Analyses**

FHV-1 virions from infected CRFK cells were purified by rate zonal centrifugation through 10 to 40% potassium tartrate gradients (Talens and Zee, 1976). The resulting polypeptides were separated by SDS-PAGE. Immunoblotting was done according to procedures described by Ausubel et al., 1988, using either rabbit anti-gB (HSV-1) antisera (gB1 or R69). Alkaline phosphatase-labeled mouse anti-rabbit conjugates along with the chromogens, BCIP and NBT were used to visualize the bands.

**Recombinant DNA Methods**

A recombinant plasmid containing the complete HSV-1 gB coding domain, pST11 was kindly provided by Dr. Joseph Glorioso (University of Pennsylvania). The external coding domain of HSV-1 gB was excised from the plasmid pST11 as a NcoI-XhoI fragment (Figure 2), radiolabelled and used extensively as a probe in reduced-stringency hybridizations of blots containing cloned restriction fragments of FHV-1. Blots were hybridized at 45°C in standard hybridization solution without formamide and washed under stringent conditions until the background bands were reduced to an acceptable level. Blots were often exposed while still wet, then rewashed and reexposed.

**RNA Isolation and Northern Analyses**

Total cellular RNA was extracted using the guanidinium isothiocyanate procedure (Ausubel et al., 1988) from mock

infected or FHV-1 infected CRFK cells. Cells were infected with FHV-1 at a m.o.i. of >1.0 pfu/cell. Lysates were prepared 10 hours later. Ten micrograms of RNA were electrophoresed in 1.2% formaldehyde gels, passively transferred to nitrocellulose and hybridized with radiolabeled probes (See Figures 3 and 5).

**DNA Isolation and Nucleotide Sequencing**

Viral DNA was prepared as described previously (Rota, et al., 1986). Plasmid DNA was isolated from bacteria by the alkaline lysis method (Sambrook et al., 1989). Single stranded DNA from M13 phage was isolated by pelleting the virions through a cushion containing 25% PEG in 3M NaCl. The pellets were then resuspended in TES buffer (20mM Tris-HCI, pH 7.5, 20 mM NaCl, 1mM EDTA) and lysed with equal volumes of water saturated phenol. The DNA was recovered after precipitation with sodium acetate and ethanol (Ausubel et al., 1988). Nucleic acid sequencing was performed by the dideoxy chain termination method (Sanger et al., 1977) with the modified T7 DNA polymerase (Sequenase, US Biochemicals) and with $^{35}S$ dATP (NEN) as the label. The analog deoxyinosine triphosphate (dITP) was often substituted for dGTP to minimize band compression. In most cases, single stranded M13 DNA was sequenced, but on occasion sodium hydroxide denatured double stranded bluescript DNA was also used. Synthetic primers, along with the universal and reverse primers of M13 were used to rapidly generate sequencing data. The oligonucleotides used

were synthesized on a 380B automated DNA synthesizer (Applied Biosystems) with a three column upgrade. Electrophoretically separated sequencing reaction products were visualized by autoradiography of dried 8% acrylamide/7M urea gels using Kodak X-AR film. The sequences of both strands of viral DNA were determined at least twice from individual clones.

**Computer Analyses of the DNA Sequence**

DNA sequence management was performed on a VAX computer using versions 5.0 and 5.3 of the University of Wisconsin package (UWGCG), (Devereux et al., 1984). Secondary structures of the predicted peptide were investigated using the methods of Chou and Fasman (1978). Graphic hydrophilicity analyses were generated by the method of Kyte and Doolittle (1982). Amino acid homology analyses were conducted using the FASTA program. The GAP program was used to align the nucleotide and amino acid sequences. Evolutionary relatedness of 12 alphaherpesviruses was analyzed using a multiple alignment of gB homologs generated by the LINEUP and PILEUP programs. The TOFITCH program was used to make the infile for the Phylogeny Inference Package (PHYLIP), version 3.2 (Felsenstein, 1985). The tree was drawn using a maximum parsimony method, PROTPARS and is based on 650 amino acids. The plotfile for the tree was drawn using DRAWGRAM.

## Characterization of the FHV-1 gB protein

Our initial evidence (Figure 1) for a HSV-1 homolog of glycoprotein B consisted of detecting FHV-1 proteins by immunoprecipitation of lysates from infected cells and virion lysates with monospecific antisera to HSV-1 gB (anti-gB1 and R69). Immunoprecipitation of FHV-1 infected cells with either R69 or anti-gB1 sera indicated the presence of crossreactive proteins with MW's of 120, 100, 64, 58, and 56 Kd. When virion lysates were immunoprecipitated with either antisera, two proteins of 64 and 58 Kd were detected. A third protein of 100 Kd was also detected on overexposed autoradiograghs. It is noteworthy that the 100, 64 and 58 Kd proteins were immunoprecipitated exclusively from virion lysates while the 56 Kd protein was immunoprecipitated when infected cellular lysates were used.

Western blot analyses provided additional evidence that FHV-1 contains a gB homolog. FHV-1 proteins from KT-gradient purified virions were separated on denaturing gels and electroblotted onto nylon membranes. After incubation with either R69 or anti-gB1, three peptides with MW's of 100 (range 99-100), 64 (range 62-66) and 58 (range 60-57) Kd could be detected with $^{125}I$ protein A.

Figure 1. **Analyses of FHV-1 B polypeptides.** (A) Lysates from FHV-1 infected cells (ICL), lanes 1 and 2; lysates from uninfected cells (UCL),lanes 3 and 4; and lysates from FHV-1 virions (VL), lanes 5 and 6, were immunoprecipitated with monospecific polyclonal HSV-1 gB specific antisera R69 (odd lanes) and anti-gB1 (even lanes). (B) Lysates from FHV-1 virions (VL) were electroblotted onto nitrocellulose and probed with R69 (lane 1) and anti-gB1 (lane 2).

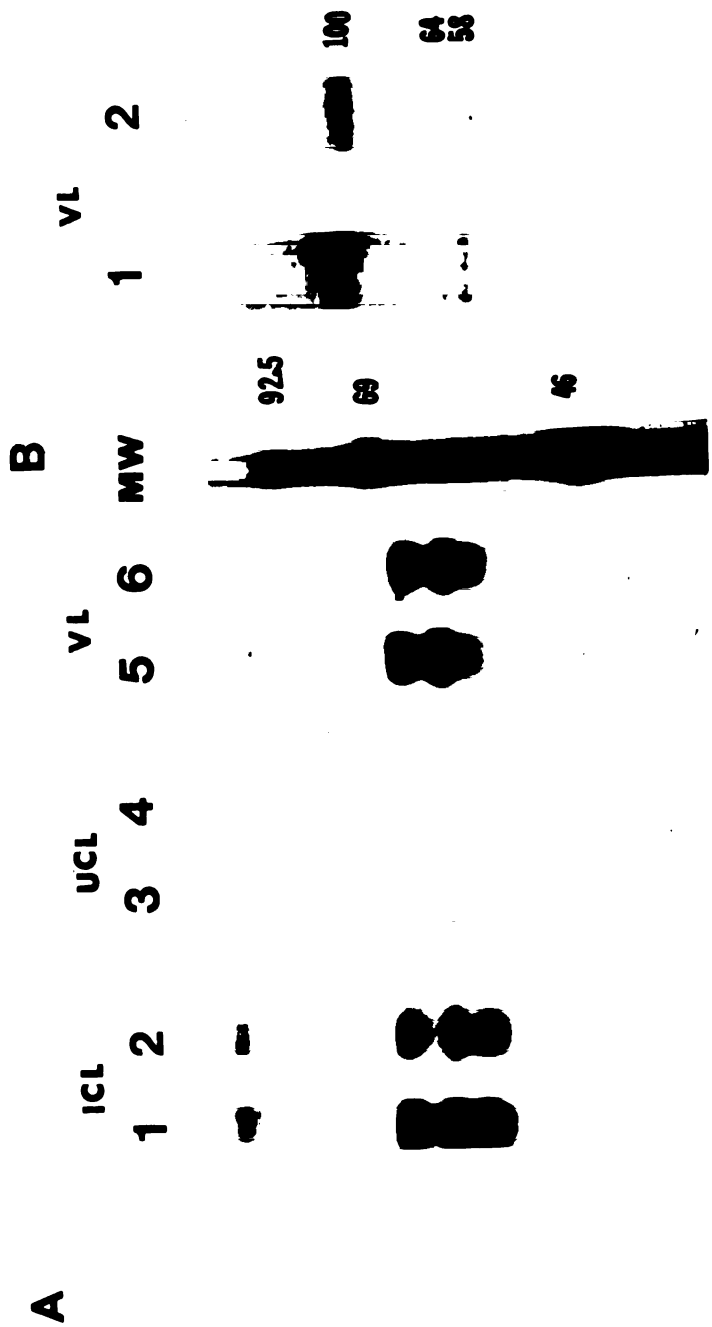


Figure 1

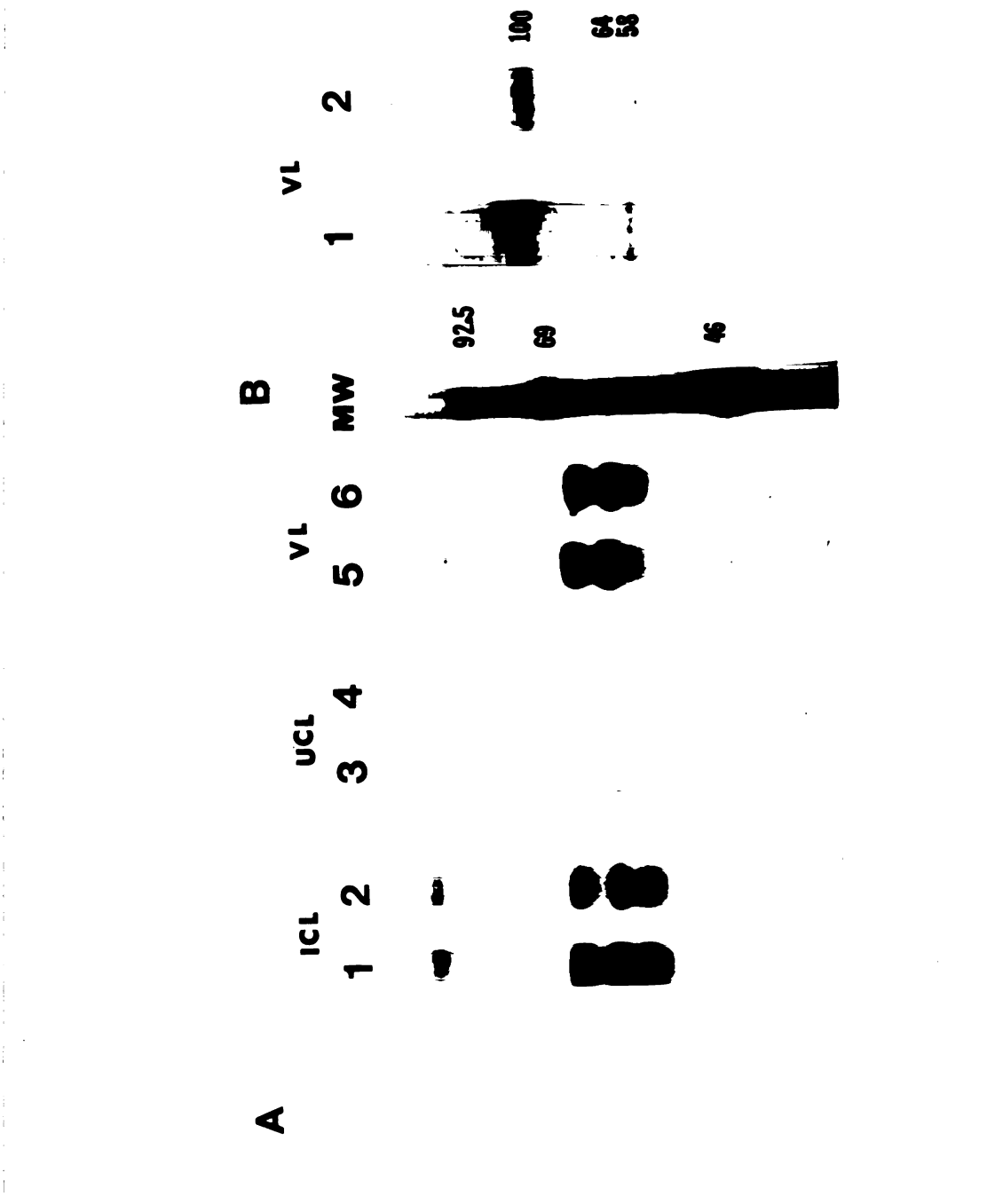


Figure 1

**Identification and sequence analysis of the FHV-1 gB gene**

Evidence for a FHV-1 glycoprotein B gene initially came from southern analyses (Figure 2) showing that a HSV-1 gB probe specific for the 5' end of the gene hybridized to an EMBL3/FHV-1 recombinant containing the 9.6 Kb SalI G fragment. Southern analyses of FHV-1 DNA further localized the gene to a 3.3 Kb SacI subfragment of the larger SalI G clone (Figure 3). The nucleotide sequence of this 3.3 Kb subfragment was then determined and analyzed for open reading frames containing amino acid stretches with similarity to gB homologs of other herpesviruses. These analyses (Figure 4) revealed two overlapping open reading frames, coding for the glycoprotein B and ICP18.5 genes. An ORF of 2,829 nucleotides capable of encoding a gB translation product of 943 amino acids was identified and there exist a TATA box (AATATATC), 148 nucleotides upstream of the initiation codon ATC**ATGT** (Kozak, 1986). The sequence ATTG was also found approximately 113 base pairs 5' of the TATA box. This sequence may function as a CAAT box, as was thought to be the case for HSV-1 gB and PRV gII (Hammerschmidt et al., 1988; Robbins et al., 1987). A potential Sp1 binding site, GGCGG was found next to the CAT box (Gidoni et al., 1984). Downstream of the ORF are two potential cis-acting elements. A polyadenylation signal, (AATAAA) was found 46 nucleotides downstream from the stop codon TAA and was followed by GT-rich sequences. Such GT-rich regions are similarly associated with many known RNA cleavage and polyadenlyation sites (Birnstiel et al., 1985).

Figure 2. **Low stringency hybridizations.** (A) The position of the XhoI-NcoI restriction sites within the gene encoding HSV-1 gB. (B) Southern blots containing restriction digested DNA isolated from a recombinant clone containing SalI fragment G (lanes 1-2) and FHV-1 infected CRFK's (lanes 3-12). Prior to electrophoresis the DNA was digested with the following restriction endonucleases: HindIII (lane 1), SalI (lane 2), BamHI (lane 3), EcoRI (lane 4), HindIII (lane 5), KpnI (lane 6), NcoI (lane 7), PstI (lane 8), XhoI (lane 9), XbaI (lane 10), SstI (lane 11) and EcoRV (lane 12).

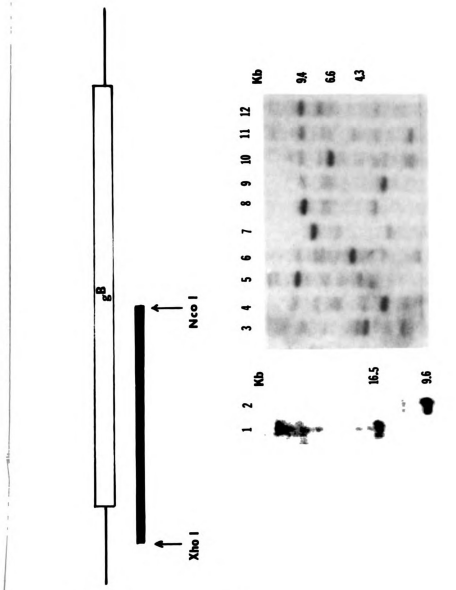


Figure 2

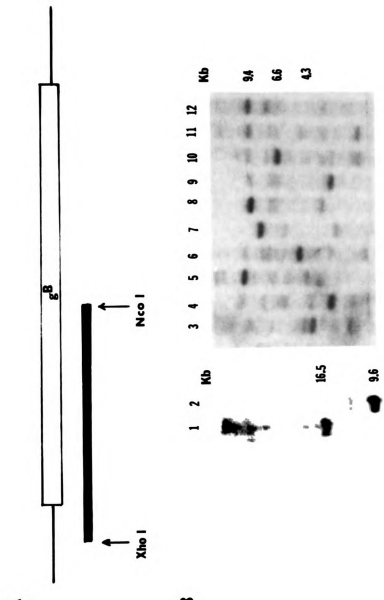


Figure 2

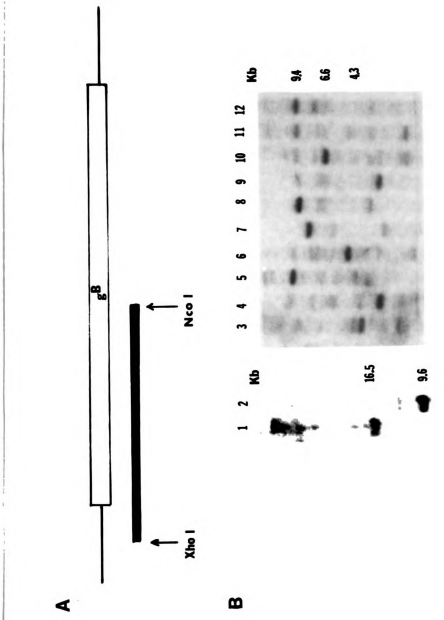


Figure 2

Figure 3. **Genomic organization of the FHV-1 glycoprotein B gene.** (A) The 134 Kb genome is represented as two unique sequences ($U_L$ and $U_s$) and two inverted repeat sequences ($IR_s$ and $TR_s$) flanking the $U_s$ region. (B) The SalI restriction map of FHV-1 (C-27) is also presented with a detailed restriction map of the 9.6 Kb SalI G fragment. Arrows indicate the location of the gB and ICP18.5 ORFs within the region sequenced. (C) The black boxes represent the gB-specific hybridization probes used to map the gB transcript.

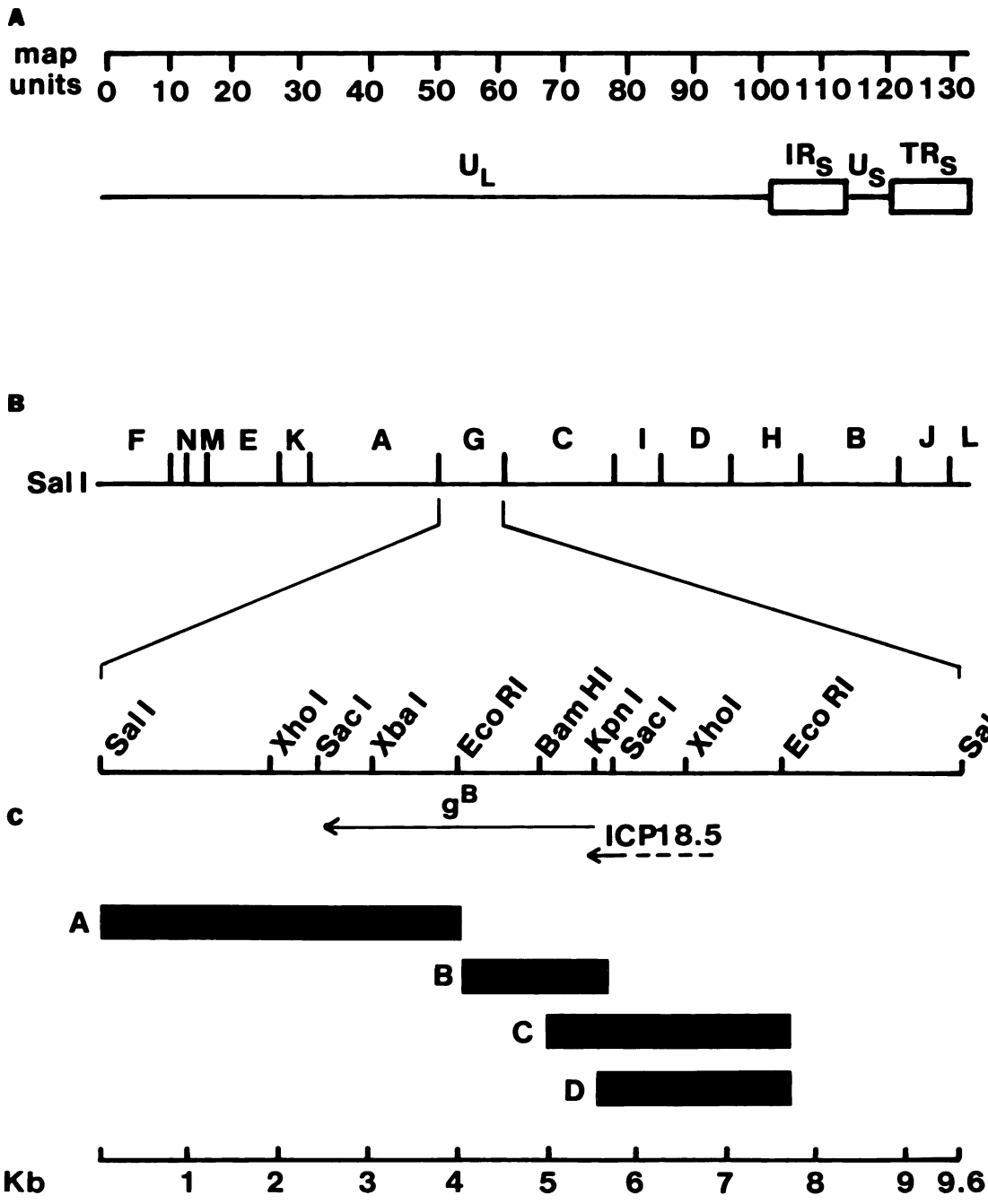
A
map units
0 10 20 30 40 50 60 70 80 90 100 110 120 130
$U_L$
$IR_S$ $U_S$ $TR_S$
B
F NM E K A G C I D H B J L
Sal I
Sal I Xho I Sac I Xba I Eco RI Bam HI Kpn I Sac I Xho I Eco RI Sal I
C
$g^B$
ICP18.5
A
B
C
D
Kb
1 2 3 4 5 6 7 8 9 9.6

Figure 3

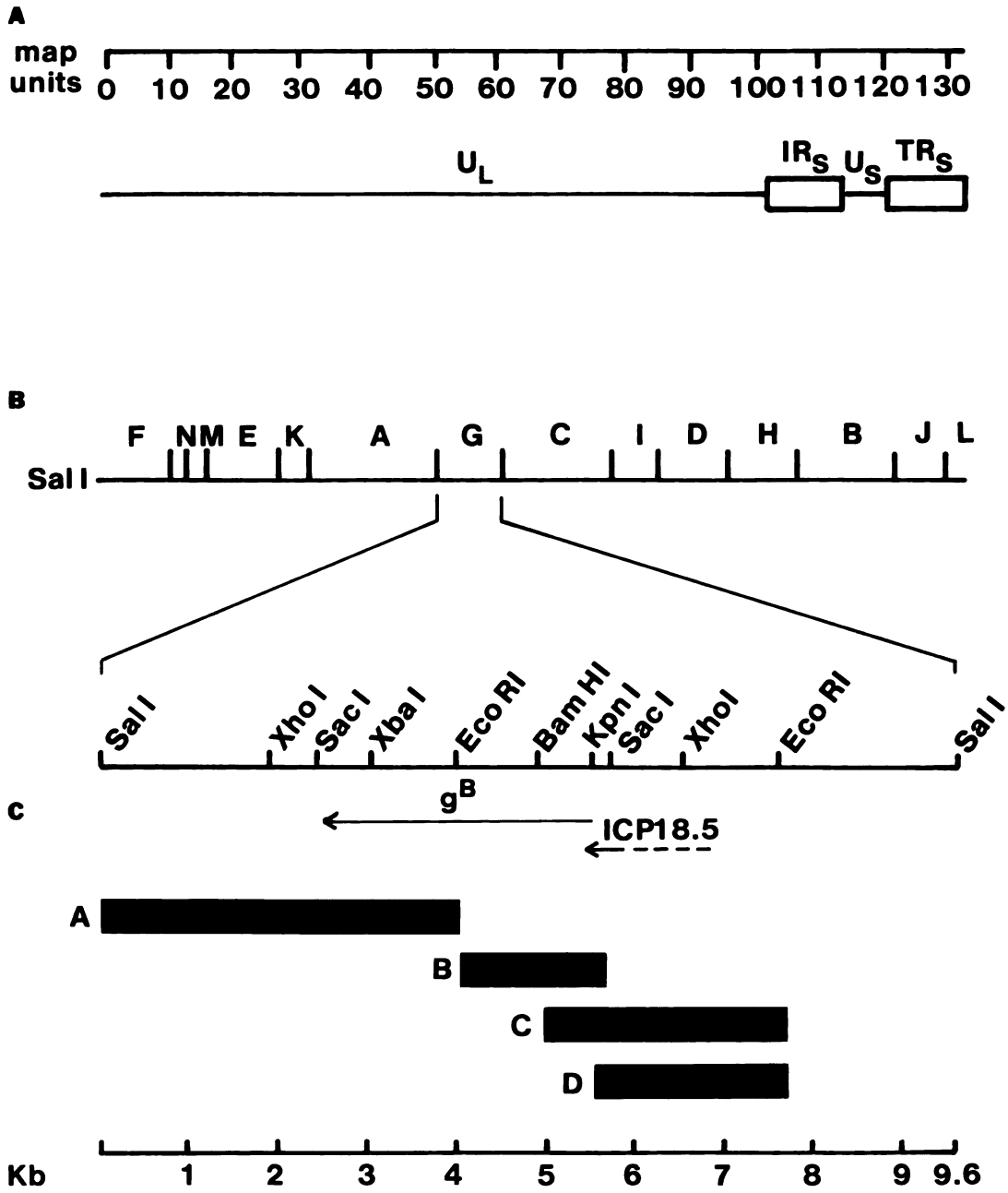
A
map units
0 10 20 30 40 50 60 70 80 90 100 110 120 130
$U_L$
$IR_S$ $U_S$ $TR_S$
B
F NM E K A G C I D H B J L
Sal I
Sal I Xho I Sac I Xba I Eco RI Bam HI Kpn I Sac I Xho I Eco RI Sal I
C
$g^B$
ICP18.5
A
B
C
D
Kb
1 2 3 4 5 6 7 8 9 9.6

Figure 3

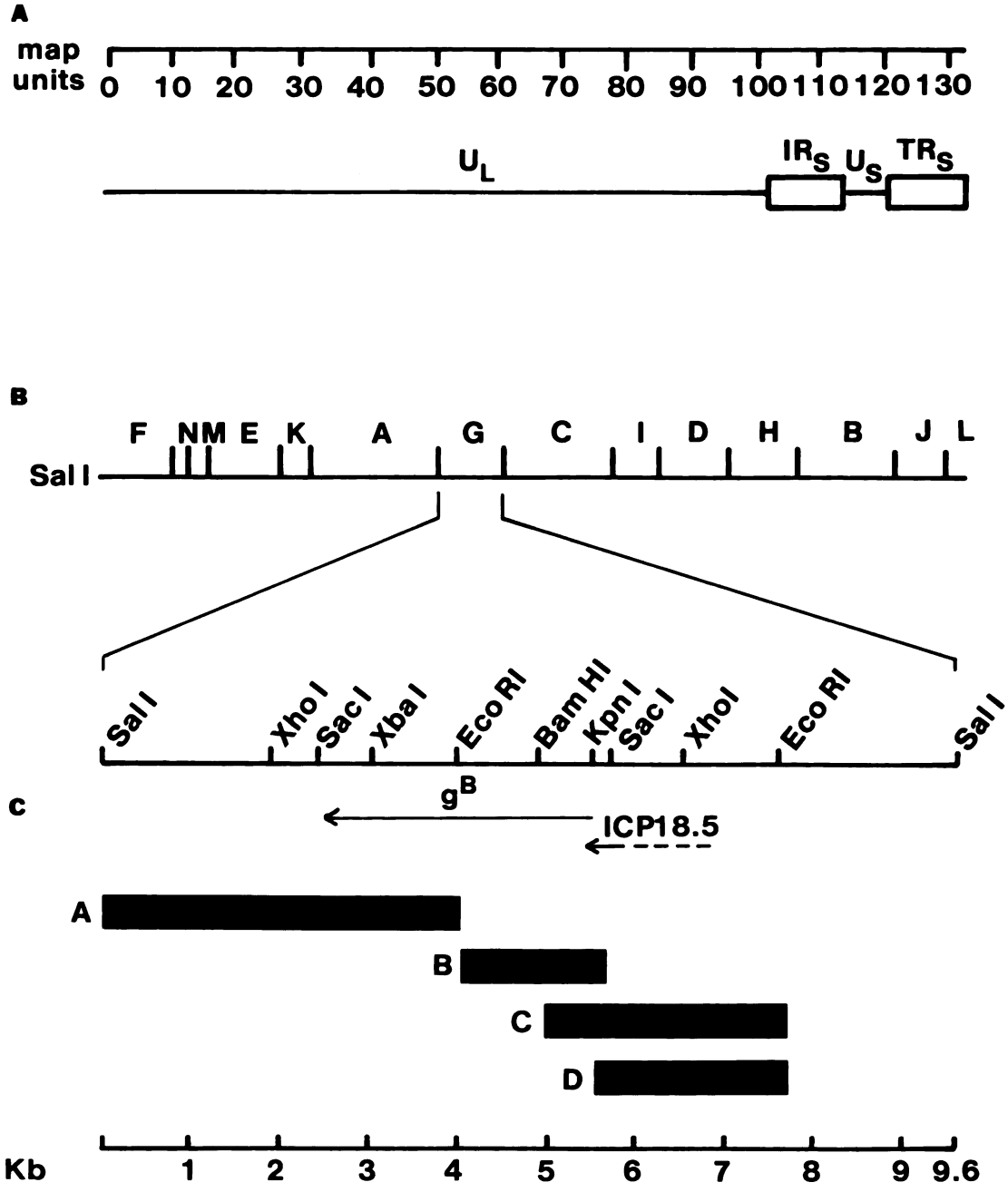
A
map units
0 10 20 30 40 50 60 70 80 90 100 110 120 130
$U_L$
$IR_S$ $U_S$ $TR_S$
B
F NM E K A G C I D H B J L
Sal I
Sal I
Xho I
Sac I
Xba I
Eco RI
Bam HI
Kpn I
Sac I
Xho I
Eco RI
Sal I
C
$g^B$
ICP18.5
A
B
C
D
Kb
1 2 3 4 5 6 7 8 9 9.6

Figure 3

Figure 4. **Nucleotide sequence and predicted amino acid sequence of the FHV-1 gB polypeptide and part of the FHV-1 gene product analogous to ICP18.5.** Putative CAT (ATTG), TATA (AATATATC) boxes and poly(A) signal sequence (AATAAA) are shown in bold. Potential N-glycosylation sites are bracketed by two lines and the predicted hydrophobic N-terminal signal peptide and C-terminal transmembrane domain are overlined. Potential proteolytic cleavage recognition sites are indicated with asterisks.

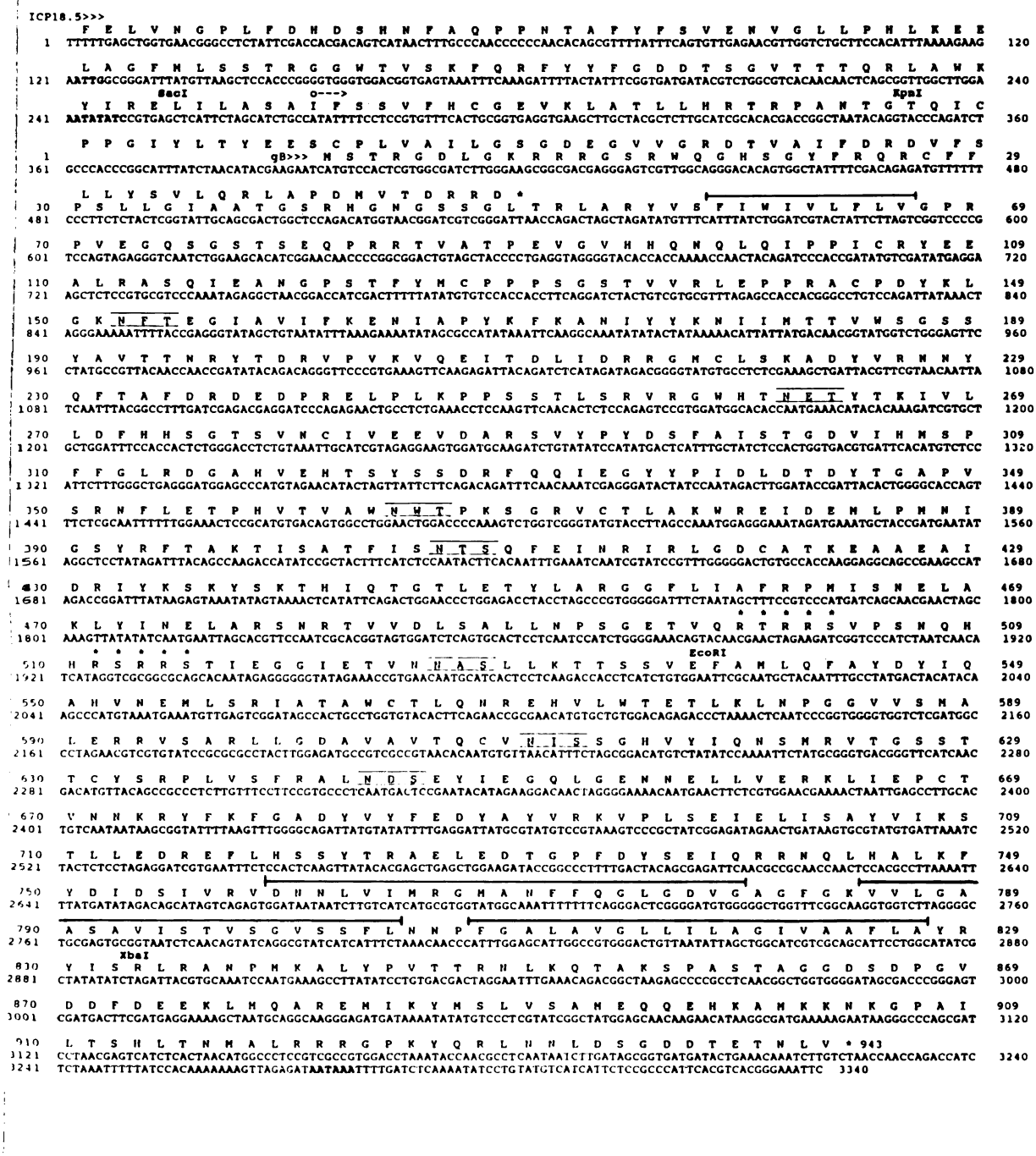


Figure 4

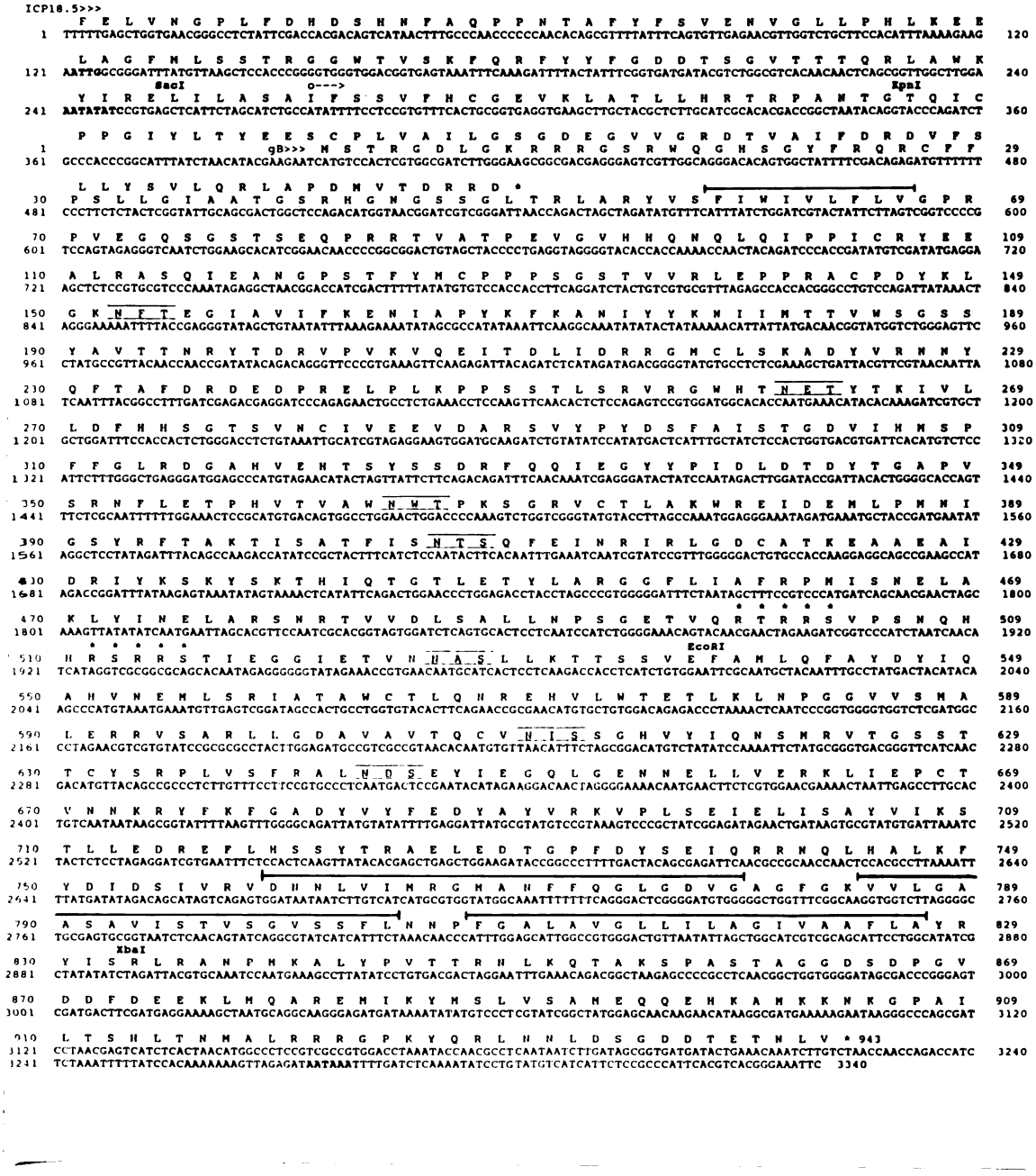


Figure 4

An ORF encoding a polypeptide with homology to ICP18.5 of HSV-1 (UL28) was found to overlap the FHV-1 gB gene by 48 codons. Since no obvious polyadenylation signal was found 3' to the FHV-1 ICP18.5 ORF, the 3' terminus of this transcript may be coterminal with that of the gB mRNA.

**Transcriptional analysis of the gB gene**

Northern blot analyses (Figure 5) using four probes that span the entire FHV-1 gB gene (Figure 3) has indicated the presence of 3 transcripts; 4.0, 3.2, and 1.5 Kb. As shown in Figure 5, both the EcoRI-KpnI fragment (probe B) and the BamHI-EcoRI fragment (probe C) hybridized to the 4.0, 3.2 and 1.5 Kb transcripts, while the KpnI-EcoRI probe (D) only hybridized to the 4.0 Kb transcript. These results indicate that the gB gene (3.2 Kb) is confined between the KpnI and SalI restriction sites and the transcription start site occurs between the KpnI and BamHI restriction sites.

**Amino acid sequence and secondary structure of gB (FHV-1)**

Hydrophilicity analyses of the 943 amino acid FHV-1 gB translation product indicated the presence of a hydrophilic surface domain at the amino-terminus with 7 potential glycosylation sites. Two hydrophobic domains were also predicted at both ends of the polypeptide (Figure 6). A signal cleavage site (residues 58 to 66) consisting of 9 consecutive hydrophobic residues, FIWIVLFLV, followed by a helix-breaking residue glycine was found near the amino-terminus. This

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Ec
hy

Figure 5. **Northern blot analyses of RNA extracted from FHV-1 infected CRFK cells.** Total cytoplasmic RNA was isolated from uninfected and FHV-1 infected cells. The RNA was fractionated in an agarose-formaldehyde gel, transferred to nitrocellulose paper and hybridized with $^{32}$P-labeled probes (Fig. 1). Lanes: 1-4, RNA extracted from FHV-1 infected cells; 5, RNA extracted from uninfected cells. The blots in lanes 1,2,3 and 4 were hybridized with $^{32}$P-labeled fragment A (SalI-EcoRI, 4.0 Kb), B (EcoRI-KpnI, 1.6 Kb), C (BamHI-EcoRI, 3.0 Kb) and D (KpnI-EcoRI, 3.1 Kb), respectively. The blot in lane 5 was hybridized with the 9.6 Kb SalI G fragment.


Figure 5

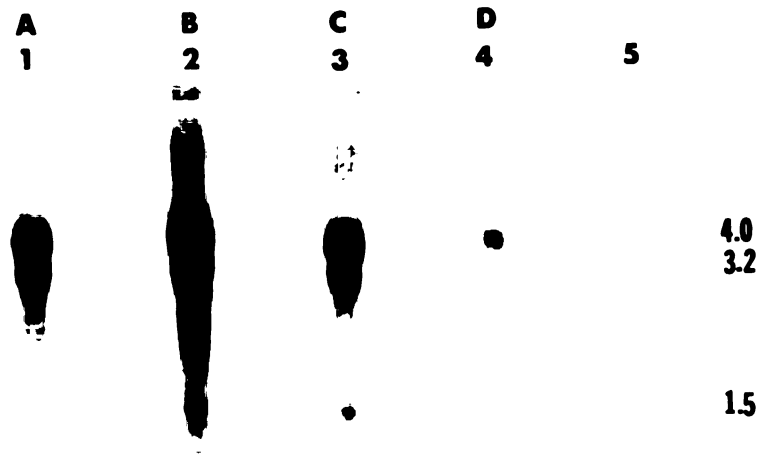



Figure 5

hydrophobic core was preceded by a region containing 10 arginine residues. A potential signal peptide cleavage site (VEG Q), residues 71-74, shares several consensus features described by von Heijne (1986) and McGeoch (1985): cleavage after a small amino acid at position -1 (glycine), a charged residue at position -2 (glutamic acid) and valine at position -3. Chou and Fasman analyses showed that the cleavage site is followed by a beta-turn (data not shown). Hydrophilicity plots (Figure 6) also indicated a second hydrophobic domain (residues 758 to 827) located near the COOH-terminus. Three distinct hydro-phobic peaks in this area fulfill the criteria for a trans-membrane region. Three similar peaks have been reported in the corresponding regions of gB homologs of other herpesviruses. Based on Chou and Fasman analyses, this transmembrane domain was predicted to contain three antiparallel hydrophobic segments. Each segment, connected to the others by very short turn regions, transverses the membrane three times and provides the anchoring sequence for glycoprotein B.

A putative cytoplasmic domain (residues 828-911), characterized by a high hydrophilicity value, was predicted at the COOH terminus and is typical of cytoplasmic regions of transmembrane glycoproteins.

Figure 6. **Hydrophilicity plot of the predicted gB protein.** The hydropathy value was calculated by the methods of Kyte and Doolittle (1982). The hydropathy window was seven amino acids, with a plus sign indicating increasing hydrophilicity and a minus sign representing increasing hydrophobicity.

Hydrophilicity Window Size = 7
Scale = Kyte-Doolittle
Hydrophilicity
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00
100
200
300
400
500
600
700
800
900

Figure 6

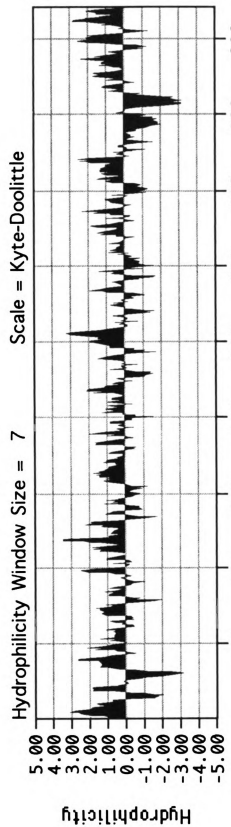

Hydrophilicity Window Size = 7
Scale = Kyte-Doolittle
Hydrophilicity
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00
100
200
300
400
500
600
700
800
900

Figure 6

**Comparison of gB (FHV-1) to gB homologs of other herpesviruses**

Compared to gB of other related herpesviruses, FHV-1 gB shows more relatedness to gB homologs of PRV, EHV-1 EHV-4, and BHV-1 (73.9, 73.5, 72.7 and 72.3% similarity, respectively) than to those of HSV-1, EBV, HCMV and HVS (64.6, 49.7, 48.9 and 47.7% similarity, respectively). There are major blocks of conserved amino acids in the alignment of the 15 herpesvirus gB homologs. The first block of conservation occurs at position 298-348 (Figures 7 and 8) with the consensus, **CiveeveArSvyPydsFalstGdivymSPFyglr.gahreht.sya.drf**. The second block, having the consensus, **mlQftYdhiqrhvNemlgriataWCelQreltw neark.NPsaiasatlgrrvsarmlGDv.avstCve.va.dnvi.lqnsmrvpgspgtCYsR Plvs**, occurs at position 601-701. When aligning FHV-1 gB to gB homologs of the other 15 herpesviruses, ten of the eleven cysteine residues are perfectly conserved. Also, the positions of the 7 glyco-sylation sites (Asn-X-Thr/Ser, with X being any amino acid except proline or aspartic acid) and proline residues are well conserved. This suggests that the secondary and tertiary structures of FHV-1 gB and other gB homologs are fairly similar.

Results from the evolutionary relatedness study are depicted in Figure 9.

Figure 7. **Comparison of gB polypeptides of 15 herpesviruses.** The number of amino acids are indicated to the right. Sizes of gB proteins are drawn to scale and aligned to maximize residue homology. Potential N-glycosylation sites are indicated by a triangle and aligned cysteine residues are indicated by vertical lines. Two highly conserved regions, indicated by closed boxes (A and B), are given in detail in Figure 8. Aligned proteolytic cleavage recognition sites are designated by an arrow.

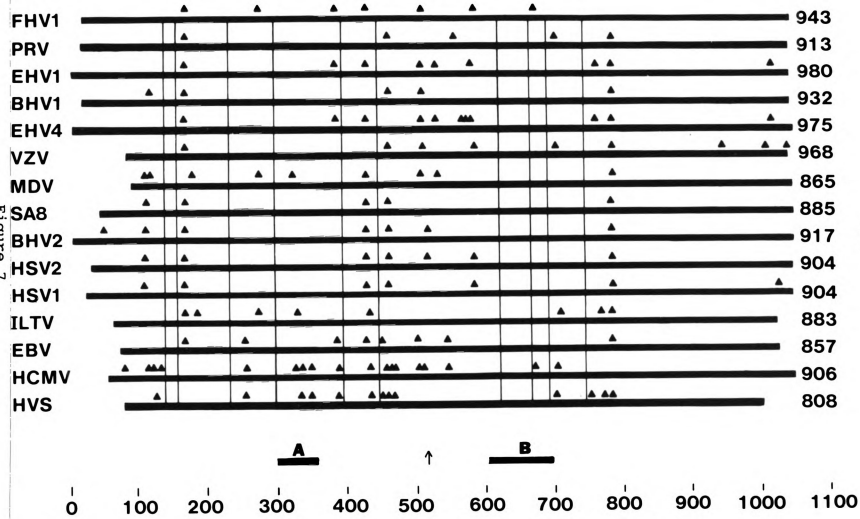

FHV1
PRV
EHV1
BHV1
EHV4
VZV
MDV
SA8
BHV2
HSV2
HSV1
ILTV
EBV
HCMV
HVS
943
913
980
932
975
968
865
885
917
904
904
883
857
906
808
A
B
0
100
200
300
400
500
600
700
800
900
1000
1100

Figure 7

Figure 8. **Amino acid sequence of two highly conserved regions in the gB proteins of 15 alpha-, gamma-, and beta-herpesviruses.** (A) Amino acids 298-348 (Box A in Fig.7). (B) Amino acids 601-701 (Box B in Fig. 7). Amino acid residues identical to those found in FHV-1 are in bold. Numbers flanking the individual sequence indicate the position of the depicted amino acid with respect to the initial methionine. A consensus sequence is indicated at the bottom of the aligned sequences. Uppercase letters represent amino acids conserved in all 15 gB homologs, while lower case letters denote semi-conservation.

**A**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| FHV1 | 236 | CIVEEVDARS | VYPYDSFAIS | TGDVIHMSPF | FGLRDGAHVE | HT.SYSSDRF | 285 |
| PRV | 235 | CIVEEVEARS | VYPYDSFALS | TGDIVYMSPF | YGLREGAHGE | HI.GYAPGRF | 284 |
| EHV1 | 313 | CIVEEVEARS | VYPYDSFALS | TGDIVYASPF | YGLRAAARIE | HN.SYAQERF | 362 |
| EHV4 | 246 | CIVEEVEARS | VYPYDSFALS | TGDIVYTSPF | YGLRSAAQLE | HN.SYAQERF | 295 |
| BHV1 | 236 | CIVEEVEARS | VYPYDSFALS | TGDIIYMSPF | YGLREGAHRE | HT.SYSPERF | 285 |
| VZV | 168 | CIIEEVEARS | IFPYDSFGLS | TGDIIYMSPF | FGLRDGAYRE | HS.NYAMDRF | 217 |
| MDV | 158 | CIVEEMDARS | VFPYSYFAMA | NGDIANISPF | YGLSPPEAAA | EPMGYPQDNF | 208 |
| Sa8 | 206 | CIVEEVEARS | VYPYDEFVLA | TGDFVYMSPF | YGYRDGSHGE | HTA.YAADRF | 255 |
| BHV2 | 294 | CIVDEVEAKS | SYPYNEFVLA | TGDFVYASPF | FGYRDGSHSE | HNA.YAADRF | 343 |
| HSV2 | 220 | CIVEEVDARS | VYPYDEFVLA | TGDFVYMSPF | YGYREGSHTE | HT.SYAADRF | 269 |
| HSV1 | 225 | CIVEEVDARS | VYPYDEFVLA | TGDFVYMSPF | YGYREGSHTE | HT.SYAADRF | 274 |
| ILTV | 185 | CVVEYLQARS | VYPYDYFGMA | TGDTVEISPF | YTKNTTGPRH | HSV.YRDYRF | 234 |
| EBV | 159 | CLITDMMAKS | NSPFDFFVTT | TGQTVEMSPF | YDGKNKETFH | ERADSFHVRT | 209 |
| HCMV | 203 | CMLTITTARS | KYPYHFFATS | TGDVVYISPF | YNGTNRNASY | FGENADK.FF | 252 |
| HVS | 201 | CEIVDMFARS | ADPYTYFVTA | LGDTVEVSPF | CDVDNSCPNA | TDVLSVQIDL | 251 |
| **gB con** | **298** | **CiveeveArS** | **vyPydsFal.** | **tGdivymSPF** | **yglr.gahre** | **ht.sya.drf** | **348** |

**B**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| FHV1 | 450 | MLQFAYDYIQ | AHVNEMLSRI | ATAWCTLQNR | EHVLWTETLK | LNPGGVVSMA | 500 |
| PRV | 532 | RLQFTYDHIQ | AHVNDMLGRI | AAAWCELQNK | DRTLWSEMSR | LNPSAVATAA | 582 |
| EHV1 | 643 | MLQFAYDHIQ | SHVNEMLSRI | ATAWCTLQNK | ERTLWNEMVK | INPSAIVSAT | 693 |
| EHV4 | 574 | MLQFAYDHIQ | SHVNEMLSRI | ATAWCTLQNK | ERTLWNEMVK | VNPSAIVSAT | 624 |
| BHV1 | 539 | ALQFTYDHIQ | DHVNTMFSRL | ATSWCLLQNK | ERALWAEAAK | LNPSAAASAA | 589 |
| VZV | 453 | MLQFTYDHIQ | EHVNEMLARI | SSSWCQLQNR | ERALWSGLFP | INPSALASTI | 503 |
| MDV | 462 | MLQFLYDHIQ | THINDMFSRI | ATAWCELQNR | ELVLWHEGIK | INPSATASAT | 512 |
| Sa8 | 480 | RLQFTYDHIQ | RHVNDMLGRI | AIAWCELQNR | ELTLWNEARR | LNPGAIASAT | 530 |
| BHV2 | 344 | RLQFTYNHIQ | KHVNEMFGRM | AVSWCELQNQ | ELTLWNEAKK | INPSAIASVT | 394 |
| HSV2 | 502 | RLQFTYNHIQ | RHVNDMLGRI | AVAWCELQNH | ELTLWNEARK | LNPNAIASAT | 552 |
| HSV1 | 505 | RLQFTYNHIQ | RHVNDMLGRV | AIAWCELQNH | ELTLWNEARK | LNPNAIASAT | 555 |
| ILTV | 467 | MLQFAYDKIQ | AHVNELIGNL | LEAWCELQNR | QLIVWHEMKK | LNPNSLMTSL | 517 |
| EBV | 460 | QIQFAYDSLR | RQINRMLGDL | ARAWCLEQKR | QNMVLRELTK | INPTTVMSSI | 510 |
| HCMV | 482 | QLQFTYDTLR | GYINRALAQI | AEAWCVDQRR | TLEVFKELSK | INPSAILSAI | 532 |
| HVS | 430 | QIQYAYDKLR | QSINNVLEEL | AITWCREQVR | QTMVWYEIAK | INPTSVMTAI | 480 |
| **gB con** | **601** | **mlQftYdhiq** | **rhvNemlgri** | **ataWCelQnr** | **eltlwneark** | **.NPsaiasat** | **651** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| FHV1 | 501 | LERRVSARLL | GDAVAVTQCV | NISSGHVYI. | QNSMRVTGSS | TTCYSRPLVS | 550 |
| PRV | 583 | LGQRVSARML | GDVMAISRCV | EVRGG.VYV. | QNSMRVPGER | GTCYSRPLVT | 631 |
| EHV1 | 694 | LDERVAARVL | GDVIAITHCA | KIE.GNVYL. | QNSMRSMDS. | NTCYSRPPVT | 741 |
| EHV4 | 625 | LDRRAAARML | GDAMAVTYCH | ELGEGRVFIE | .NSMRAPG.. | GVCYSRPPVS | 672 |
| VZV | 494 | LDQRVKARIL | GDVISVSNCP | ELGSDTRIIL | QNSMRVSGST | TRCYSRPLIS | 544 |
| MDV | 513 | LGRRVAAKML | GDVAAVSSCT | AIDAESVTL. | QNSMRVITST | NTCYSRPLVL | 562 |
| Sa8 | 531 | VGRRVSARML | GDVMAVSTCV | PVAPDNV.IM | QNSIGVAARP | GTCYSRPLVS | 580 |
| BHV2 | 395 | LHRRVSACML | GDVLAISTCV | AVPAENV.IM | QNSMRIPSKP | GTCYSRPLLS | 444 |
| HSV2 | 553 | VGRRVSARML | GDVMAVSTCV | PVAPDNV.IV | QNSMRVSSRP | GTCYSRPLVS | 602 |
| HSV1 | 556 | VGRRVSARML | GDVMAVSTCV | PVAADNV.IV | QNSMRISSRP | GACYSRPLVS | 605 |
| ILTV | 518 | FGQPVSARLL | GDIVAVSKCI | EIPIENIR.M | QDSMRMPGDP | TMCYTRPVLI | 567 |
| EBV | 511 | YGKAVAAKRL | GDVISVSQCV | PVNQATVT.L | RKSMRVPGSE | TMCYSRPLVS | 560 |
| HCMV | 533 | YNKPIAARFM | GDVLGLASCV | TINQTSVKVL | RD.MNVKESP | GRCYSRPVVI | 582 |
| HVS | 481 | YGKPVSRKAL | GDVISVTECI | NVDQSSV..S | IHKSLKTENN | DICYSRPPVT | 529 |
| **gB con** | **651** | **lgrrvsarml** | **GDv.avstCv** | **.va.dnvi.l** | **qnsmrvpgsp** | **gtCYsRPlvs** | **701** |

Figure 8

Figure 9. **Evolutionary tree compiled using 12 alphaherpesvirus gB amino acid sequences.** Individual gB polypeptides were aligned using the LINEUP and PILEUP programs (UWGCG). Evolutionary relatedness was analyzed using the Phylogeny Interference Package (PHYLIP).

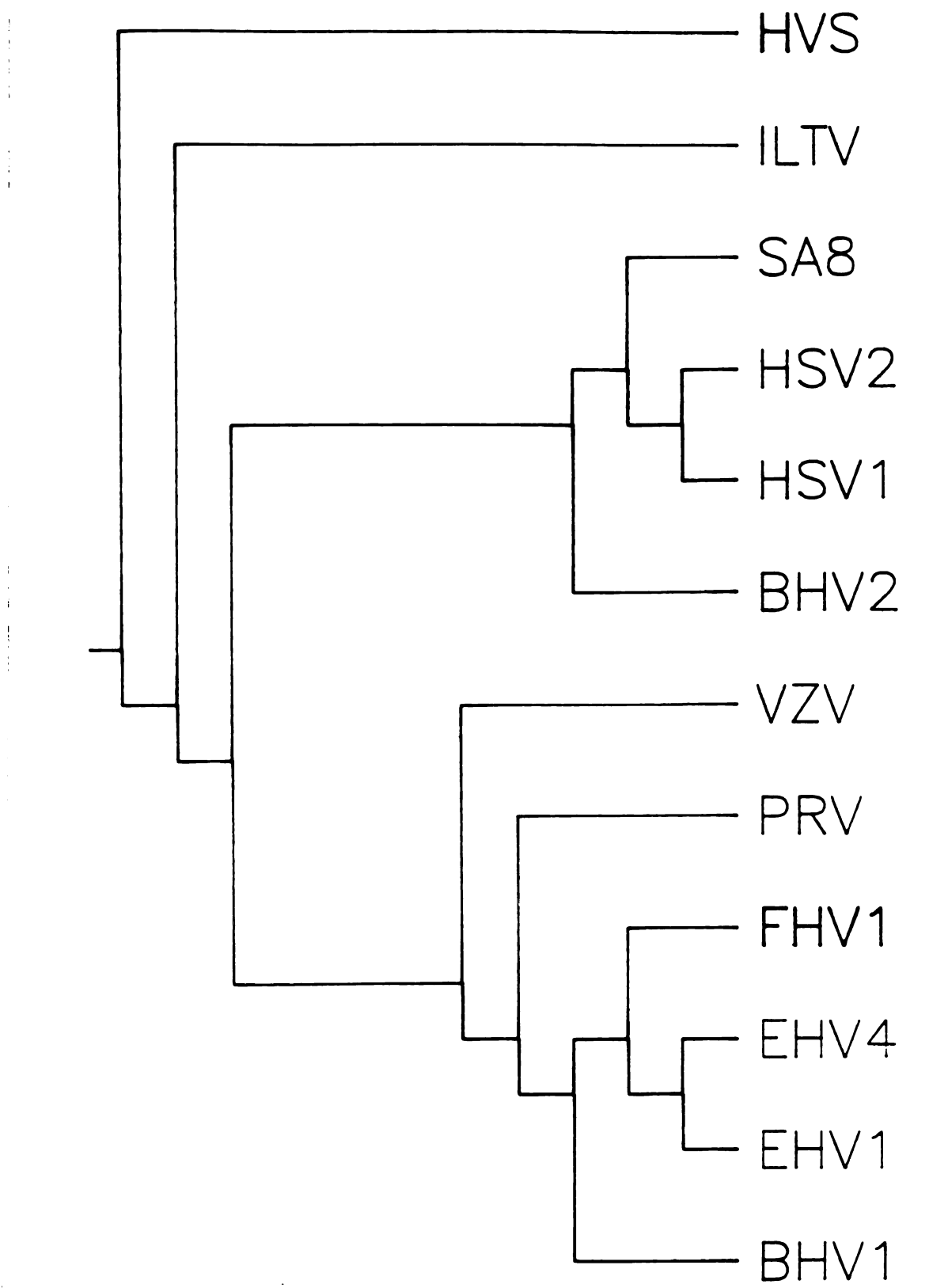



Figure 9

## DISCUSSION

The FHV-1 homolog of the HSV-1 gB gene was first identified using a 5' HSV-1 gB DNA probe in reduced-stringency hybridization experiments. The gene localized to a 3.3 Kb SacI subfragment of the larger SalI G fragment. Nucleotide sequencing of the SacI fragment has identified two open reading frames, one with characteristics typical of a glycoprotein gene. A unique characteristic of the FHV-1 gB polypeptide is the unusually long signal sequence of 73 residues. Although this is a deviation from the consensus length (16-29 amino acids) of a class I glycoprotein, it is not unprecedented for gB polypeptides. The constraints imposed by the gB gene and an overlapping gene (ICP18.5) (Pederson et al., 1991; Addison et al., 1990) has allowed for such a long signal sequence as evidence, in the genomes of PRV, BHV-1 and EHV-1. The genomes of these viruses contain overlapping genes with unusually lengthy signal sequences for the gB homologs. Using N-terminal sequencing, Wolfer et al., (1990) has reported the cleavage of gII of PRV occurs after residue 58. Likewise, cleavage of gB of EHV-1 is predicted to occur after residue 85 on the nascent polypeptide (Whalley et al., 1989).

Northern analyses have indicated that the 3.2 Kb transcript is most likely to represent the RNA encoding for gB for two reasons: the ORF of FHV-1 is 2.8 Kb and mRNA of gB homologs are generally 3.0 Kb. It is proposed that the larger 4.0 Kb transcript and the 3.3 Kb gB transcript share common 3'

termini, since no obvious poly(A) signal was evident downstream from FHV-1 ICP18.5 ORF. A similar transcription pattern for glycoprotein B and ICP18.5 has been reported for herpesvirus genomes containing a similar ICP18.5/gB allele (Bell et al., 1990). ICP18.5 has been reported to be a nuclear protein essential in capsid maturation, therefore it is logical that FHV-1 would contain a homolog (Pederson et al., 1991; Addison et al., 1990).

We compared 15 gB homologs of various herpesviruses and present a schematic diagram in Figure 7. Gaps were introduced to maximize amino acid similarities. The 10 cysteine residues, typical of glycoprotein B, can be perfectly aligned in all 15 homologs. Thirty eight additional residues can also be aligned. Although the number of potential glycosylation sites varies from 5 (PRV and BHV-1) to 19 (HCMV), the relative position of 4 sites (triangles in Figure 7) seems to be largely conserved. Since these positions also occur in areas of hydrophilicity, there is a high probability that these sites are used and important for glycan addition. Two major blocks of conserved amino acids are also present at positions 298-348 and 601-701 (Figure 7). Chou and Fasman predictions indicated that these stretches parallel each other. Recent studies involving epitope mapping of HSV-1 gB have indicated that amino acids surrounding Box 1, Figure 7 are involved in the rate of virion penetration and spread to adjacent cells (Highlander et al., 1988; Qadri et al., 1991: Navarro et al., 1992). Specifically, residues 241-441 on the HSV-1 gB

polypeptide are involved in this function. Amino acid residues 600-690 of HSV-1 gB (just right of Box 2, Figure 7) have been shown to be involved in dimerization. This region is highly antigenic and contains residues that specify 8 continuous epitopes which affect the conformation of 12 discontinuous epitopes (Qadri et al., 1991; Navarro et al., 1992). As is typical of a glycoprotein, two hydrophobic regions are evident: the signal peptide sequence at the amino terminus and the transmembrane sequence at the carboxyl terminus. When comparing the gB homologs of alphaherpesviruses, a high degree of amino acid similarity can be found in the transmembrane domain. The degree of similarity, within this area, decreases substantially, when gB homologs found in gamma and beta herpesviruses are included. Kyte and Doolittle analyses have indicated that gB homologs probably transverse the membrane three times.

With respect to evolutionary relatedness, a tree diagram is presented in Figure 9. Since glycoprotein B is the most conserved glycoprotein among herpesviruses, it is the prime candidate for such an analysis. From the evolutionary tree and the GAP analyses, FHV-1 diverged from a pseudorabies lineage and through a common ancestral virus, is related to EHV-1 and BHV-1.

Glycoprotein B homologs in VZV, EHV-1, EHV-4, PRV, and HCMV have been shown to be processed by an internal proteolytic cleavage at the sequence [RX(K/R)R'S]. Using the gene encoding glycoprotein gII of PRV, Whealy et al., (1990)

localized the cleavage recognition sequence to an 11 amino acid stretch containing the sequence PAAARRARRSP, with cleavage occurring between the arginine and the serine residues. This cleavage site was also determined by N-terminal sequencing the precursor and cleavage products of gII (PRV) (Wolfer, 1990). Two sequences, at positions 499 and 511 (RTRRS and RSRRS, respectively) are present in gB of FHV-1. Similar sequences have been found in VZV (RSRRS, 427), EHV-1 (RRRRS, 517), EHV-4 (RTRRS, 512), PRV (RARRS, 499), and HCMV (RTRRS, 455). This recognition signal is generally located in the middle of gB and is absent in the gB-equivalent sequences of MDV, HSV-1, HSV-2, EBV, and HSV. The latter four herpesviruses have the uncleaved gene product in the envelope, while the gB homolog of Marek's Disease herpesvirus appears to be cleaved into two peptides (62 and 47 Kd). Recently, Misra and Blewett (1990) using pseudodiploid recombinants of gB of BHV-1 and HSV-1 reported that cleavage and oligomerization is not necessary for virion production. On the other hand, Brucher, et al., (1990) demonstrated that cleavage of gB of HCMV was inhibited by palitoylated peptidly-chloromethyl ketone and release of infectious virus from human fibroblasts was impaired, although production of intracellular infectious viral progeny was unaffected.

It is interesting to speculate the FHV-1 gB, like gII of PRV and VZV, could exist as a disulfide linked dimer resulting from proteolytic cleavage at either recognition site. Based on immunoprecipitation and western blot analyses it is possible

to speculate that the 120 Kd protein is the glycosylated form, which subsequently gets trimmed to the 100 Kd form. The 64 and 58 Kd proteins are likely cleavage products of the 100 Kd trimmed precursor. The proteolytic cleavage of the 100 Kd precursor is probably incomplete, since both the uncleaved (100 Kd) and the cleaved (64 and 58 Kd) forms were detected when virion lysates were used in the immunoprecipitation and western blot analyses. Incomplete cleavage patterns have also been noted for other cleaved-gB homologs using pulse-chase experiments (Whealy et al., 1990). The 56 Kd protein immunoprecipitated from FHV-1 infected cellular lysates, appears to be a viral or cellular protein induced upon infection. Similarly, a protein of 44 Kd was detected in MDV (Marek's disease virus) infected cellular lysates when immunoprecipitated with antisera specific for MDV glycoprotein B (Chen and Velicer, 1992).

Based on the assumption that gB of FHV-1 contains 943 residues, cleavage of the signal peptide occurs after amino acid 69 and that 7 potential glycosylation sites are used, it is possible to calculate a MW of 114.5 Kd. If proteolytic cleavage occurs at amino acid 502, then two peptides with MW's of 60.5 and 54.0 Kd could result. If cleavage occurs at amino acid 513 then the two resulting peptides would have the MW's of 61.9 and 52.6 Kd. These numbers are in good agreement with the observed Mr values obtained by the immunoprecipitation and western blotting experiments.

Interestingly, a similar protein profile was achieved by

Horimoto et al., (1990) during attempts to isolate the hemagglutinin of FHV-1. Using Con-A chromatography on detergent-soluble protein lysates from FHV-1 infected fcwf-4 cells, they found three proteins, which showed HA activity and had MW's of 59Kd, 65Kd and 105Kd were detected using SDS-PAGE analysis of protein purified by Con A-chromatography. Two major HA-proteins (59Kd and 105 Kd), purified by ion-exchange chromatography were also visualized using silver staining. Using the third chromatographic technique, gel-exclusion chromatography, two fractions showed HA activity with MW's estimated to be approximately 110-130 Kd and 68 Kd. Although it is not known whether gB is the hemagglutinin, the similarities in the protein profiles are most striking.

Based upon these results and observations, it is now possible to express this glycoprotein, to assess its possible role as a hemagglutinin and to define its role in the induction of humoral and cell-mediated immunity in cats, the natural host of FHV-1.

Chapter 3

# Sequence Analysis of the Unique Short Region of Feline Herpesvirus-1: Identification of the Genes Encoding Glycoproteins G, D, I and E

Stephen J. Spatz

**ABSTRACT**

Feline herpesvirus-1, a common viral pathogen of cats, has been reported to contain a group D genome. Restriction mapping studies have indicated that the size of the $U_S$ region is approximately 8.0 Kb. We now report the nucleotide sequence of a 6.2 Kb portion of this region. Analyses of this sequence has identified 5 open reading frames capable of encoding homologs to HSV-1 Protein kinase and glycoproteins gG, gD, gI and gE. Hydropathic analysis has shown that FHV-1 glycoprotein G, D, I and E exhibit features typical of a membrane-bound glycoprotein: a hydrophobic signal sequence at the N-terminus, potential N-linked glycosylation sites and a hydrophobic transmembrane domain near the C-terminus. Homologs to these glycoproteins have been found in a number of other alphaherpesviruses and at the amino acid level the $U_S$ gene products of FHV-1 are most similar to those of EHV-1. The exception is glycoprotein D, which shows more homology with gD of BHV-1. Although glycoprotein G of FHV-1 has features displayed by membrane proteins, it maybe a secreted protein. Glycoprotein G homologs of the varicelloviruses (EHV-4 and PRV) have been reported to be secreted and extensive homology exist between this secretory glycoprotein and gG of FHV-1. Surprisingly, homology between the individual polypeptides of gG, gD and gI can be demonstrated which may indicate that these genes evolved as a result of duplication and divergence of an ancestral gene family. Northern analyses of the unique short genes of FHV-1 point to the likelihood of numerous

co-terminal transcripts. One gene cluster (3.5 and 1.8 Kb) consists of the PK/gG genes, another cluster is the gD/gI, while gE appears to be encoded in a monocistronic 2.5 Kb transcript.

## INTRODUCTION

Feline herpesvirus-1, an alphaherpesvirus is a predominant cause of upper respiratory disease in cats. As is typical of other herpesviruses, numerous FHV-1 glycoproteins are synthesized and incorporated into cell membranes of infected cells and in the virion envelope. Functionally, these glycoproteins have been shown to be involved in membrane attachment, penetration of the virion into cells via cell-to-cell spread, complement binding, virus neutralization and immune destruction of infected cells (Courtney, 1991).

Immunological and biochemical studies of the polypeptides of FHV-1 have shown the presence of at least 7 glycoproteins. In studies involving $^{14}C$- and $^{3}H$-glucosamine, Maes et al., (1984) and Compton et al., (1989) have identified a group of closely migrating glycoproteins with molecular weight ranging from 103-107 kd. Three additional glycoproteins (85, 68 and 59 Kd) were also identified, while two glycoproteins (107 and 75 Kd) were detected in the culture medium harvested from FHV-1 infected cells. Similar protein profiles have also been observed by Fargeaud et al., (1984) and Limcumpao et al., (1990). In addition, Horimoto and coworkers (1990) have identified a 60 Kd protein that elicits virus neutralizing antibodies and is capable of hemagglutination. Recently, we have identified the gene encoding FHV-1 glycoprotein B and have characterized its gene product. Immunoblot and immunoprecipitation data have indicated FHV-1 virions contain a

cleaved gB polypeptide with the MW's of 105, 64, and 58 Kd.

In order to expand the genetic characterization of FHV-1 glycoprotein genes, we sought to identify the genes encoded in the unique short region of the FHV-1 genome. Based on the complete nucleic acid sequences of the genomes of HSV-1, VZV and EHV-1, a glycoprotein gene cluster in the $U_S$ region appears to be conserved throughout the subfamily of Alphaherpesvirinae (McGeoch et al., 1985; Davison, 1984; Telford et al., 1992; Elton et al., 1991; Flowers et al., 1991; Audonnet et al., 1990). In addition, partial DNA sequencing of the $U_S$ region of PRV, MDV and BHV-1 have revealed minor differences in the genetic organization of the $U_S$ gene cluster (Petrovskis et al., 1986; Ross and Binns, 1991). These variations range from the lack of a gD homolog in VZV to the presence of additional glycoprotein genes in MDV and EHV-1. The majority of these $U_S$ glycoprotein genes have been reported to be dispensable for replication of the virus in cell culture (Mettenleiter et al., 1990). In the case of HSV-1, 11 of the 12 $U_S$ genes can in fact be deleted, (Longnecker, 1987). Glycoprotein D is the only $U_S$ glycoprotein essential for virion production. Through continuous passage in tissue culture, many of the $U_S$ genes of animal herpesviruses have been naturally deleted, resulting in reduced virulent strains (Kimman et al., 1992; Petrovskis et al., 1986).

In this communication, we report the nucleotide sequence of a 6.2 Kb fragment from the unique short region of the FHV-1 genome and the identification of 5 major open reading frames.

Four of these ORF's display homology to HSV-1 gG, gD, gI and gE and partial sequencing analysis of the fifth ORF reveals significant homology to the COOH- terminus of the HSV-1 $U_S$ protein kinase.

## MATERIAL AND METHODS

### Viral and bacterial strains

FHV-1 (strain C-27) was obtained from the American Type Culture Collection and propagated in Crandell Reese Feline Kidney (CRFK) cells as described previously (Maes et al., (1984). Cellular lysates from FHV-1-infected cells were used as the source for viral DNA. *Escherichia coli* strain JM 101 and JM101 were grown in LB medium and used to propagate recombinant M13 mp18 and mp19 clones.

### Cloning and DNA Sequencing

The complete nucleotide sequence of a 6,208 bp portion of the $U_S$ region was determined (Figure 1). The 4.3 Kb EcoRI-EcoRI fragment and the adjacent 1.9 Kb EcoRI-SalI fragment located at the right terminus of the SalI B fragment were chosen for DNA sequence analyses. Hybridization analysis have indicated that these two restriction fragments solely contain unique short region DNA.

In order to rapidly generate sequencing data, 4 individual M13 libraries were created using HAEIII, RSAI, TAQI and SAU3A restriction digestions of the 4.3 Kb EcoRI-EcoRI and 1.9 Kb EcoRI-SalI fragments. Single stranded DNA from recombinant M13 phage was isolated according to Ausubel et al., (1988) and sequenced using standard dideoxynucleotide chain termination reactions with the modified T7 polymerase, Sequenase (US Biochemical). $^{35}$S-dATP (NEN) was used as the

label and dITP was used to resolve band compressions. Synthetic oligonucleotides, along with the Universal and 17'mer M13 primers were used to obtain sequencing information from both strands. Reaction products were electrophoretically separated and visualized by autoradiography of dried 8% acrylamide/7.0 M urea gels on Kodak X-AR film.

**Analyses of Sequence Data**

DNA sequences were compiled on a VAX computer using versions 6.2 and 7.0 of the University of Wisconsin GCG package (Devereux et al., 1984). Computer management of the sequences verified that both strands of the 6.2 Kb fragments were sequenced. Hydrophilicity analyses of individual predicted translation products were generated by the method of Kyte and Doolittle (1982). Amino acid homology searches of the Swissprot (Release 18.0, 5/91) data bases were conducted using the FASTA program (UWGCG). The GAP, LINEUP, PILEUP programs were used to generate multiple alignments between FHV-1 $U_S$ predicted polypeptides and homologs found in related herpesviruses.

**Northern Analysis of FHV-1 $U_S$ Transcripts**

Crandell-Reese feline kidney cells were infected with plaque-purified FHV-1 using a m.o.i. of >1.0. At 12 hours post infection, infected cells were harvested and RNA isolated using the guanidium thiocyanate-CsCl method (Ausubel et al., 1988). Gradient-purified RNA was denatured in formamide and

formaldehyde and electrophoresed in formaldehyde-agarose gels. Separated RNA was passively transferred to nytran and hybridized to radiolabeled plasmid probes (Figure 1). RNA was also isolated from thymidine kinase producing mouse L cells and subjected to a similar analysis. Northern bolts were visualized using a Betagen betascope and standard autoradiography.

## RESULTS

### Restriction map of the Unique Short Region of the FHV-1 Genome

As shown in Figure 1, the 14.5 Kb SalI B fragment contains 3 EcoRI restriction sites. The complete restriction maps of the 4.3 EcoRI-EcoRI and the 1.9 Kb EcoRI-SalI sub-fragments were generated using the results from digestions with the restriction endonucleases EcoRI, NdeI, BamHI, XbaI and EcoRV.

### DNA Sequence Analysis of a 6.2 Kb portion of FHV-1 $U_S$ DNA

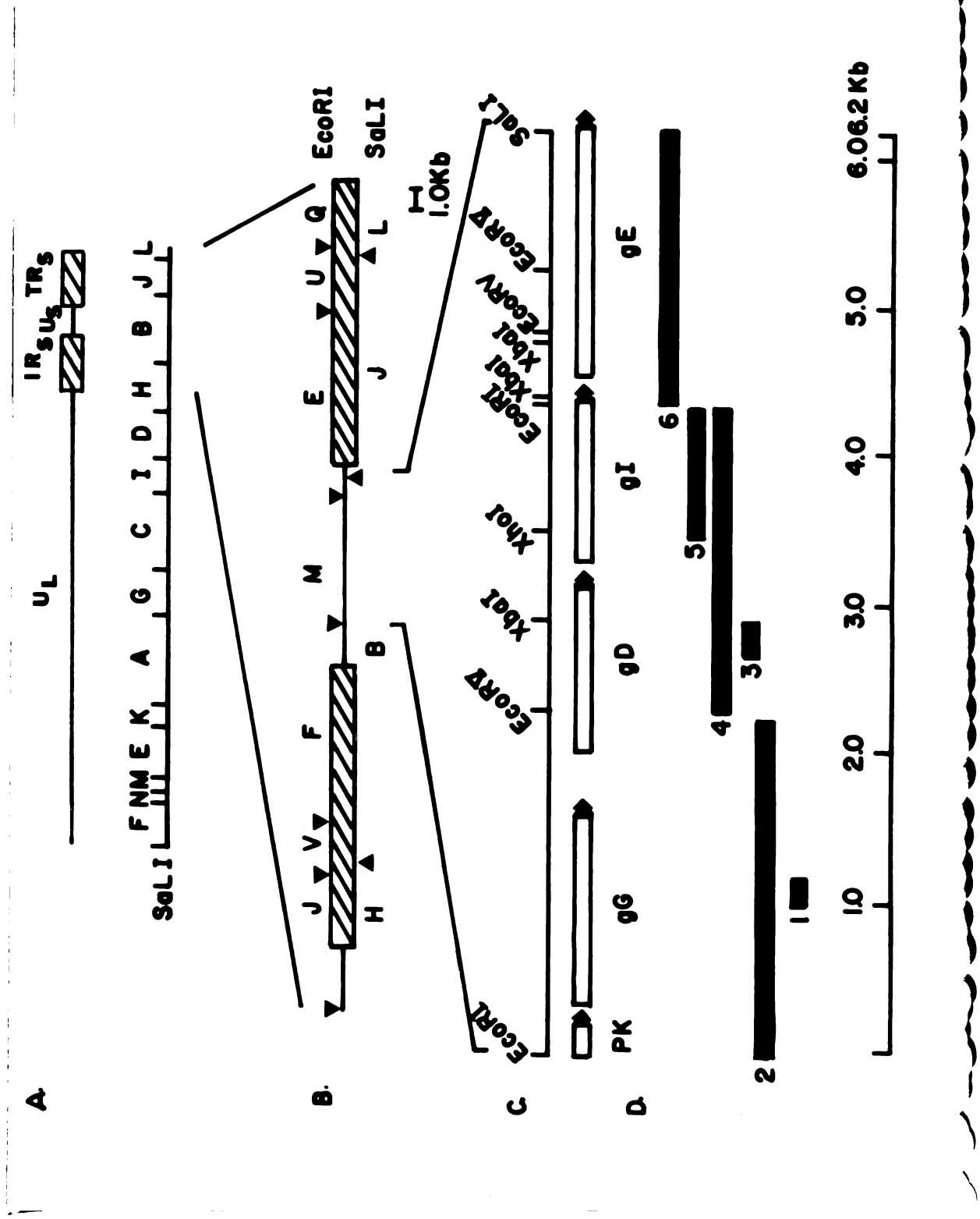
Sequence data obtained from the 6.2 Kb region of the SalI B fragment are presented in Figure 2. Examination of the nucleotide sequence revealed the presence of 5 major open reading frames (ORF's) and 3 minor ORF's. Although these minor ORF's are >80 and <100 amino acids and contain the appropriate cis-acting transcription regulatory sequences, they shown no homology to peptides found in the data base of Swissprot.

### Analysis of the Major ORF's

(a) Protein Kinase

The first reading frame extending from the EcoRI site at position 1 to position 211 encodes the last 69 amino acid residues of a suspected protein kinase. A search for amino acid similarities using FASTA and the Swissprot database have shown that this ORF contains the sequence RPSA, a sequence found in all known $U_S$ protein kinases. FASTA scores were

Figure 1. **Genomic organization of the FHV-1 unique short genes encoding a putative protein kinase and glycoproteins gG, gD, gI, and gE.** (A) The 134 Kb genome is represented as two unique sequences ($U_L$ and $U_S$) and two inverted repeat region ($I_R$ and $T_R$) flanking the unique short region. (B) The SalI and EcoRI restriction maps of 13 Kb of FHV-1 DNA including the $U_S$ and inverted repeats. (C) A detailed restriction map of the unique short region is presented along with the position and transcriptional direction of the genes encoding the putative PK , gG, gD, gI and gE. (D) The black boxes (1-6) represent the hybridization probes used to map the $U_S$ transcripts.


A.
B.
C
D.
$U_L$
$IR_S$ $U_S$ $TR_S$
SaLI
F N M E K A G C I D H B J L
SaLI
EcoRI
SaLI
I
1.0Kb
Q U L
E
J
M
F
B
V J
H
Sall
EcoRV
EcoRV
XbaI
XbaI
EcoRI
XhoI
XbaI
EcoRV
EcoRI
PK
gG
gD
gI
gE
1
2
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1.0
2.0
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4.0
5.0
6.0 6.2 Kb

Figure 1

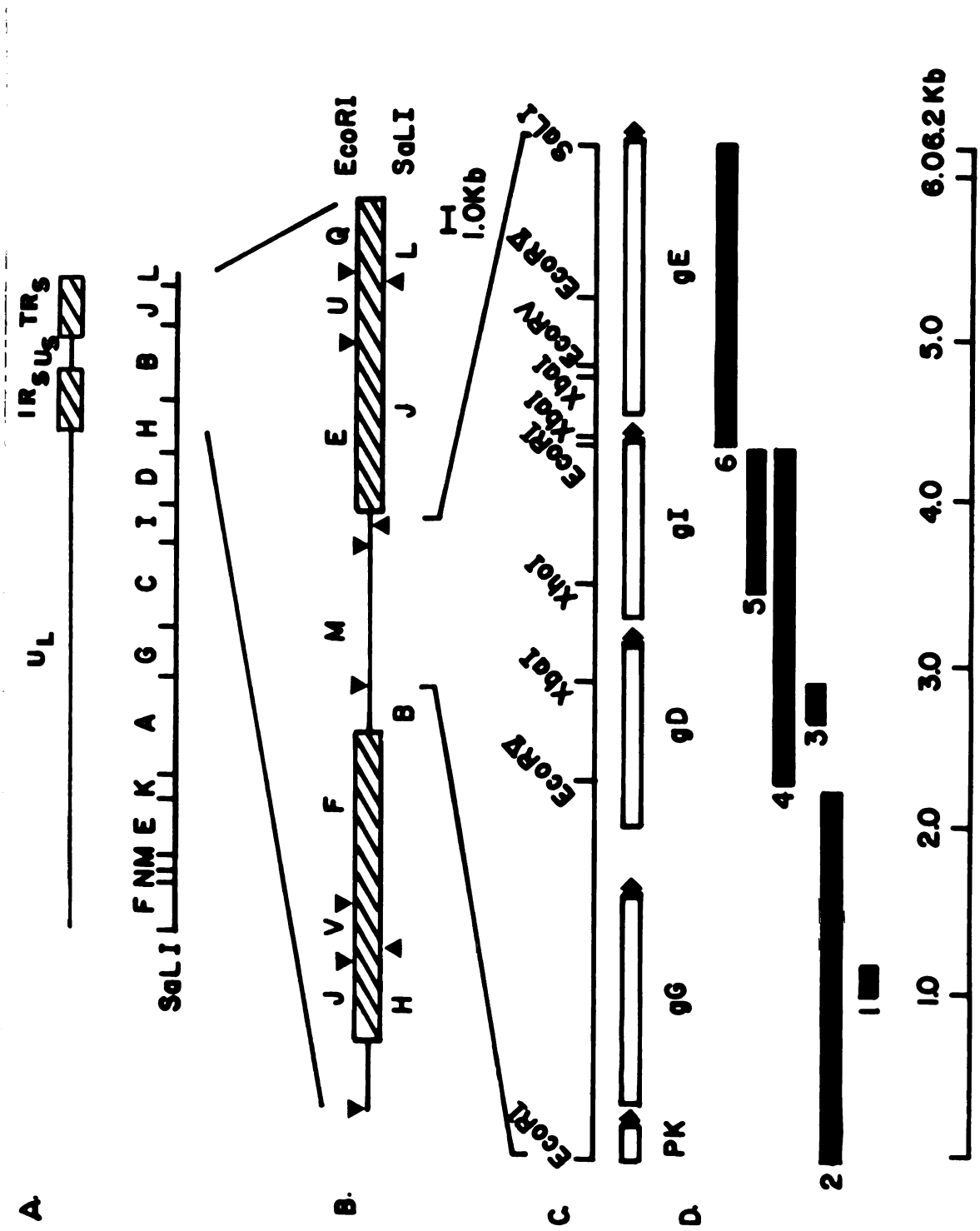

A
B
C
D
$U_L$
$IR_S$
$U_S$
$TR_S$
SalI
EcoRI
1.0Kb
PK
gG
gD
gI
gE
6.0
6.2Kb
5.0
4.0
3.0
2.0
1.0

Figure 1

Fi

se

pa

ac

sh

br

GT

Figure 2. **Nucleotide sequence and predicted amino acid sequences of the FHV-1 polypeptides, gG, gD, gI and gE and part of the putative threonine/serine protein kinase.** Cis-acting sites (CAAT, TATA) boxes and polyadenylation sites are shown in bold. Potential N-linked glycosylation sites are bracketed by two lines. Direct repeats of the sequence GGG GCT GTG GGG ACG A are indicated with a partitionary line.

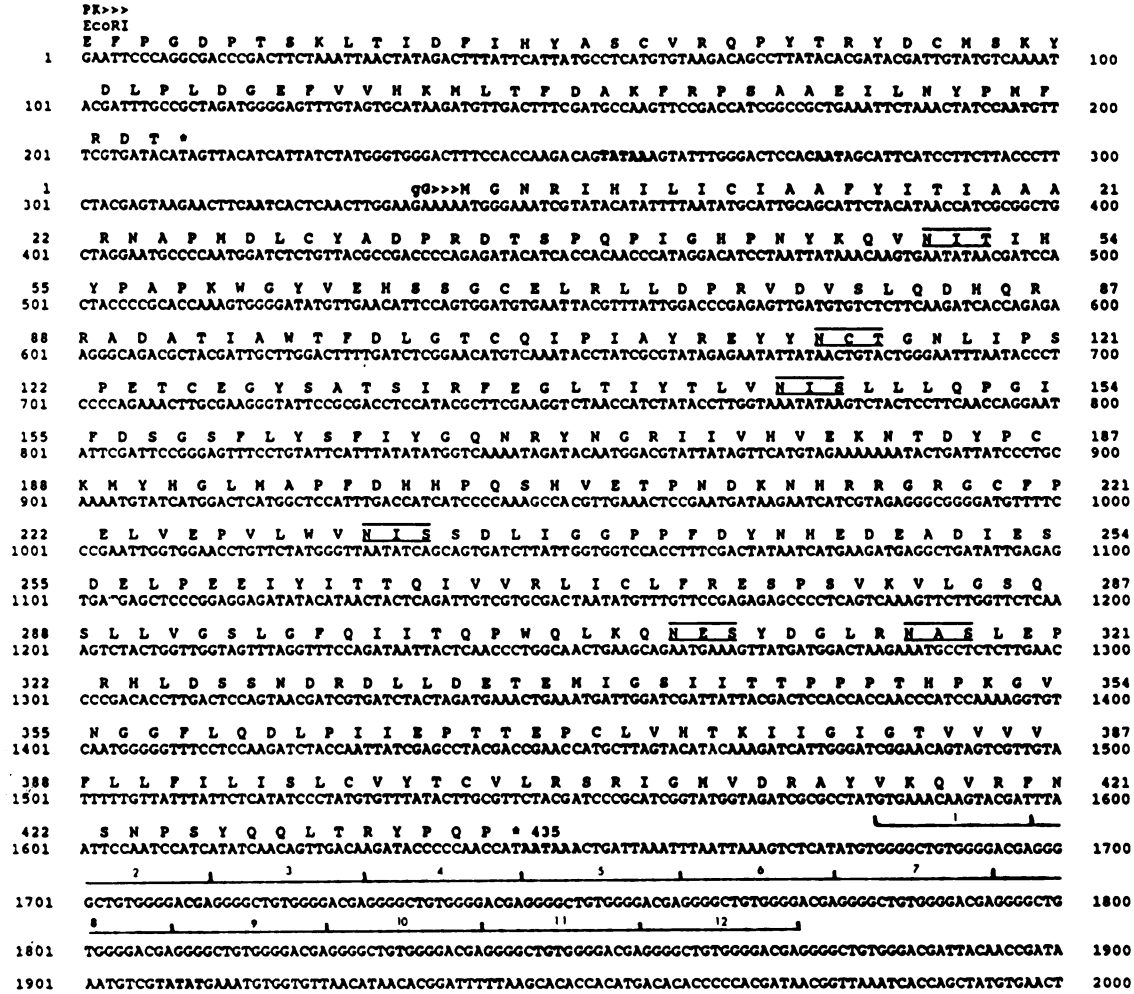



Figure 2

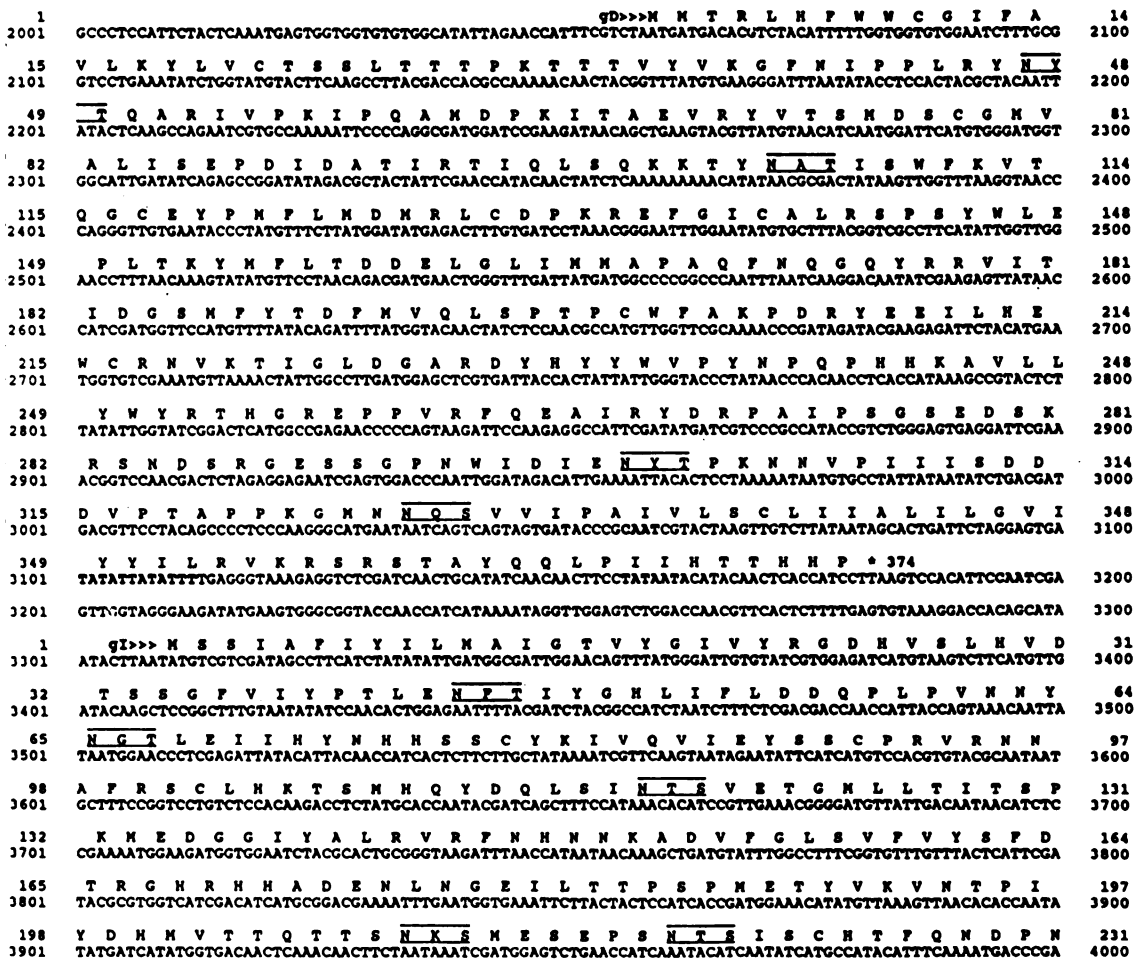


Figure 2 (Cont)

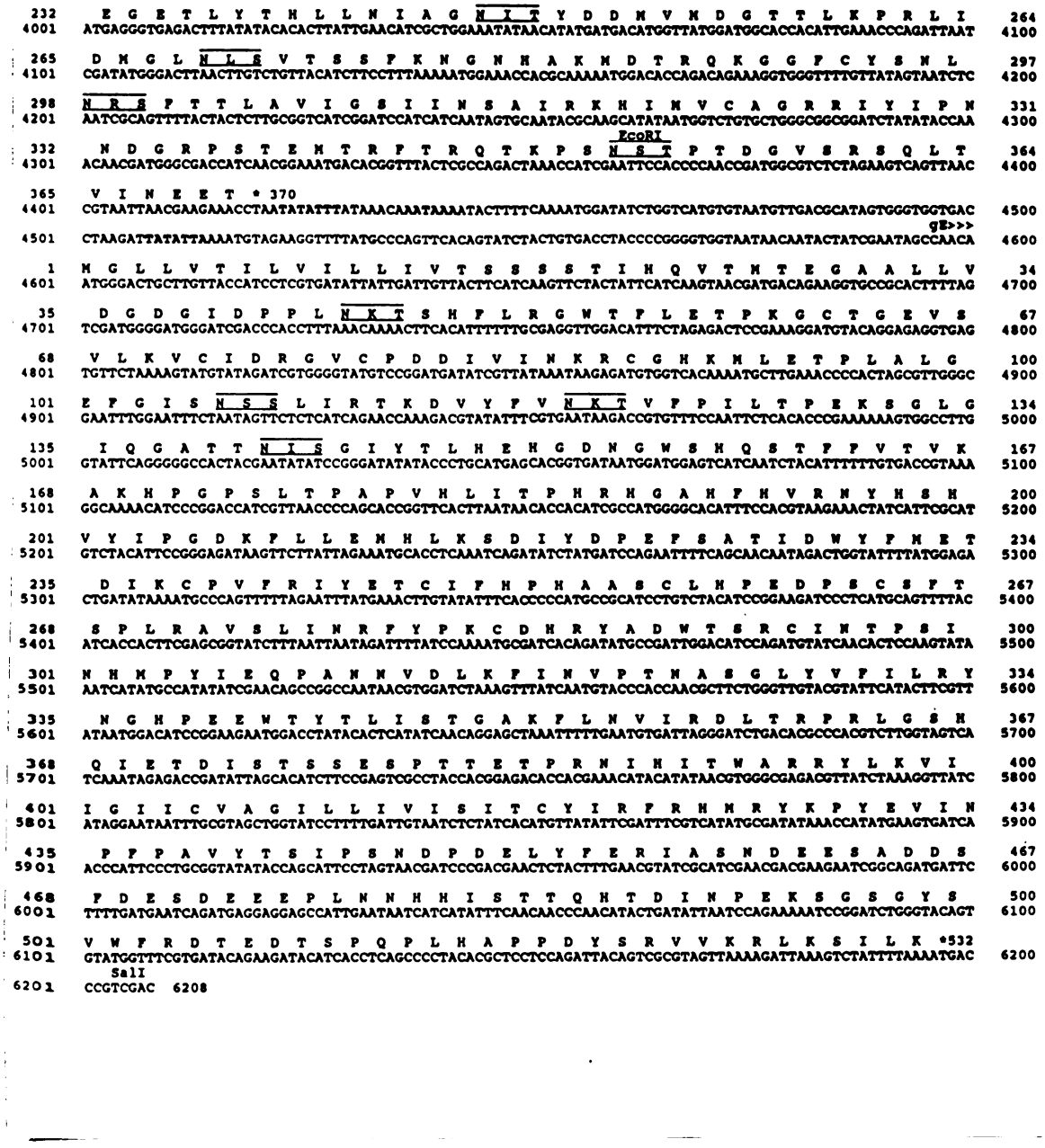



Figure 2 (Cont)

greater than 100 when compared to the protein kinases of PRV, HSV-1, HSV-1 and VZV. Other stretches of amino acid similarities could also be demonstrated. No evidence for a polyadenylation site was found downstream of the termination codon, TAG. It is estimated that the gene encoding FHV-1 PK is at least 1.2 Kb.

(b) Glycoprotein G

The deduced amino acid sequence of ORF 2 consists of 435 amino acids from nucleotide positions 340-1645. This ORF has several features in common with glycoprotein G of HSV-1 and gX of PRV. Two possible initiation codons (AAAATGG and CCAATGG) were located at positions 340-415. However, only the initiation codon at 340 is in favored by Kozak's rules (purine residue in the -3 position). No major cis-acting transcription sites (TATA-like elements) were found 5' to the gene, although 3 CAAT boxes were apparent. A polyadenylation signal AATAAA was found 3' to the stop codon TAA. Hydrophilicity analyses of the 435 amino acid polypeptide have identified two hydrophobic sequences at both termini of the polypeptide. Six potential N-linked glycosylation sites were also predicted from the deduced amino acid sequence which has a calculated MW of 57 Kd. This is assuming that cleavage of the signal peptide occurs between $Ala_{21}$ and $Arg_{22}$.

(c) Glycoprotein D

Open reading frame 3, capable of encoding a polypeptide of 374 amino acid residues, extends from nucleotide positions 2062-3180. Two potential initiation codons (CTAATGA and ATGATGA), which are adjacent to each other, can be used a start codons, although the latter sequence has the critical purine at position -3. If this second initiation codon is used, then the expected polypeptide would be 373 amino acids long. A TATA-like element at position 1908 to 1912 is the only potential cis-acting promoter element. A stop codon TAA at positions 3181-3183 is present, in the absence of any downstream polyadenylation signal. Hydropathy plots have also indicated the presence of two hydrophobic sequences close to the N- and C-termini. The first region FWWCGIFAVL (position 2077-2104) corresponds to the signal sequence and the second region VVIPAIVLSCLIIALILGVI near the C-terminus could function as a membrane anchoring sequence. Four potential N-linked glycosylation sites are possible in the predicted translation product of ORF 3. An MW of 46 Kd can be calculated, assuming cleavage of the nascent polypeptide occurs between $Ala_{13}$ and $Val_{14}$

Comparison of the amino acid sequence of ORF 3 with proteins in the Swissprot data base has revealed extensive homology with gD analogs of other alphaherpesviruses. FASTA scores greater than 400 were achieved when these analyses included gD of BHV-1, PRV and EHV-1.

(d) glycoprotein I

A 370 amino acid residue predicted protein product of the ORF 4 shares common features of glycoproteins. In fact, amino acid similarity studies have indicated that this ORF encodes a protein with extensive homology to gI of HSV-1 and to gp63 of PRV. An initiation codon (AATATGT) at position 3307 - 3312 is favored by Kozak's rules. Three potential TATA-like elements at positions 3101-3112 and 3156-3162 exist with a CAAT box at position 3056. A poly (A) site (AATAAA) at position 4437-4442 is located downstream from the termination codon TAA at position 4420. As is typical of an anchored membrane protein, two hydrophobic amino acid stretches were apparent from analyses of the translation product. These stretches are likely to encode the signal sequence and the transmembrane domains. Also, 9 potential N-glycosylation sites can be determined yielding a calculated MW of 57.7 Kd, assuming signal sequence cleavage occurs after $Gly_{18}$.

(e) glycoprotein E

The fifth ORF (ORF 5) extends from nucleotide position 4601-6200 and encodes a polypeptide exhibiting similarities to gE of HSV-1 and g1 of PRV. Two initiation codons, (ACAATGG) at position 4598 and (ACGATGA) at position 4673, were predicted. The former is favored by Kozak's rules. Putative transcription regulatory signals were found 5' of the initiation codon at position 4423 and 4508. A termination codon is located at position 6197-6199, three base pairs 5' of the SalI site.

Since this is the limit of the sequencing analysis, no information about polyadenylation of the gE transcript is available. ORF 5 encodes a protein of 532 amino acids, contains 4 potential N-linked glycosylation sites and if cleavage of the signal sequence occurs between $Ser_{16}$ and $Ser_{17}$ then a MW of 61.6 Kd can be calculated.

**Comparison of the FHV-1 $U_S$ glycoproteins: gG, gD, gI and gE.**

Similarities at the amino acid level between the individual $U_S$ glycoproteins of FHV-1 and those homologous polypeptides of related herpesviruses, investigated using the UWCGC program GAP are given in Table 1. Overall, extensive homology could be demonstrated between predicted translation products of the genes encoding gG, gD, gI and gE of FHV-1 and those of the related varicelloviruses, equine herpesvirus type 1 and pseudorabies (Figure 3).

**Analysis of the transcripts encoding PK, gG, gD, gI. gE of FHV-1**

Northern analyses, using probes specific for each of the genes encoding gG, gD, gI and gE, has added additional support for co-terminal $U_S$ transcripts. As shown in Figure 3, only three transcripts could be detected with probes spanning the entire 6.2 Kb region. One transcript (2.5 Kb), thought to encode gE, was localized to the 1.7 Kb EcoRI-SalI fragment at the right terminus of the 14.5 Kb SalI B fragment. Two transcripts (3.5 and 1.8 Kb) were detected using probes specific

for the genes encoding gG, gD and gI. The three 1.8 Kb transcripts are thought to encode monocistronic transcripts specifying gG, gD and gI. It is not known if the 1.8 Kb transcript, detected with probes specific for gD, is polyadenylated. Northern analysis of the $U_S$ region of FHV-1 has been difficult to interpret due to the similar sizes of the transcripts and suspected bicistronic nature of the transcripts.

Table 1. **GAP analyses of putative glycoproteins whose genes map within the $U_S$ region of the FHV-1 genome.** Values are represented as the percentage similarities/percentage identities.

**HOMOLOGY COMPARISON OF THE US GLYCOPROTEINS OF FELINE HERPESVIRUS -1**

| | gG | gD | gI | gE |
|---|---|---|---|---|
| EHV-1 | 57/36 | 49/28 | 56/40 | 65/47 |
| EHV-4 | 59/36 | N/A | N/A | N/A |
| PRV | 56/33 | 50/29 | 49/29 | 53/30 |
| BHV-1 | N/A | 54/33 | N/A | N/A |
| VZV | D/C | D/C | 51/30 | 49/28 |
| HSV-2 | 40/21 | 47/25 | 40/24 | 43/22 |
| HSV-1 | 42/18 | 47/25 | 43/26 | 47/24 |
| MDV | D/C | 47/24 | 47/24 | 43/22 |

N/A = NOT AVAILABLE

D/C = DOESN'T CONTAIN THIS GLYCOPROTEIN

Table 1

Figure 3. **Northern blot analyses of RNA with probes representative of the $U_S$ glycoprotein genes.** Total cytoplasmic RNA isolated from FHV-1 infected cells was separated in agarose/formaldehyde as described in methods. Strips blots were hybridized with radiolabeled restriction fragments as depicted in Figure 1,D. Blots 1-6 were probed with the following restriction fragments: Blot 1, 0.16 Kb TaqI-TaqI (gG-specific), Blot 2, 2.3 Kb EcoRI-EcoRV (PK/gG/gD-specific), Blot 3, 0.42 Kb RsaI-RsaI (gD-specific), Blot 4, 2.0 Kb EcoRV-EcoRI (gD/gI-specific), Blot 5, 0.85 Kb XhoI-EcoRI (gI-specific) and Blot 6, 1.8 Kb EcoRI-SalI (gI/gE specific).

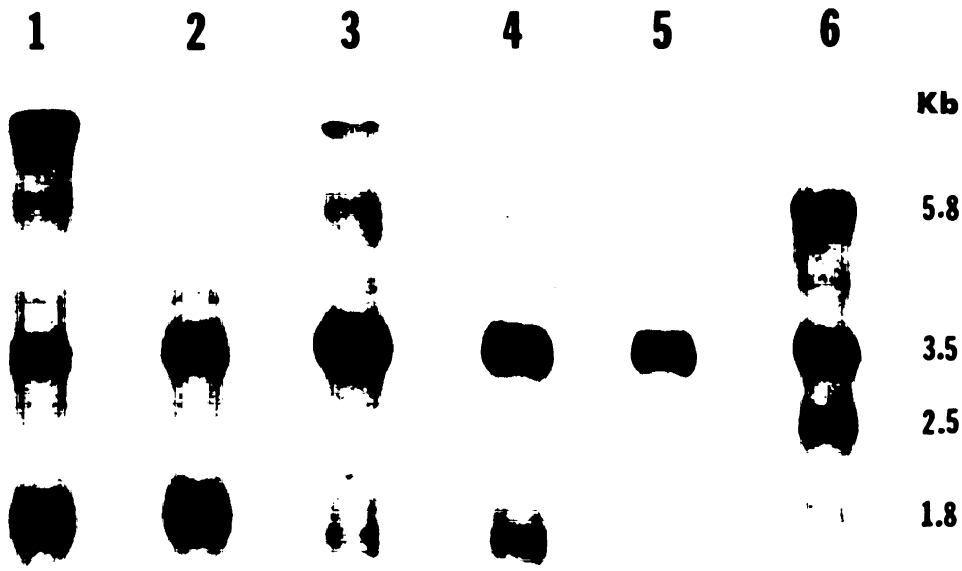



Figure 3

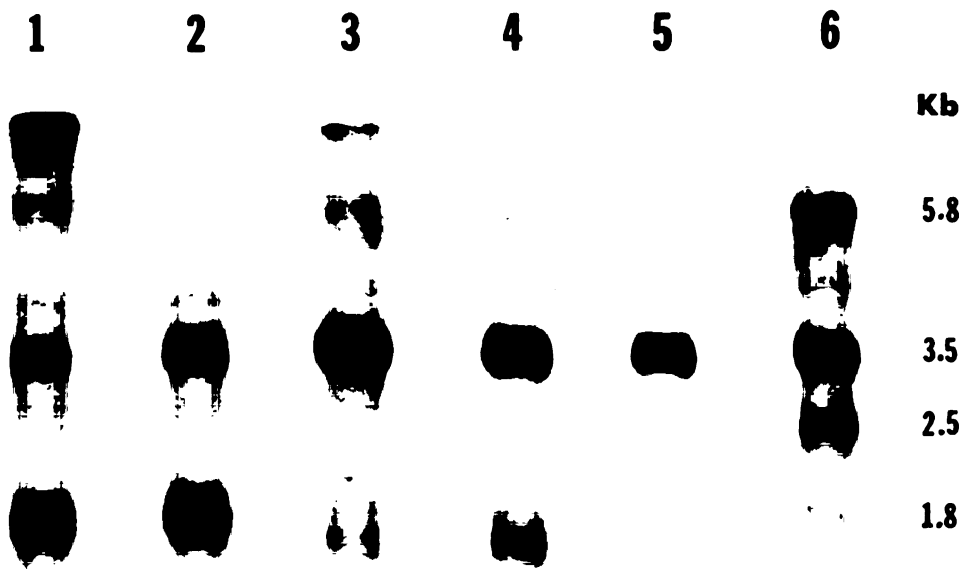



Figure 3

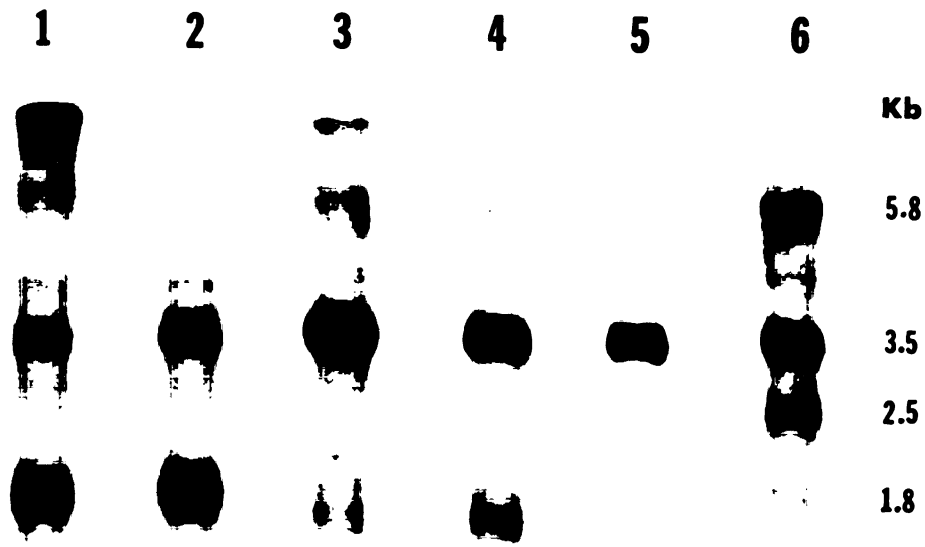



Figure 3

Figure 4 (Parts A-D). **Multiple alignments of $U_S$ glycoproteins of alphaherpesviruses.** Conserved residues are shown in bold. Amino acid residues conserved throughout (*) and semiconserved residues (.) are illustrated at the bottom of the alignment.

```
            1                                                                                                   100
Hsv1gg  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
Hsv2gg  MHAIAPRLLL  LFVLSGLPGT  RGGSGVPGPI  MPPNSDVVFP  GGSPVAQYCY  AYPRLDDPGP  LGSADAGRQD  LPRRVVRHEP  LGRSFLTGGL  VLLAPPVRGF
Ehv4gg  ..........  ..........  ..MLAVGATL  CLLSFLTGAT  GRLAPDDLCY  AEPRKTGPWP  RSKPWMQPL.  ...LFEAPKV  ALTAESKGCQ  LILDLPPTDM
Ehv1gg  ..........  ..........  ..MLTVLAAL  SLLSLITSAT  GRLAPDELCT  AEPRRTGSPP  NTQPERPPV.  ...IFEPPTI  AIKAESKGCE  LILDPPIDV
Fhv1gg  ..........  .....MGNR  IHILICIAAF  ....YITIAA  ARHAPMDLCY  ADPRDTSPGP  IGHPNYKQVM  ITIHYPAFKW  GVVEHSSGCE  LRLLDPRVDV
 Prvgx  ..........  ..........  MKWATWILAL  GLLVVETRV.  AREAPRELCT  GHPVNDDSRP  VGPATDAGPV  MP..LAPARA  TGTDYSRGCE  MRLLDPPLDV
                                                  ..          ..**. *.    *                  . *.    *..*

            101                                                                                                 200
Hsv1gg  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
Hsv2gg  G..APWATYA  ARVTYVRLTR  ACRQPILLKQ  YGGCRGGEPP  SPRTC.GSTY  YTYQGGGPPT  RYALVRAAEL  VPIWDRAAET  FEYQIELGCE  LHVELLMVTV
Ehv4gg  GYR.LEDKIN  ASIAWFFDPG  NCRMPIAYRE  YYDCVGNAIP  SPETC.DGTD  FTLVKTEGVV  EPTIVMNSLL  LQPGIYDSGS  FIYSALLDMD  VLTGRVILMV
Ehv1gg  SYR.REDKVN  ASIAWFFDPG  ACRMPIAYRE  YYGCIGNAVP  SPETC.DAYS  FTLIRTEGIV  EPTIVMNSLL  FQPGIYDSGH  FIYSVLLDVH  IFTGRVILMV
Fhv1gg  SLQDHQRRAD  ATIAWTFDLC  TCQIPIAYRE  YYNCTGNLIP  SPETC.EGYS  ATSIRFEGLT  IYTLVWIELL  LQPGIYDSGS  FLYSFIYGQN  RYNGRIIVMV
 Prvgx  SSRS.SDPVW  VTVAWFFDGG  HCKVPLVHRE  YYGCPGDAMP  SVETCTGGYS  YTRTRIDTLM  EYALYWAALV  LQPGLYDAGL  YIVVLVFGDD  AYLGTVSLSV
                .  ..... .  * *. *. *. * *  * *..** *  *          ** **.  ... . .  . *          *

            201                                                                                                 300
Hsv1gg  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
Hsv2gg  GGSEPGFTAP  PQAARAEGGP  CVPPVPAGRP  WRSVPPVWYS  APNFGFRGLR  FRERCLPPQT  PAAPSDLPRV  AFAPQSLLVG  ITGRTFIRMA  APTEVDVDLP
Ehv4gg  ENDTNYPCGM  TRGLTADGMI  NVDET.T...  ..........  ..........  ..........  ..........  ..........  ....HTT  PHPRAVGCFF
Ehv1gg  EKDTNYPCGM  IRGLTAYGNI  NVDETMD...  ..........  ..........  ..........  ..........  ..........  ....NAS  PHPRAVGCFF
Fhv1gg  EKNTDVPCKM  YHGLAAPTGN  HPQSHVETP.  ..........  ..........  ..........  ..........  ..........  ....NDK  MHRRGRGCFF
 Prvgx  EANLDYPCGM  KHGLTITRPG  ATLPPIA.P.  ..........  ..........  ..........  ..........  ..........  ....TAG  DHQRWRGCFF
                .  .*. .  ... .  .                                                                          .  *..*

            301                                                                                                 400
Hsv1gg  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
Hsv2gg  PHWAPCALDD  GPYAPFPPRP  RFRRALRTDP  EGVDPDVRAP  RTGRRLMALT  EDTSSDSPTS  APEKTPLPVS  ATAMAPSVDP  SAEPTAPATT  TPPDEMATQA
Ehv4gg  ELINFDAWEN  VTFEDMGI..  ..........  ..........  .....PDP  MSFLDDESDV  PN........  .TMDCTSWDL  YTVPRSLK.Q  AEGPQTTLL.
Ehv1gg  EPINEAWAAN  VTFTELGI..  ..........  ..........  .....PDP  NSFLDDEGDV  PN........  .ISDCHSWES  YTYPNTLR.Q  ATGPQTTLL.
Fhv1gg  ELVEPVLWVN  ISSDLIGG..  ..........  ..........  .....PPP  DYNHEDEADI  ESDELPEEIY  ITTQIVVRLI  CLPRESPSVK  VLGDQSSLL.
 Prvgx  S.TDEGAMEN  VTAADKGLSD  DYADYYDVNI  FRSESDDEVV  HGDAPEAPEG  EEVTEEAEAL  TSSDLDNIEI  EVVGSPAAPA  EGPATEEGNG  AEEDEELTSS
                .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .

            401                                                                                                 500
Hsv1gg  ..........  ..........  ..........  ..........  .MSQGAMRAV  VPIIPFLLVL  VGVSGVPTMV  SSTTQPQLQT  TGRPSHEAPH  MTQTGTTDSP
Hsv2gg  ATVAVTPEET  AVASPPATAS  VESSPLPAAA  AATPCAGH.T  NTSSASAAKT  PPTTPAPTTP  PPTSTHATPR  PTTPGPQTTP  PGPATPGPVG  ASAAPTADSP
Ehv4gg  .IGAVGLRIL  A.........  ...QAWKFVE  NETYSQNTRT  VTRDAKEVDV  TQPSPVQADS  VLAKKRTSMK  KNPIV.....  ..SEGKPHAK  PFSTIDSIHT
Ehv1gg  .VGAVGLRIL  A.........  ...QANKFVG  DETYD...TT  IRAEAKKLET  HVPSSLAAES  SLENQSTQEE  SNS.......  .....PEVA  NLRSVNS...
Fhv1gg  .VGSLGFQII  T.........  ..QPWQLKQ  NESYD.......  .....GLRN  ASLEPRHLDS  SNDRDLLDET  EMIGS.....  .IITTPPPT  HPKGVNG...
 Prvgx  DLDNIEIEVV  GSPRPPASSP  PPPPPRPNPR  GRDHDNDHGH  HRADDRGPQR  HHRLPPEPTF  VSPSDIPVTP  TGSPALLIGF  LGSALASRPL  HLTAGETAQH
                    ***                                 .  .  .                                 ---  .

            501                                                                                                 600
Hsv1gg  TAISLTTPDH  TPPMPSIGLE  EEEEEEGAGD  GEHLEGGD..  ....GTRDTL  PQSPGPA...  ........FPL  AEDVE....K  DKPNRPVVPS  PDPNN.SPAR
Hsv2gg  LTASPPATAP  GPSAAPVSVA  ATTATPGTRG  TARTPTDPPK  TNPHGPADAP  PGSPAPPPPE  HRGGPEEPEG  AGDGEPPEDD  DSATGLAFRT  PNPPKEPPAR
Ehv4gg  EGMKNNPVYS  ESLMLMVQMS  DSITTGGVLM  GLQDCDNQLK  T.VYICLALI  GLAHVP....  ..........  ..........  ..........
Ehv1gg  ..........  .....D      DSTHTGGASN  GIQDCDSQLK  T.VYACLALI  GLGTCAMIGL  IVYICVLRSK  LSSRNFSRAQ  MVKHRMYQRL  EYVA......
Fhv1gg  ..........  .GFLQDLPII  EPTTEPCLVN  TKIIGIGTVV  V.VFLLFILI  SLC.......  .YFTCVLRSR  IGNVDRAYEV  QYKPNSMPEY  QQLTRVPQP.
 Prvgx  VREAQQKSRH  IRSLGGLQLS  VETETTMTTT  TQTGLSGDIR  TSIYICVALA  GLVVVGIVIM  CLMAAIIRA  ENRDVGYRHV  SA......  .........

            601                                                                                                 700
Hsv1gg  PETSRPKTPP  TIIGPLA...  TRPTTRLTSK  GRPLVPTPQH  TPLPSFLTAS  PALDTLFVVS  TVIHTLSPLC  IGAMATHLCG  GWSRRGRRTH  PSVRYVCLPS
Hsv2gg  PGPIRPTLPP  GILGPLAPHT  PRPPAQAPAK  DMPSGPTPQH  IPLPWFLTAS  PALDILFIIS  TTIHTAAPVC  LVALAAQLWR  GRAGRRRVAR  PSVRYVCLPP
Ehv4gg  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
Ehv1gg  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
Fhv1gg  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
 Prvgx  .  **  **  * ****  **  *  * *****  *** ***** *** ** * * **  * * *  *  * * * **********

            701
Hsv1gg  ERG
Hsv2gg  ERD
Ehv4gg  ...
Ehv1gg  ...
Fhv1gg  ...
 Prvgx  ...
        **
```

Figure 4


Hsv1gg
Hsv2gg
Ehv4gg
Ehv1gg
Fhv1gg
Prvgx

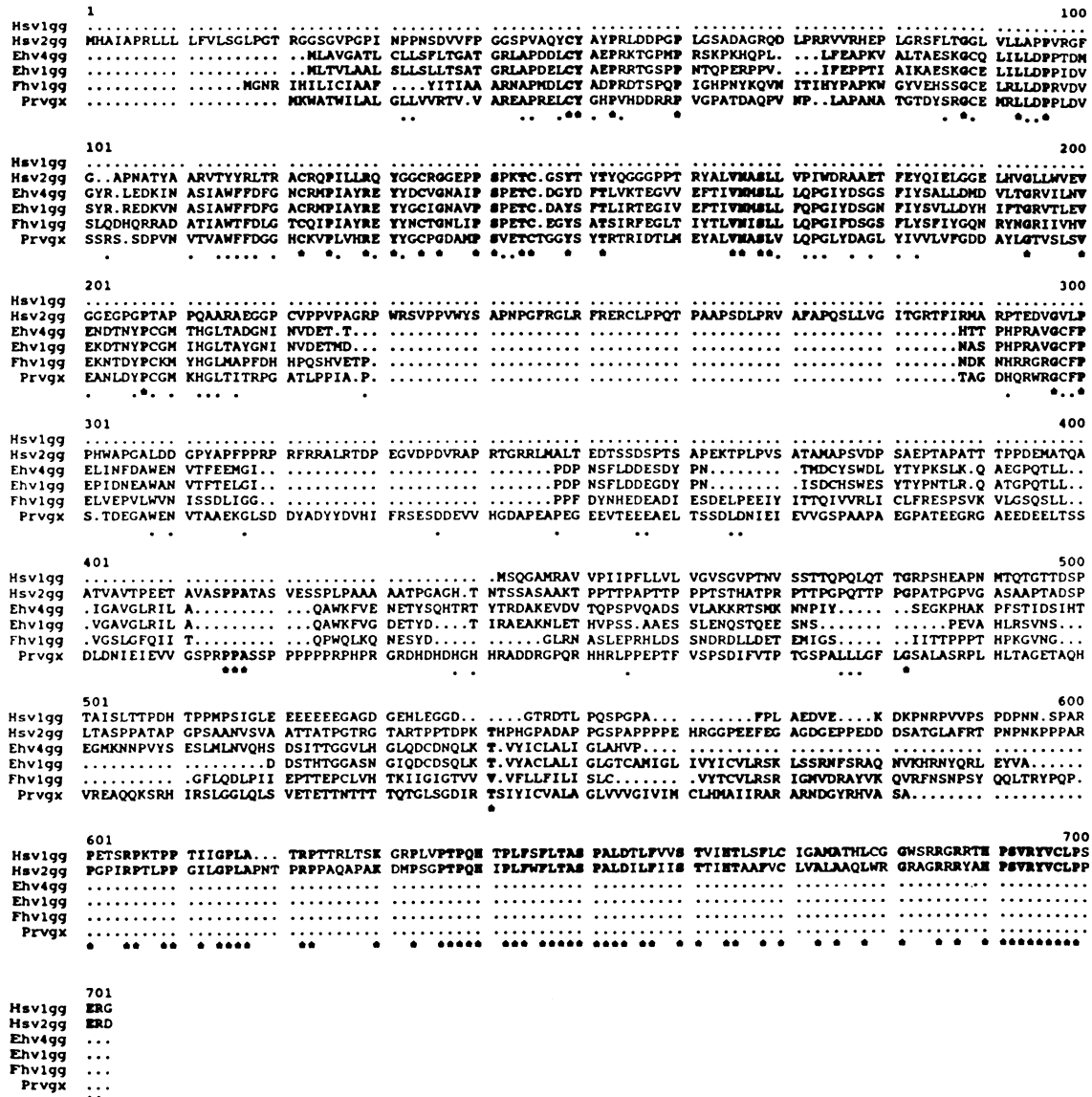


Figure 4

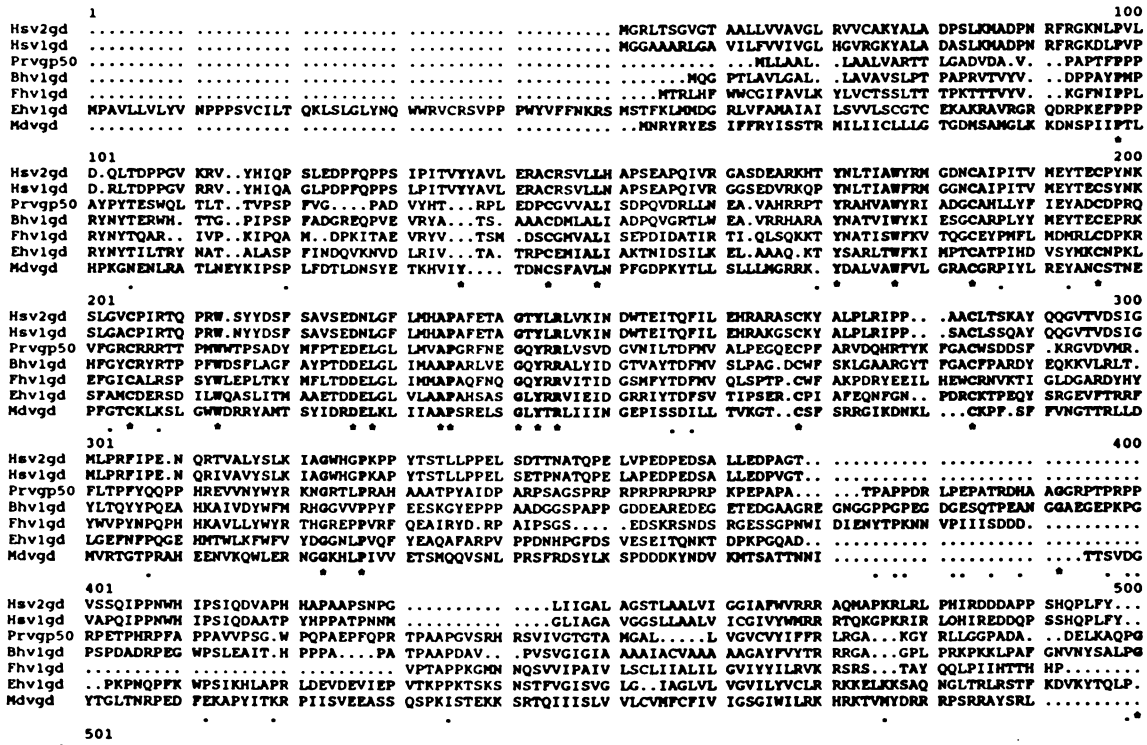


Figure 4 (Cont)

Figure 4 (Cont)

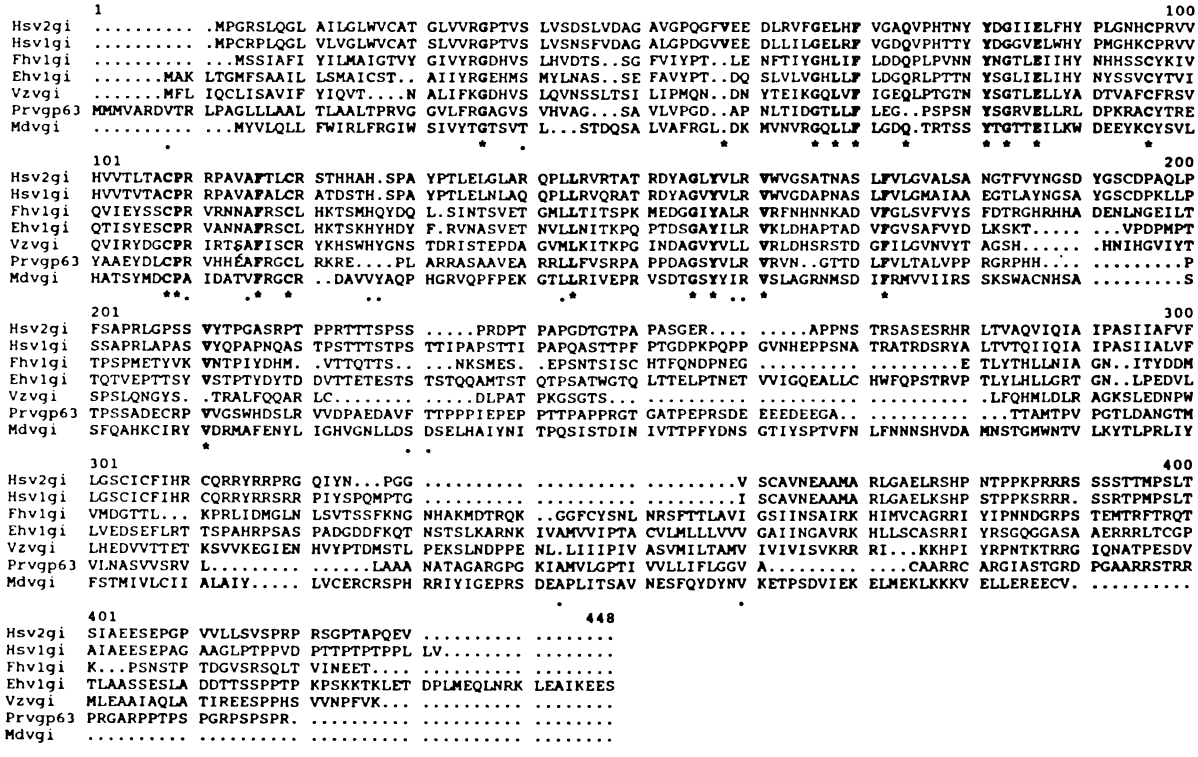


Figure 4 (Cont)

```
          1                                                                                                  100
Hsv2gi    .......... .MPGRSLQGL AILGLWVCAT GLVVRGPTVS LVSDSLVDAG AVGPQGFVEE DLRVFGELHF VGAQVPHTNY YDGIIELFHY PLGNHCPRVV
Hsv1gi    .......... .MPCRPLQGL VLVGLWVCAT SLVVRGPTVS LVSNSFVDAG ALGPDGVVEE DLLILGELRF VGDQVPHTTY YDGGVELWHY PMGHKCPRVV
Fhv1gi    .......... ...MSSIAFI YILMAIGTVY GIVYRGDHVS LHVDTS..SG FVIYPT..LE NFTIYGHLIF LDDQPLPVNN YNGTLEIIHY NHHSSCYKIV
Ehv1gi    .......MAK LTGMFSAAIL LSMAICST.. AIIYRGEHMS MYLNAS..SE FAVYPT..DQ SLVLVGHLLF LDGQRLPTTN YSGLIELIHY NYSSVCYTVI
Vzvgi     .......MFL IQCLISAVIF YIQVT....N ALIFKGDHVS LQVNSSLTSI LIPMQN..DN YTEIKGQLVF IGEQLPTGTN YSGTLELLYA DTVAFCFRSV
Prvgp63   MMMVARDVTR LPAGLLLAAL TLAALTPRVG GVLFRGAGVS VHVAG...SA VLVPGD..AP NLTIDGTLLF LEG..PSPSN YSGRVELLRL DPKRACYTRE
Mdvgi     .......... ...MYVLQLL FWIRLFRGIW SIVYTGTSVT L...STDQSA LVAFRGL.DK MVNVRGQLLF LGDQ.TRTSS YTGTTEILKW DEEYKCYSVL
                    .                          *  .                    *       * * *    *       * * *         *
          101                                                                                                200
Hsv2gi    HVVTLTACPR RPAVAFTLCR STHHAH.SPA YPTLELGLAR QPLLRVRTAT RDYAGLYVLR VWVGSATNAS LFVLGVALSA NGTFVYNGSD YGSCDPAQLP
Hsv1gi    HVVTVTACPR RPAVAFALCR ATDSTH.SPA YPTLELNLAQ QPLLRVQRAT RDYAGVYVLR VWVGDAPNAS LFVLGMAIAA EGTLAYNGSA YGSCDPKLLP
Fhv1gi    QVIEYSSCPR VRNNAFRSCL HKTSMHQYDQ L.SINTSVET GMLLTITSPK MEDGGIYALR VRFNHNNKAD VFGLSVFVYS FDTRGHRHHA DENLNGEILT
Ehv1gi    QTISYESCPR VANNAFRSCL HKTSKHYHDY F.RVNASVET NVLLNITKPQ PTDSGAYILR VKLDHAPTAD VFGVSAFVYD LKSKT..... ...VPDPMPT
Vzvgi     QVIRYDGCPR IRTSAFISCR YKHSWHYGNS TDRISTEPDA GVMLKITKPG INDAGVYVLL VRLDHSRSTD GFILGVNVYT AGSH...... .HNIHGVIYT
Prvgp63   YAAEYDLCPR VHHEAFRGCL RKRE....PL ARRASAAVEA RRLLFVSRPA PPDAGSYVLR VRVN..GTTD LFVLTALVPP RGRPHH..... .........P
Mdvgi     HATSYMDCPA IDATVFRGCR ..DAVVYAQP HGRVQPFPEK GTLLRIVEPR VSDTGSYYIR VSLAGRNMSD IFRMVVIIRS SKSWACNHSA .........S
                 **.     .*  *       ..                    .*          * * .. *          *
          201                                                                                                300
Hsv2gi    FSAPRLGPSS VYTPGASRPT PPRTTTSPSS .....PRDPT PAPGDTGTPA PASGER.... .....APPNS TRSASESRHR LTVAQVIQIA IPASIIAFVF
Hsv1gi    SSAPRLAPAS VYQPAPNQAS TPSTTTSTPS TTIPAPSTTI PAPQASTTPF PTGDPKPQPP GVNHEPPSNA TRATRDSRYA LTVTQIIQIA IPASIIALVF
Fhv1gi    TPSPMETYVK VNTPIYDHM. .VTTQTTS.. ...NKSMES. .EPSNTSISC HTFQNDPNEG ..........  .........E TLYTHLLNIA GN..ITYDDM
Ehv1gi    TQTVEPTTSY VSTPTYDYTD DVTTETESTS TSTQQAMTST QTPSATWGTQ LTTELPTNET VVIGQEALLC HWFQPSTRVP TLYLHLLGRT GN..LPEDVL
Vzvgi     SPSLQNGYS. .TRALFQQAR LC........ .....DLPAT PKGSGTS... .......... .......... .......... .LFQHMLDLR AGKSLEDNPW
Prvgp63   TPSSADECRP VVGSWHDSLR VVDPAEDAVF TTPPPIEPEP PTTPAPPRGT GATPEPRSDE EEEDEEGA.. .......... ...TTAMTPV PGTLDANGTM
Mdvgi     SFQAHKCIRY VDRMAFENYL IGHVGNLLDS DSELHAIYNI TPQSISTDIN IVTTPFYDNS GTIYSPTVFN LFNNNSHVDA MNSTGMWNTV LKYTLPRLIY
                    *                      . .
          301                                                                                                400
Hsv2gi    LGSCICFIHR CQRRYRRPRG QIYN...PGG .......... .......... .........V SCAVNEAAMA RLGAELRSHP NTPPKPRRRS SSSTTMPSLT
Hsv1gi    LGSCICFIHR CQRRYRRSRR PIYSPQMPTG .......... .......... .........I SCAVNEAAMA RLGAELKSHP STPPKSRRR. SSRTPMPSLT
Fhv1gi    VMDGTTL... KPRLIDMGLN LSVTSSFKNG NHAKMDTRQK ..GGFCYSNL NRSFTTLAVI GSIINSAIRK HIMVCAGRRI YIPNNDGRPS TEMTRFTRQT
Ehv1gi    LVEDSEFLRT TSPAHRPSAS PADGDDFKQT NSTSLKARNK IVAMVVIPTA CVLMLLLVVV GAIINGAVRK HLLSCASRRI YRSGQGGASA AERRRLTCGP
Vzvgi     LHEDVVTTET KSVVKEGIEN HVYPTDMSTL PEKSLNDPPE NL.LIIIPIV ASVMILTAMV IVIVISVKRR RI...KKHPI YRPNTKTRRG IQNATPESDV
Prvgp63   VLNASVVSRV L......... ......LAAA NATAGARGPG KIAMVLGPTI VVLLIFLGGV A......... ....CAARRC ARGIASTGRD PGAARRSTRR
Mdvgi     FSTMIVLCII ALAIY..... LVCERCRSPH RRIYIGEPRS DEAPLITSAV NESFQYDYNV KETPSDVIEK ELMEKLKKKV ELLEREECV. .........
                                                          .                   .
          401                                          448
Hsv2gi    SIAEESEPGP VVLLSVSPRP RSGPTAPQEV .......... ........
Hsv1gi    AIAEESEPAG AAGLPTPPVD PTTPTPTPPL LV......... ........
Fhv1gi    K...PSNSTP TDGVSRSQLT VINEET.... .......... ........
Ehv1gi    TLAASSESLA DDTTSSPPTP KPSKKTKLET DPLMEQLNRK LEAIKEES
Vzvgi     MLEAAIAQLA TIREESPPHS VVNPFVK... .......... ........
Prvgp63   PRGARPPTPS PGRPSPSPR. .......... .......... ........
Mdvgi     .......... .......... .......... .......... ........
```

Figure 4 (Cont)

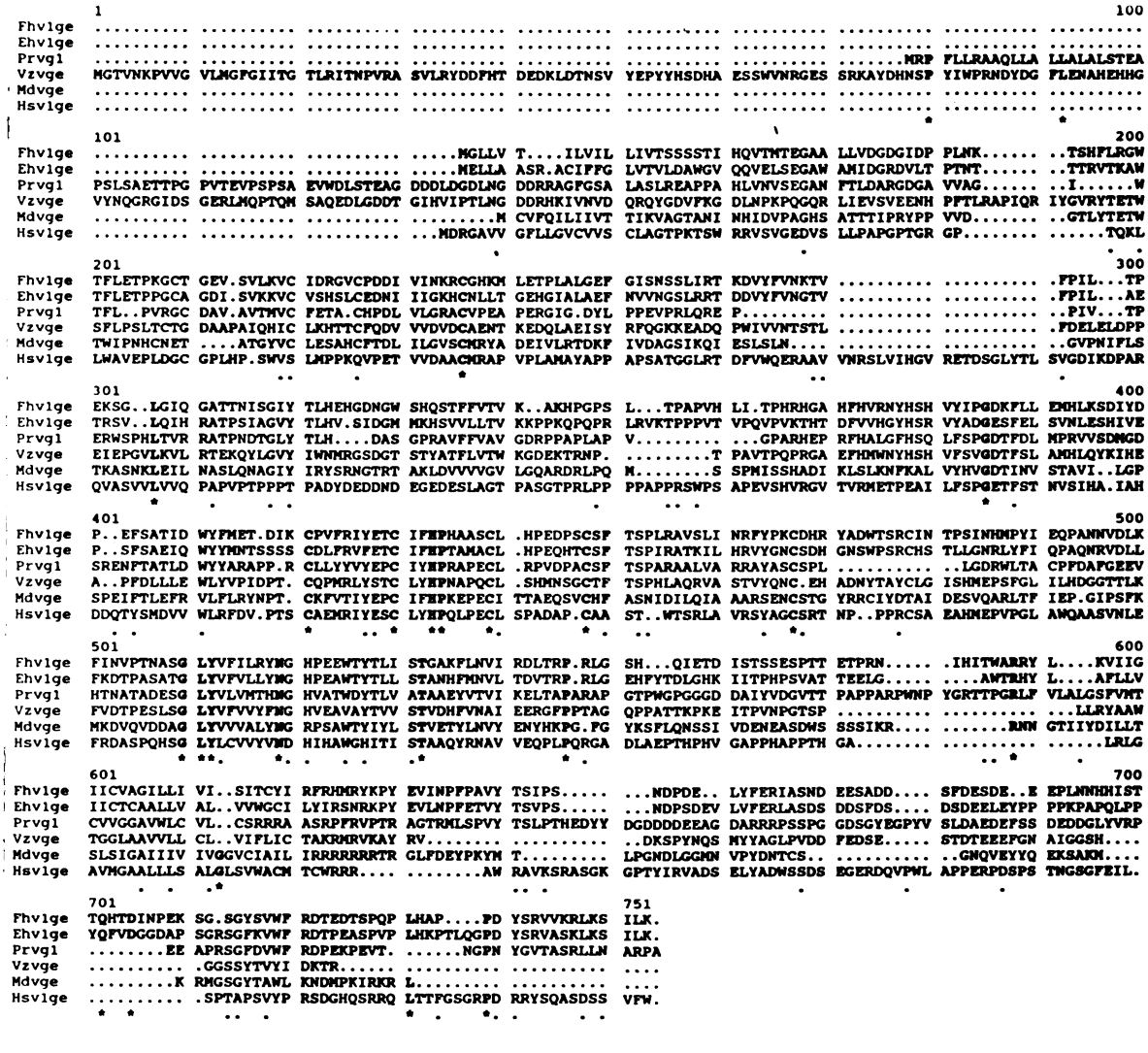


Figure 4 (Cont)

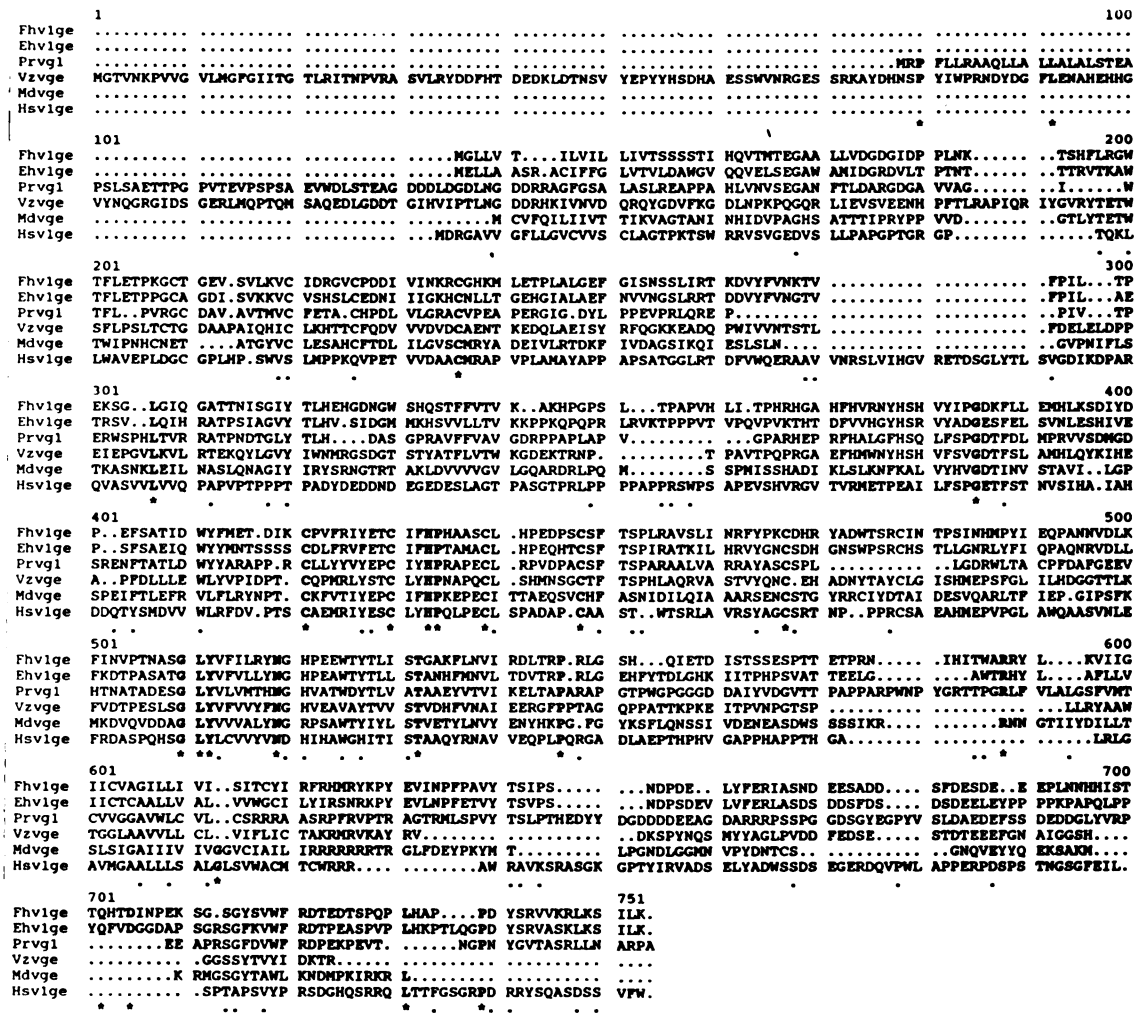


Figure 4 (Cont)

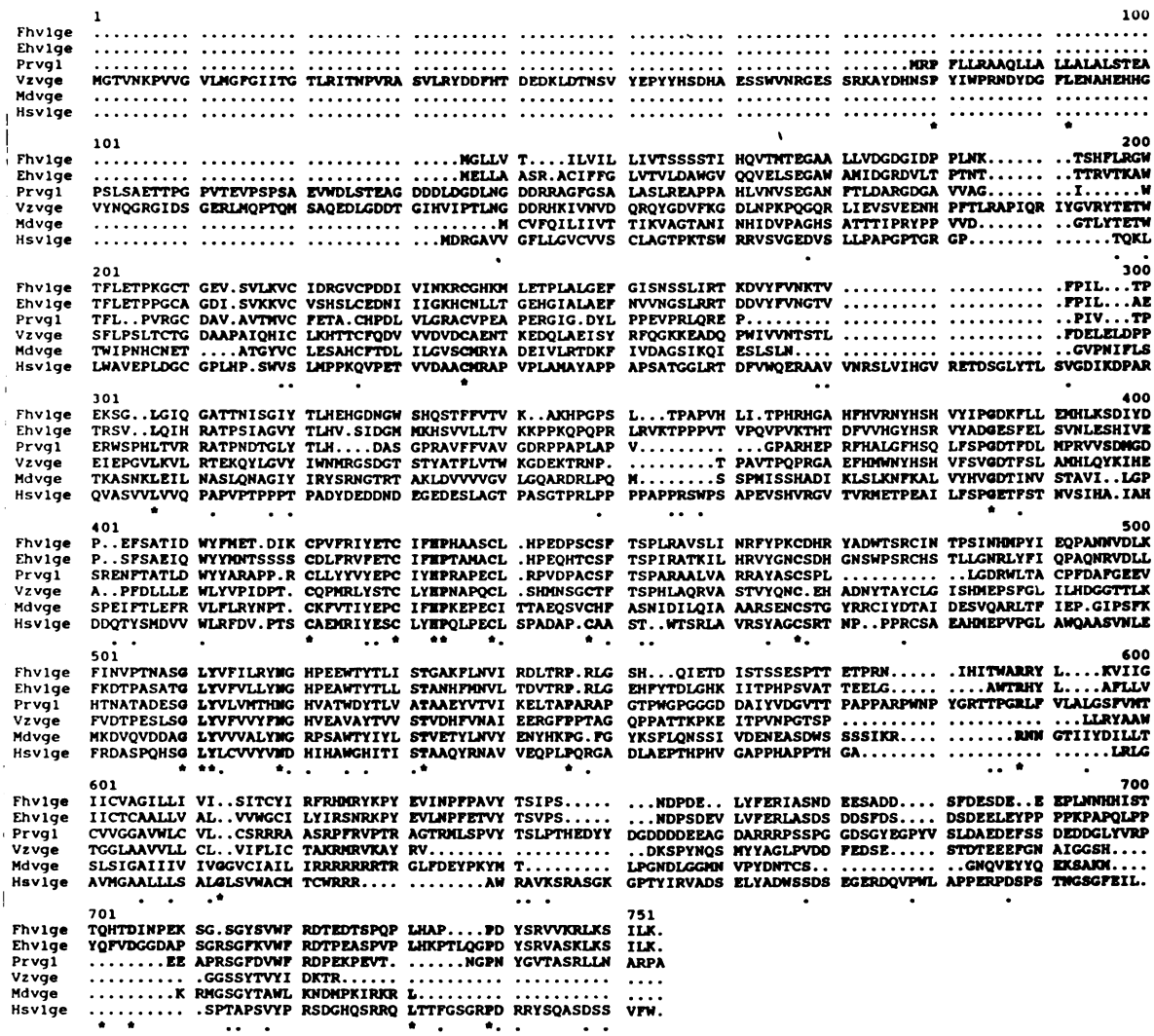


Figure 4 (Cont)

## DISCUSSION

In this report, we present 6.2 kb of DNA sequence located within the 8.0 kb unique short region of the FHV-1 genome. This sequence contains ORFs capable of encoding polypeptides with homology to the protein kinase and glycoproteins G, D, I and E of HSV-1. All five open reading frames for these glycoproteins are encoded by the same strand of DNA and are oriented in the same direction. The gene order is identical to that of Pseudorabies virus. Based on these results and great homology to related alphaherpesvirus proteins, we propose to designate the 5 putative FHV-1 gene products as the protein kinase (ORF 1) and glycoprotein G, D, E, and I (ORF's 2, 3, 4 and 5).

FHV-1 ORF 1 encodes a truncated polypeptide of 69 amino acid residues which exhibits homology to a serine/threonine protein kinase. FASTA and GAP analyses of the truncated polypeptide have indicated that the FHV-1 PK is more closely related to kinases of alphaherpesviruses than those of cellular kinases.

Inspection of the multiple alignments of the protein kinases from the $U_S$ region of HSV-1, HSV-2, EHV-1, PRV, VZV and MDV has revealed good overall conservation of residues at the COOH-terminus. Eight amino acids are perfectly conserved in the last 70 amino acids of the $U_S$ protein kinases. Since no poly(A) signal was found 3' to the PK termination codon, it is probable that the PK mRNA overlaps the gG (the downstream

gene) mRNA and terminates at the same polyadenylation signal. A similar transcriptional organization has been reported for HSV-1, in which many families of overlapping mRNA with unique 5' ends share common 3' ends (van Zijl et al., 1990; Rixon, 1985; Wagner, 1985). In a study by van Zijl et al., (1990) the protein kinase of PRV is encoded in a mRNA of 2.7 Kb while a 1.6 Kb message encodes gX (HSV-1 gG homolog). Both co-terminate at the poly(A) signal downstream from the gX gene.

The reading frame downstream of the PK gene encodes a protein with homology to gG of HSV-2 and gX of PRV. A TATA-like element (TATAAAG) was found 5' to the methionine start codon. A poly(A) signal was found downstream of the termination codon. This site is likely to be used both the PK and gG transcripts: one transcript originating from the promoter region of the PK gene and the other originating upstream of the gG initiation codon.

Another group of suspected co-terminal transcripts encode glycoprotein D and I. As in the case of the PK/gG gene cluster, there is no AATAAA or ATTAAA polyadenylation/ processing signal between these two glycoprotein genes. Likewise, TATA elements were found 5' to the gG gene and numerous TATA transcriptional elements could be identified upstream of the initiation codon of the gI transcript.

Over the last few years, a large amount of nucleic acid sequencing information concerning the $U_S$ regions of HSV-1, VZV, EHV-1, PRV and MDV has become available (McGeoch et al., 1985; Davison and Scott, 1986; Telford et al., 1992;

Petrovskis et al., 1986; Petrovskis et al., 1986). Review of the genes encoded within these regions have revealed many similarities. The greatest similarity is that the gene order is always Protein Kinase, (glycoprotein G), (glycoprotein D), glycoprotein I and glycoprotein E (5'>3'). All alpha-herpesviruses sequenced to date, excluding channel catfish herpesvirus, contain genes encoding homologs to gI and gE (Davison, 1992). Genes encoding homologs to HSV-1 gD are also highly represented, the exception being VZV. Besides the absence of a gD gene, VZV also lacks the gene encoding the semiconserved homolog, glycoprotein G (Davison, 1984). HSV-1 and the oncogenic herpesvirus MDV (previously classified as a gammaherpesvirus) contain similar genetic organization within their $U_S$ regions. Each contain genes encoding homologs to PK, gD, gI and gE and contain potential glycoprotein genes between the protein kinase and glycoprotein D. In HSV-1, this region contains $U_S$ 4 (gG) and a short gene called $U_S$ 5. A short open reading frame (sorf 4) is located between the protein kinase and gD genes of MDV (Peter Brunovskis, Michigan State University, Personal Communication).

Cis-acting transcriptional regulatory sequences of FHV-1's $U_S$ region are highly collinear with sequences in the regulatory region of genes in the $U_S$ region of alpha-herpesviruses. This allows for many co-terminal transcripts. The polyadenlyation sequences, AATAAA/ATTAAA are absent in the transcript termination regions of genes encoding homologs to the protein kinase and glycoprotein D. VZV does contain a

poly(A) signal downstream of its PK gene, but this genome also lacks a homologous gene to glycoprotein G. All TATA-like elements of the $U_S$ genes of FHV-1 contain the consensus TATA(A/T)N(T/A), with N=T in most cases. Polyadenylation signals are only apparent downstream of the termination codons for the gG, gI and gE genes of FHV-1.

This duplication of promoter elements for downstream genes in a gene cluster encoding co-terminal transcripts may be important in expression of $U_S$ genes. Northern analysis of $U_S$ transcripts of FHV-1, using a radioimaging system, has indicated that there are noticeable differences in the amounts of bicistronic vs. monocistronic transcripts specific for a gene cluster. Two transcripts, 3.0 and 1.8 Kb can be detected using probes specific for either the PK/gG or the gD/gI gene clusters. The full length transcripts were determined to be quantitatively more abundant then the individual 1.8 Kb transcripts. The 1.8 Kb transcript was weakly detected with the XhoI-EcoRI (gI) probe, while the 3.0 and 1.8 Kb transcripts detected with a probe specific for gD appear to be present in equal amounts late in infection.

The most striking result in homology analyses (Table 1) was the fact that reasonable homology could be demonstrated between individual glycoproteins (G, D and I) within a specific virus. GAP analyses of the homologs of gG, gD and gI have revealed a conserved area of 110 amino acid residues representing external coding domains of the glycoproteins. As depicted in Figure 5, three cysteine residues can be aligned

without the introduction of major blocks of spaces. Twelve amino acid residues exist between the first and the second cysteine residues, while 11 residues are between the second and the third cysteine. In this region, numerous stretches of similar amino acids can be noted between the protein products of these genes.

This conservation between gG, gD, and gI has led to the hypothesis that gG, gD and gI arose as a result of gene duplication and divergence. In a recent study, Ross et al., (1991) illustrated evolutionary relationships of MDV $U_S$ glycoproteins using the CLUSTRAL program (Higgins and Sharp, 1988) Dendrograms revealed three main glycoprotein families; (i) the gI family (ii) the gG/gD family and (ii) the HSV-1 gG/MDV ORF4. Clusters of gI homologs and those of gG/gD homologs are thought to have evolved independently from a common ancestral gene family. The HSV-1 gG/MDV ORF4 cluster is likely to have evolved from this common ancestral gene family. As illustrated in Figure 5, gG and gD homologs share many amino acid similarities. This homology diminishes when gI homologs are included. This could indicate that the gD gene family may have evolved as a duplication of a gG-like gene family. The gI gene family, in turn, could have evolved independently from the common gG-like gene family. The attractiveness of this model is based upon the presence of (i) conserved cysteine residues between gG, gD and gI, homologs that are involved in penetration of the virion and neurovirulence and (ii) the tissue tropisms of individual

Figure 5. **Multiple alignment of conserved regions of glycoproteins gG, gD and gI of the subfamily Alphaherpesviridae.**

```
Hsv2gg (88-185)  G.GLVLLAPP VR..... GFGAPNATYA ARVTYYRLTR ACRQPILLRQ YGGCRGGEPP SPKTC..GSY TYTYQGGGPP TRVALVNASL LVP.IWDRAA ETFEYQIELG
  Prvgx (65-164)  GCEMRLLDPP LDV..... SSRS.SDPVN VTVAWFFDGG HCKVPLVHRE YYGCPGDAMP SVETCT.GGY SYTRTRIDTL MEYALVNASL VLQ.PGLYDA GLYIVVLVFG
 Ehv1gg (62-160)  GCELILLDPP IDV..... SYR.REDKVN ASIAWFFDPG ACRMPIAVRE YYGCIGNAVP SPETC..DAY SFTLIRTEGI VEPTIVNMSL LFQ.PGIYDS GNFIYSVLLD
 Fhv1gg (68-194)  GCELRLLDPR VDV..... SLQDHQRRAD ATIAWTFDLG TCQIPIAYRE YYNCTGNLIP SPETC..EGY SATSIRFEGL TITTLVNISL LLQ.PGIFDS GSFLYSFIYG

 Hsv1gd (90-145)  ACRSVLLHAP SEAPQIVRGA SDEARKHTYN LTIAWYRMGD NCAIPITVME YTECP.YNK. SLGVCPIRTQ PRW.SYYDSF SAVSEDWLGF LWHAPAFETA GTY.LRLVKI
 Hsv2gd (90-145)  ACRSVLLHAP SEAPQIVRGA SDEARKHTYN LTIAWYRMGD NCAIPITVME YTECP.YNK. SLGVCPIRTQ PRW.SYYDSF SAVSEDWLGF LWHAPAFETA GTY.LRLVKI
  Mdvgd (87-192)  NCSFAVLNPF GDPKYTLLSL LLMGRRK.YD ALVAWFVLGR ACGRPIYLRE YANCS.TNE. PFGTCKLKSL GWTDRRYAMT SYIDRDELKL IIAAPSRELS GLY.TRLIII
 Ehv1gd (137-241)  PCEMIALIAK TNIDSILKEL .AAAQ.KTYS ARLTWFKIMP TCATPIHDVS YMKCN.PKL. SFAMCDERSD ILWQASLITM AAETDDELGL VLAAPAHSAS GLY.RRVIEI
 Fhv1gd (76-181)  SCGMVALISE PDIDATIRTI .QLSQKKTYN ATISWFKVTQ GCEYPMFLMD MRLCD.PKR. EFGICALRSP SYWLEPLTKY MFLTDDELGL IWMAPAQFNQ GQY.RRVITI
 Bhv1gd (74-179)  ACDMLALIAD PQVGRTLWEA .VRRHARAYN ATVIWYKIES GCARPLYYME YTECE.PRK. HPGVCRYRTP PFWDSFLAGF AYPTDDELGL IWAAPARLVE GQY.RRALYI
Prvgp50 (65-170)  PCGVVALISD PQVDRLLWEA .VAHRRPTYR AHVAWYRIAD GCAHLLYFIE YADCD.PRQ. VPGRCRRRTT PWTWTPSADY MFPTEDELGL LWVAPGRFNE GQY.RRLVSV

  Mdvgi (47-144)  VRGQLLFLGD Q.TRTSSYTG TTE..... ..ILWDEEY KCYSVLHATS YWDCPAIDAT VFRGCR..DA VVYAQPHGRV QPFPEKGTLL RIVEPRVSDT GSYYIRVSLA
Prvgp63 (59-153)  IDGTLLFLEG ..PSPSNYSG RVE..... ..LLRLDPKR ACYTREYAAE YDLCPRVHHE APRGCLRKRE ....PLARRA SAAVEARRLL FVSRPAPPDA GSYVLRVRVN
   Vzvgi (51-151)  IKGQLVFIGE QLPTGTNYSG TLE..... ..LLYADTVA FCPRSVQVIR YDGCPRIRTS APISCRYKHS WHYGNSTDRI STEPDAGVML KITKPGINDA GVYVLLVRLD
 Ehv1gi (51-150)  LVGHLLFLDG QRLPTTNYSG LIE..... ..LIHYNYSS VCVTVIQTIS YESCPRVANN APRSCLHKTS KHVHDYF.RV NASVETTVLL NITKPQPTDS GAYILRVKLD
 Fhv1gi (47-146)  IYGHLIFLDD QPLPVNNTNG TLE..... ..IIHYNHHS SCYKIVQVIE YSSCPRVRNN APRSCLHKTS MHQYDQL.SI NTSVETGHLL TITSPKMEDG GIYALRVRFN
 Hsv1gi (53-152)  ILGELRFVGD QVPHTTYYDG GVE..... ..LWHYPMGH KCPRVVHVVT VTACPRRPAV APALCRATDS TH.SPAYPTL ELNLAQQPLL RVQRATRDYA GVYVLRVWVG
 Hsv2gi (53-152)  VFGELHFVGA QVPHTNYYDG IIE..... ..LFHYPLGN HCPRVVHVVT LTACPRRPAV APTLCRSTHH AH.SPAYPTL ELGLARQPLL RVRTATRDYA GLYVLRVWVG

                  .cg.lll... .......y.. ..e.....y. a..awy.... .C..pi...e y..Cp..... sf..C..... ..W..... ....edn1.l ...ap....a g.y.lrv...
                                                  <----12----|----11 ---->
```

Figure 5

herpesviruses. The ability of viruses to infect a broad range of tissue types or subtypes is dependent on virion receptors present in the envelope. Some of these receptors, gD, gI, gE for example, have been reported to be involved in neurovirulence (Izumi and Stevens, 1990; Card et al., 1992; Zuckermann et al., 1988; Petrovskis et al., 1986). While the function of gG is unknown, it is conceivable that duplication of an ancestral gG gene enabled the mutant to productively infect and, perhaps, even establish latency in a new range of tissue types.

Recently, the functional importance of individual herpes virus glycoproteins is being addressed by generation of chimeric herpesviruses. For example, Kopp and Mettenleiter (1992) have created a gB- PRV mutant by incorporating the BHV-1 gB gene into the PRV genome. This recombinant expressed the BHV-1 glycoprotein and in cells of pig, rabbit, canine, monkey, or human origin, had growth characteristics, similar to its PRV parent. However, altered penetration kinetics of the gB(BHV-1) recombinant PRV were reported in Madin-Darby bovine kidney (MDBK) cells. The exchange of gB(PRV) for gB(BHV-1) slowed the penetration of the viruses to a level intermediate between those of wild-type PRV and BHV-1. Similarly, a gC(PRV) recombinant BHV-1 has been generated by Liang et al., 1991. In penetration studies with MDBK cells, this recombinant had significantly higher penetration rates than wt or gC-(BHV-1) viruses. The generation of recombinants, with altered envelope receptors, will provide the tools for a

thorough investigation of glycoprotein functional domains. These recombinants will also aid in defining which glycoproteins are involved in various cell tropisms. Since FHV-1 can only infected cells of feline origin and PRV can infect a wide variety of cells, it would be interesting to use the genome of FHV-1 as a host for various combinations of PRV glycoprotein gene. Deleting the endogenous FHV-1 homolog gene before addition of PRV homologs can facilitate determination of viral receptors necessary to bind and infect certain cells, previously nonpermissive to FHV-1.

There is little doubt that the $U_S$ glycoproteins are important in the survival and spread of the virus in infected animals. Avirulent strains of PRV (i.e. Bartha) and EHV-1 (i.e. KyA) containing deletions in the $U_S$ glycoprotein genes have been generated and their protective immunity is well documented (Mettenleiter et al., 1985; Vandeputte et al., 1990; van Oirschot et al., 1991; Wardley et al., 1991). Interestingly, all these vaccine strains contain deletions in the gE gene and have reduced neurovirulence (Petrovskis et al., 1986: Flowers and O'Callaghan, 1992). Animal studies with the Bartha strain of PRV (Card et al., 1992) have demonstrated that this virus is unable to infect certain neurons that were readily susceptible to the parent stain containing gE. Although PRV mutant viruses containing deletions in the PK, gX, gI and gE genes have been engineered and replicate *in vitro*, these viruses do not replicate well in the host (Mettenleiter et al., 1990). Genetic analyses of DNA

isolated from vaccine strains of FHV-1 (Solvay and Fermenta) have indicated the lack of genetic alterations (i.e.deletions) within the gE gene (unpublished data).

The role of the unique short glycoproteins of FHV-1 in the pathogenesis of FVR is currently being addressed by the generation of modified live vaccines containing deletions in both the gI and gE genes and poxvirus recombinants expressing gD. Assessment of these gE deletion mutants of FHV-1 in kitten may provide useful information on the role of this suspected neurovirulence factor in FVR. Infection of the CNS and generalization of the virus in the lung and liver, are rarely observed in adult cats but often seen in naturally or experimentally infected kittens (Shields and Gaskin, 1977).

The identification of the genes encoding these important glycoproteins has laid down the foundation for the immunological characterization of their gene products and the role of these products in feline viral rhinotracheitis.

# Chapter 4

## The Nucleotide Sequence of the Gene encoding Glycoprotein H of Feline Herpesvirus-1

Stephen J. Spatz

**ABSTRACT**

Feline herpesvirus-1, which is classified as an alpha-herpesvirus causes a major respiratory disease in cats and is often fatal in kittens. Similar to other herpesviruses, FHV-1 contains immunologically important glycoproteins. Recently, the genes encoding glycoproteins homologous to gB, gD, gI, gE and gG of HSV-1, have been localized within the genome of FHV-1 (C-27). To further expand this work, we defined the genomic position of the essential and conserved glycoprotein gene encoding gH. In all herpesviruses characterized thus far, the gene coding for glycoprotein H is located downstream of the thymidine kinase gene. The selectability of the FHV-1 thymidine kinase gene in transfected mouse TK- cells, allowed for the localization of the TK/gH gene cluster. DNA from recombinant EMBL3 clones, representing 85% of the genome, was transfected into mouse TK- cells under HAT selection. Colonies of cells only appeared with cells transfected with DNA from the SALI A clone. DNA from a 6.6 Kb EcoRI subfragment of SalI A was then used in transfection assays to pinpoint the TK/gH genes. Nucleic acid sequencing of this subfragment has indicated the presence of 2 open reading frames. Computer predicted translation products from each reading frame were shown to share similarities with the gene products of the TK and gH genes of VZV. The 813 amino acid translation product (glycoprotein H of FHV-1) shows many features typical of a glycoprotein. Northern blot analyses of RNA isolated from

FHV-1 infected CRFK cells had indicated the likelihood of co-terminal transcripts. Two transcripts (4.0 and 1.2 Kb) were detected with probes specific for the thymidine kinase gene. Similarly, two transcripts (4.0 and 2.7 Kb) were detected with gH-specific probes. It is likely that the 2.7 and 1.5 Kb transcripts encode gH and TK, respectively. The larger 4.0 Kb transcript may be a bicistronic transcript. On northern blots containing RNA isolated from the TK/gH-transfected mouse cells, only the 4.0 and the 1.2 Kb transcripts could be detected.

## INTRODUCTION

Glycoprotein H (gH) of herpesvirus simplex -1 (HSV-1), as well as glycoproteins B and D, are three envelope proteins that are essential for virus penetration (Spear, 1989; Klupp and Mettenleiter, 1991). Their immunological importance has been shown by their ability to elicit complement independent virus-neutralizing antibodies (Fuller and Spear, 1985; Long et al., 1984; McDermott et al., 1989; Britt et al., 1990; Buckmaster and Minson, 1984; Fuller et al., 1989. Temperature-sensitive gH mutants of HSV-1, have been generated and produce noninfectious viruses at the nonpermissive temperature (Desai et al., 1988). Likewise, deletion mutants of gH (HSV-1) have been constructed that are noninfectious in non-complementing cell lines. Biologically, this glycoprotein appears to be involved in fusion of the virion envelope to the plasma membrane. Using the fusogenic agent polyethylene glycol, Forrester and coworkers (1992), reported that phenotypically gH-negative mutants could be obtained by a single growth cycle in non-complementing Vero cells.

Electron microscopy studies by Fuller et al.,(1989) have also demonstrated the role of gH(HSV-1) in cell fusion. Electron micrographs of infected cells in the presence of anti-gH monoclonal antibodies, have revealed neutralized virions bound to the cell surfaces and the absence of nucleocapsids within the cytoplasm of susceptible cells. Although fusion bridges could be demonstrated, no expansion of

these bridges nor rearrangement of the envelope or the tegument was observed.

Proteins homologous to glycoproteins H and B are the only two essential membrane proteins which have been described in all three subfamilies of Herpesvirinae (Buckmaster et al., 1984; Davison and Scott, 1986; Cranage et al., 1988; Gompels, et al., 1988; Heineman et al., 1988; Joseph et al., (1991); Keller et al., 1987; Klupp and Mettenleiter, 1991; McGeoch and Davison, 1986; Meyer et al., 1991; Nicolson et al., 1990; Pachl et al., 1989). A high degree of amino acid conservation exists between homologs of glycoprotein H, second only to glycoprotein B homologs (Klupp and Mettenleiter, 1991). Attempts to express glycoprotein H in mammalian expression systems have generally resulted in the expression of a recombinant gH that requires interactions with other herpes viral factors for proper formation of its tertiary antigenic structure and cell surface localization (Gompels and Minson, 1989).

In mammalian expression systems, gH of HSV-1 was reported to be antigenically different from gH produced during infection. Only one out of three monoclonal antibodies that recognize conformational epitopes of gH could immunoprecipitate the expressed gH. However, equal recognition of the transfected gH product by all three monoclonal antibodies could be demonstrated if the gH-transfected cells were previously superinfected with HSV-1 or HSV-2 (Gompels and Minson, 1989; Foa-Tomasi et al., 1991). Superinfection

resulted in the proper transport of expressed gH to the infected cell surface, a result not noticed in the cytoplasm of gH expressing cells (Gompels and Minson, 1989). Similar results have been found with vaccinia viruses expressing either gH of HSV-1 or HCMV (Forrester et al., 1991; Cranage et al., 1988). In HCMV-infected cells, gH is present on the nuclear and cytoplasmic membranes. However, when recombinant gH is expressed in cells, gH accumulates predominantly on the nuclear membrane.

The failure to obtain surface expression of gH in vaccinia virus and mammalian expression systems suggests that there is a block in the transport of gH to the cell surface, as evident by pronounced nuclear membrane staining. This block could be overcome by superinfection, leading to the hypothesis that the correct synthesis, processing and antigenic presentation of gH is dependent on additional viral factors, perhaps other glycoproteins. Using glycoprotein mutants of HSV-1, Roberts et al., (1991) and Foa-Tomasi et al., (1991) reported that glycoproteins gB, gC, gD, gG, gE, and gI are not required for antigenic maturation and cell surface transport of gH. Recently, the gene encoding a likely factor needed for the correct folding and processing of gH has been identified. Huthinson et al., (1992) reported that coexpression of HSV-1 gH and the Ul 1 gene product (gL) in vaccinia virus resulted in the correct cellular localization and antigenic presentation of the recombinant protein.

The objective of the work described here was to localize

the thymidine kinase/glycoprotein H gene cluster of FHV-1 and sequence the gene encoding gH. During the course of this work, Nunberg et al., (1989) reported the location of the TK gene within the genome of FHV-1. Expanding on this work, we present the complete nucleic acid sequence of the gH gene and similarities of its gene product to gH polypeptides of herpesviruses.

## METHODS

### Cells and Viruses

Crandell Reese feline kidney cells were grown in Dulbecco's modified Eagle medium (Gibco Laboratories), containing 100 Units/ml of Penicillin, 100 ug/ml of Streptomycin and 10% heat-inactivated fetal bovine serum. FHV-1 strain (C-27) was obtained from the American Type Culture Collection.

### Recombinant DNA

The 6.6 Kb EcoRI subfragment was purified from a recombinant FHV-1/lambda EMBL3 clone containing the 19 Kb SalI A fragment and subcloned into pBluescript-KS. Various restriction fragments were generated and cloned into M13 mp18 and mp19. These recombinants were then used as probes in northern analyses and as templates for nucleic acid sequencing.

### Transfection of Mouse and Human TK- cells

Initially, SalI-digested DNA fragments of FHV-1 (SalI A, B, C, D, E, G, H, I, J, and K) were used to transfect both mouse L (TK-) and human (TK-) cells (Rota et al., 1986). This approach was extended using subclones of the recombinant EMBL3/SalI A construct to pinpoint the TK/gH gene cluster. Transfections were done by the calcium phosphate technique (Graham and van der Eb, 1973). Plasmid and EMBL3 DNA were

isolated via standard procedures (Ausubel et al., 1988). Mouse and human cells were plated 48 hours prior to transfection at a density which gave 80 -90% confluency. $Ca(PO_4)$ precipitates of 20.0 ug of DNA were added to cells in 25 $cm^2$ tissue culture flask. TK+ colonies were selected in HAT-supplemented medium (hypoxanthine 1 X $10^{-4}$ M, aminopterin 4 X $10^{-5}$ M, thymidine 1.6 X $10^{-5}$ M).

**Northern Blot analyses**

Crandell-Reese feline kidney cells were infected with plaque-purified FHV-1, using a m.o.i. of >1.0. At 12 hours post infection, infected cells were harvested and RNA was isolated using the guanidium thiocyanate-CsCl method (Ausubel et al., 1988). Gradient-purified RNA was denatured in formamide and formaldehyde and electrophoresed in formaldehyde-agarose gels. Separated RNA was passively transferred to Nytran membranes and hybridized to radiolabeled plasmid probes. RNA isolated from TK+ transfected mouse L cells was also subjected to northern blot analysis.

**Nucleotide Sequence Determination**

Single-stranded M13 DNA was used as a template for dideoxynucleotide sequencing. The sequence from both strands was determined with Sequenase (US Biochemicals) using $^{35}S$-dATP as the radiolabel. The reaction products were separated on TBE-buffered acrylamide gels containing 7.0 M Urea. Synthetic oligonucleotides were used to extend sequencing information.

These oligonucleotides were synthesized by solid phase phosphoramidite chemistry on a 380 A DNA Synthesizer (Applied Biosystems), based on previously determined FHV-1 sequences.

**Analysis of Sequence Information**

Nucleic Acid sequences were assembled and analyzed using a VAX computer and versions 5.0 and 5.3 of the University of Wisconsin Genetic Computer Package (UWGCG), (Devereux et al., 1984). The GAP program was used to align the nucleotide and amino acid sequences. Graphic hydrophilicity analyses were generated by the method of Kyte and Doolittle (1982). Amino acid homology analyses were conducted using the FASTA and GAP programs. The LINEUP and PILEUP programs were used to generate multiple alignments of gH polypeptides, representative of the three herpesvirus subfamilies.

## RESULTS

### Localization of the genes encoding thymidine kinase and glycoprotein H.

The thymidine kinase gene of FHV-1 was functionally localized to a 19.0 Kb SalI A restriction fragment (Rota et al., 1986). TK activity was only produced in transfected mouse L cells and not in the human cell line. A restriction subfragment (EcoRI-EcoRI, 6.6 Kb) of the 19.0 Kb SalI A fragment further pinpointed the TK gene. A large portion of this subfragment was sequenced using dideoxynucleotide chain termination (Sanger et al., 1977). An overall map of the corresponding genomic region is shown in Figure 1. Analysis of the computer predicted open reading frames revealed two major ORF's, capable of encoding the thymidine kinase and glycoprotein H. The complete sequence for the glycoprotein H gene is presented in Figure 2. The DNA sequence for the thymidine kinase gene has already been reported by Nunberg et al., (1989).

### Sequence analysis of the gH gene of FHV-1

The nucleotide sequence of the gH (FHV-1) gene is given in Figure 2. Examination of the DNA sequence analysis for cis-acting transcriptional elements revealed a TATA box (TATAAGC) (Corden et al., 1980) , 90 bp 5' to the initiation codon, (CTTATGATGTG). The sequence, TAATTGA, located 125 nucleotides upstream of the initiation codon, when reverse complemented, show similarities to the CAAT box consensus, TCAATCT. A

Figure 1. **Genomic organization of the gene encoding gH of FHV-1.** (A) The 134 Kb genome is represent as a group D genome. (B) The SalI restriction map of FHV-1 (C-27) DNA. A restriction map of the EMBL3 recombinant representing SalI A (19 Kb) is also presented with a detailed map (C) of a 6.6 Kb EcoRI-EcoRI subfragment. (D) Solid boxes represent the hybridization probes used to map the gH and TK transcripts.

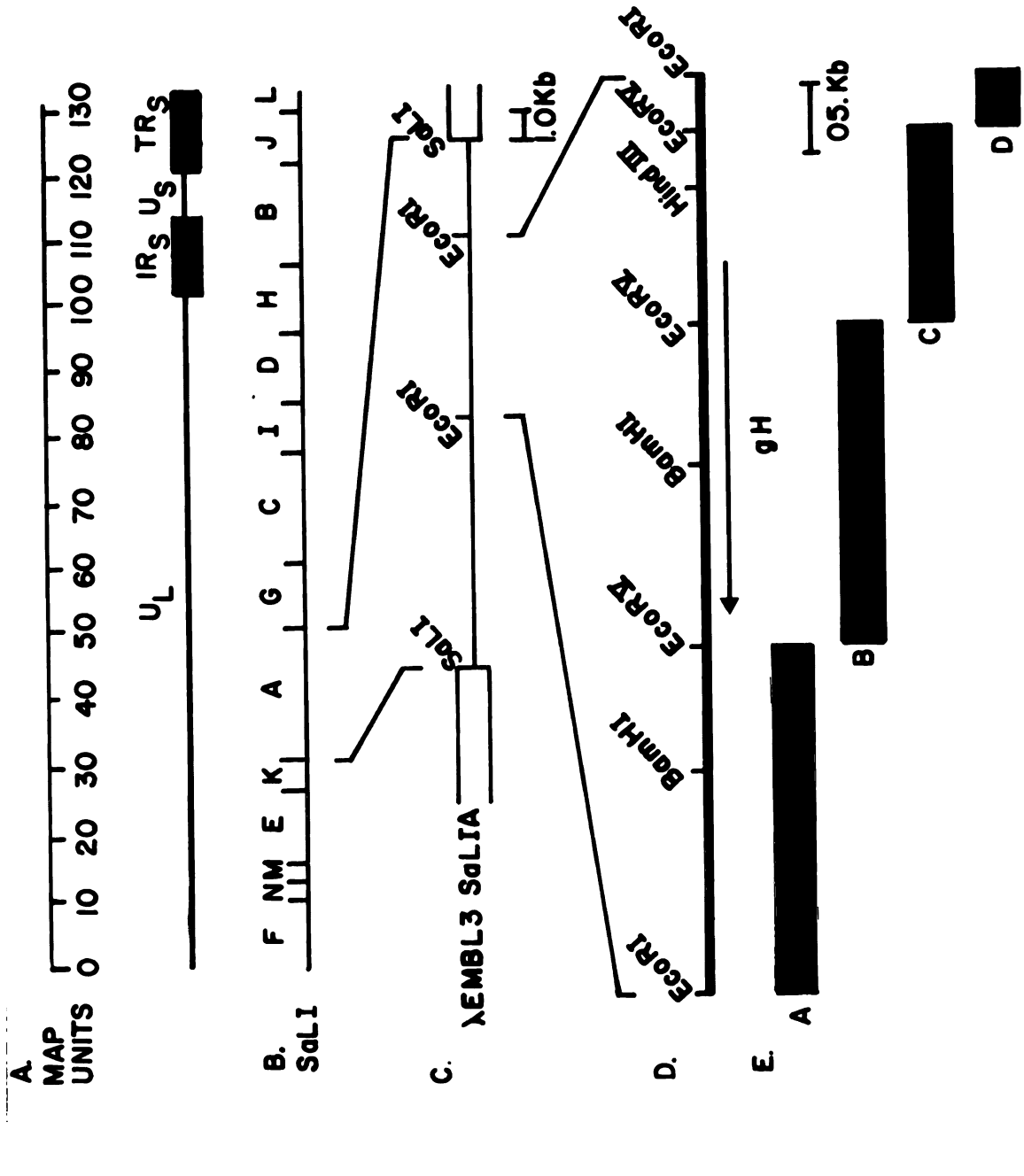

A.
MAP
UNITS
0 10 20 30 40 50 60 70 80 90 100 110 120 130
$U_L$
$IR_S$ $U_S$ $TR_S$
B.
SaLI
F NM E K A G C I D H B J L
C.
λEMBL3 SaLIA
SaLI
EcoRI
SaLI
D.
EcoRI
BamHI
EcoRX
BamHI
EcoRX
BamHI
EcoRX
Hind III
EcoRI
EcoRI
1.0Kb
gH
E.
A
B
C
D
0.5Kb

Figure 1

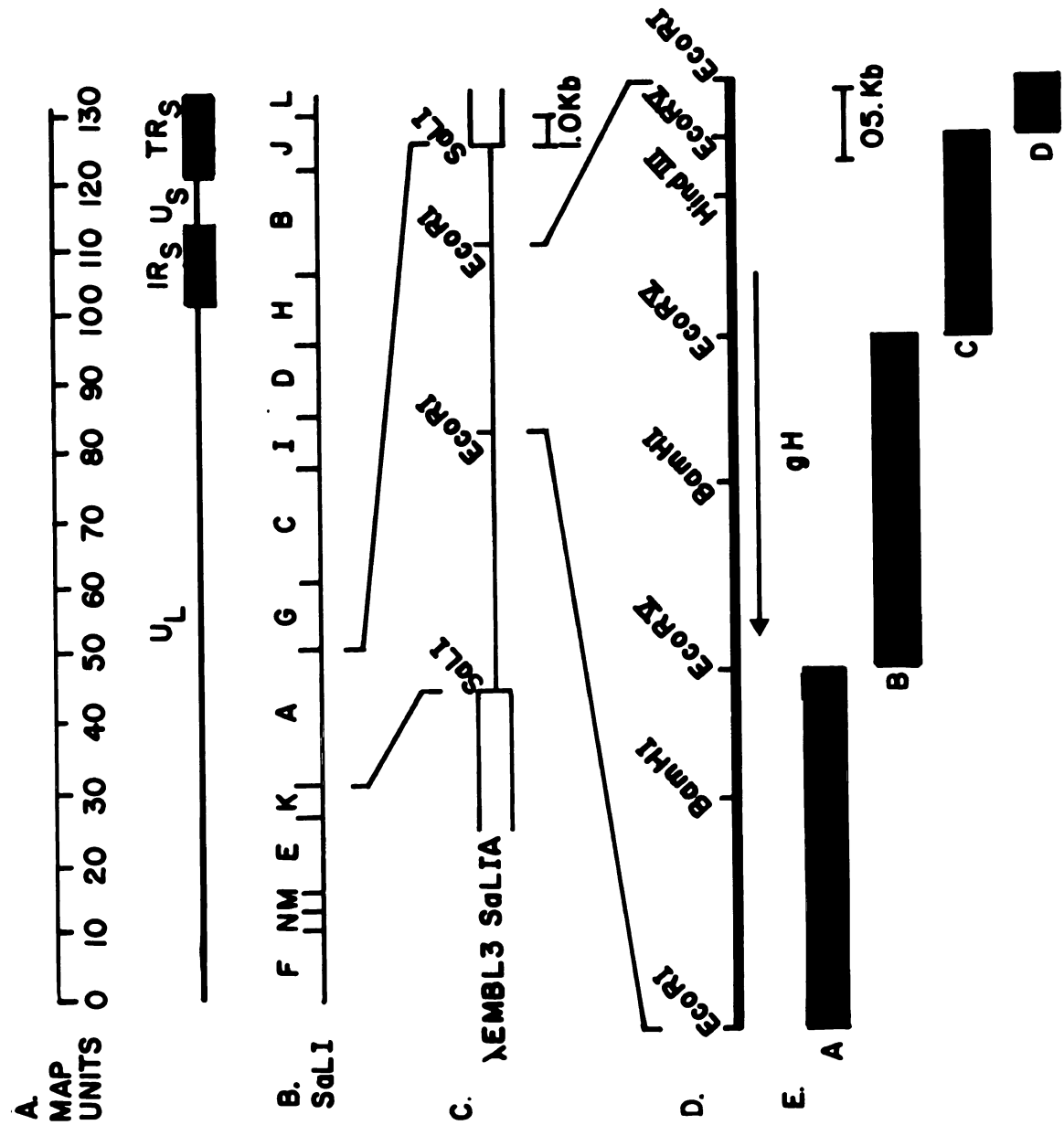

A.
MAP
UNITS
0 10 20 30 40 50 60 70 80 90 100 110 120 130
$U_L$
$IR_S$ $U_S$ $TR_S$
B.
SaLI
F NM E K A G C I D H B J L
C.
λEMBL3 SaLIA
SaLI
EcoRI
EcoRI
SaLI
1.0Kb
D.
EcoRI
EcoRI
HindIII
EcoRI
BamHI
EcoRI
BamHI
EcoRI
gH
E.
A
B
C
D
0.5Kb

Figure 1

Figure 2. **Nucleotide sequence and predicted amino acid sequence of the FHV-1 gH polypeptide and part of the FHV-1 gene product analogous to the thymidine kinase.** Putative CAAT (TAATTGA), TATA (TTTATAA) boxes and polyadenylation sites (AATAAA;TK) and (TATTAAA;gH) are shown in bold. Potential N-glycosylation sites are bracketed by two lines. Two stretches of hydrophobic amino acid residues representing the N-terminal signal peptide and the C-terminal transmembrane domain are overlined.

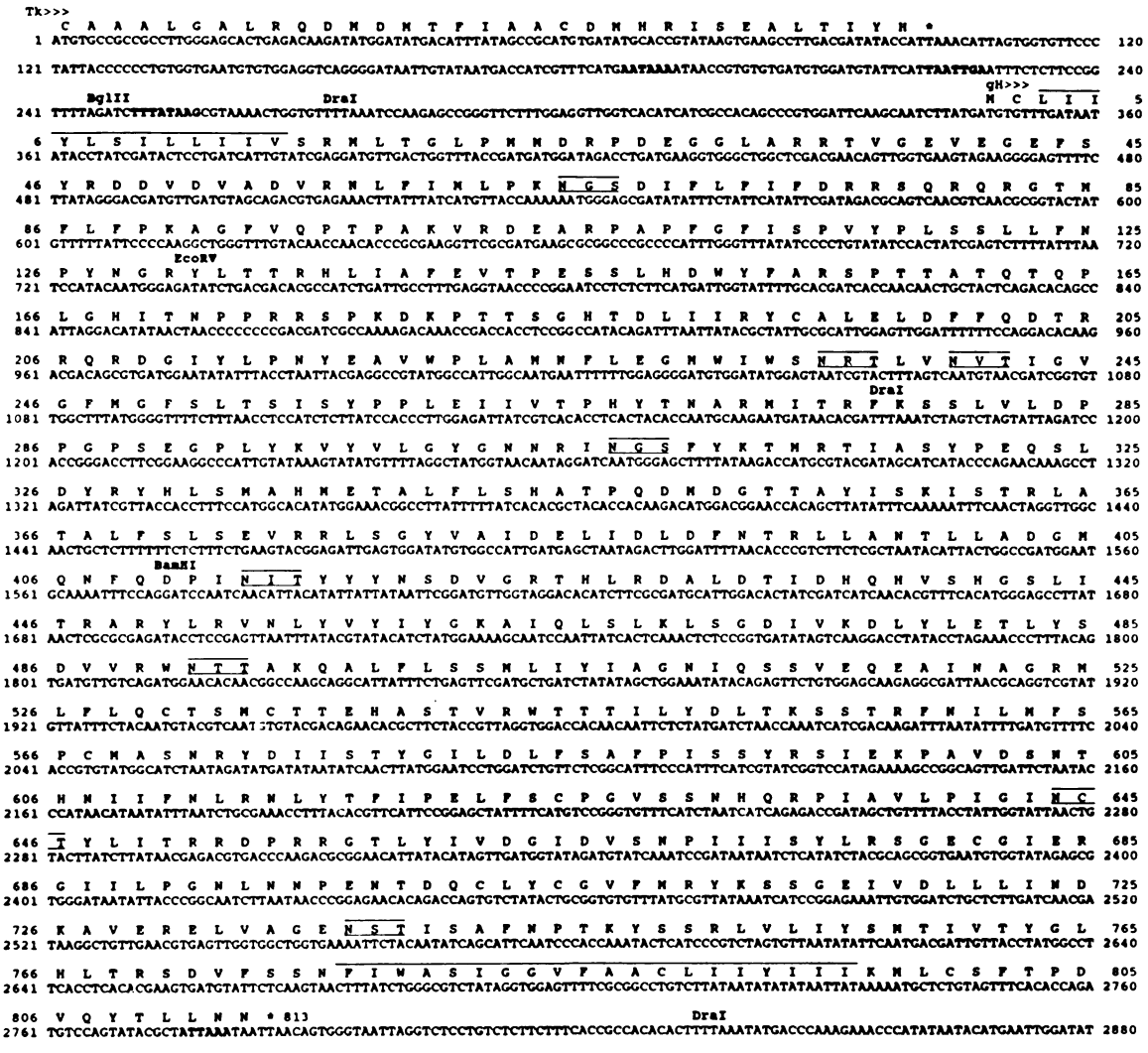



Figure 2

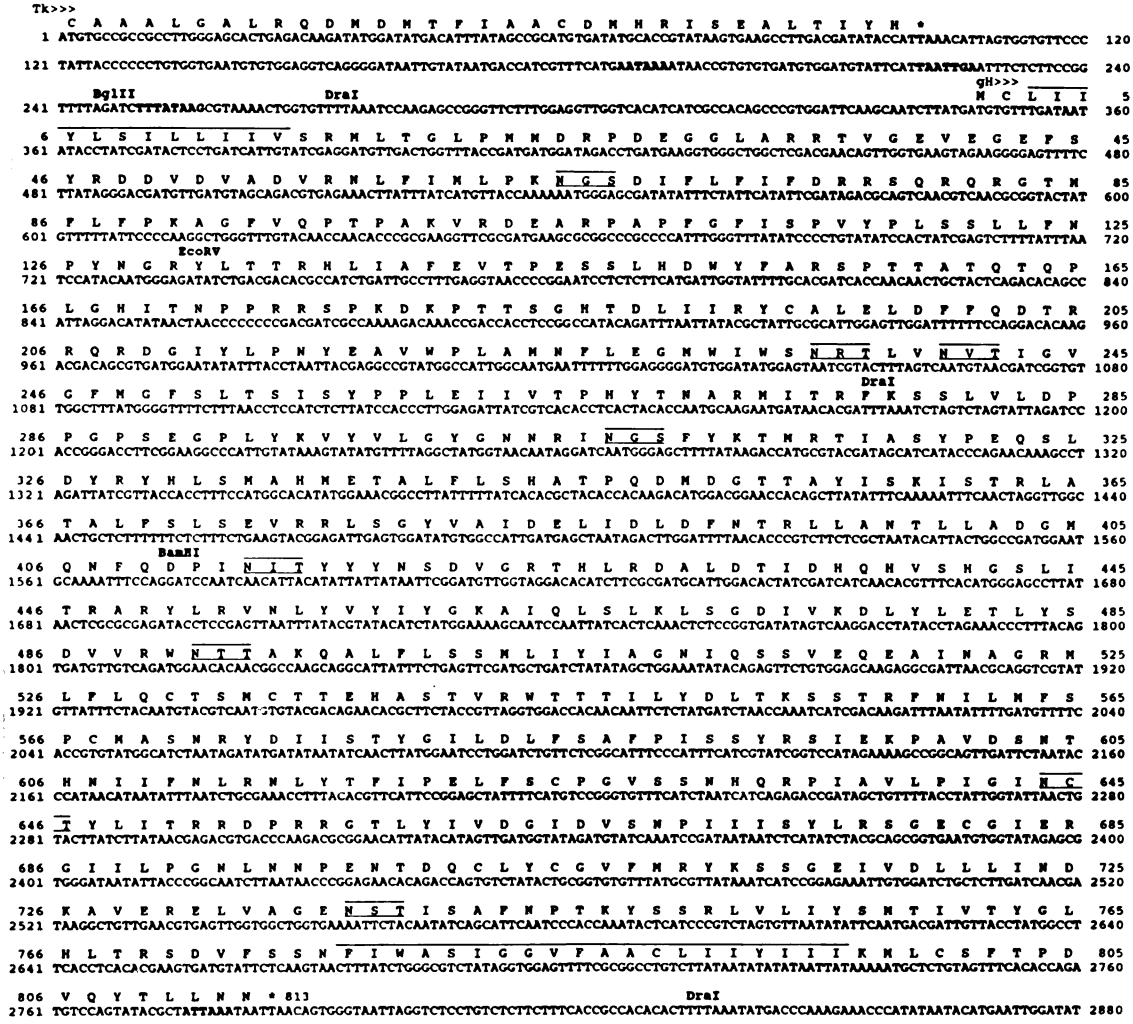



Figure 2

distance of 85 to 95 bp between the CAT box and the mRNA start site has been observed for HSV mRNA (Wagner, 1983). The polyadenylation signal AATAAA is not specified within the 3' noncoding region proximal to the termination codon, TAA. However, a minor polyadenylation signal, (CTATTAAAT), was specified within the extreme 3' terminal coding region of the gene.

**Amino acid sequence and comparison to gH analogous proteins in Herpesviridae**

Two initiation codons, (CTTATGATGTG) were predicted, however only the second codon exhibits features of a strong translation initiation signal: a purine at position -3 (Kozak, 1986). The 2,439 bp ORF encodes a protein 813 amino acids in length. The translated sequence has many characteristics of a transmembrane glycoprotein. Hydrophobicity analyses has identified a hydrophobic sequence near the amino-terminus corresponding to the signal sequence and a region of hydrophobic amino acids (residues 778-796) close to the carboxyl-terminus which may function as a transmembrane anchor sequence.

The putative signal sequence of FHV-1 gH (positions 1-20) is similar in length and composition to other described eukaryotic signal sequences (McGeoch, 1985, von Heijne, 1985). Application of the weight matrix developed by von Heijne (1986) for the prediction of signal cleavage sites indicate that cleavage might occur at $Gly_{20}$, with leucine, threonine, and glycine at positions -3, -2 and -1, respectively. Cleavage

at this site would result in a nonglycosylated protein of 793 amino acids with a predicted MW of 87300. The proposed gH (FHV-1) hydrophilic external domain, residues 21-777, contains eight potential N-glycosylation sites, Asn-X-Thr/Ser, with X being any animo acid except proline and aspartic acid. These sites are bracketed in Fig. 2. One potential gH glycosylation site (NGTV), highly conserved among 10 other herpesviruses, is absent from the polypeptide of gH of FHV-1 (Fig. 3). The polypeptide contains twelve cysteine residues, 9 of which are located in the proposed extracellular domain. Like other gH proteins, the peptide sequence of gH(FHV-1) has a short carboxyl-terminal cytoplasmic region.

Comparison of the predicted amino acid sequence of glycoprotein H of FHV-1 to gHs of other herpesvirus (Table 1) has revealed similarities (GAP program;UWGCG) as follows: 56% with either gH homologs of equine herpesviruses type 1 and 4, 53 to 50% with the gHs of BHV-1, PRV and VZV, and 45% with HSV-1 gH. Comparison to gamma- and betaherpesviruses indicated similarities of 44% with EBV, HVS, and HCMV and of 42% with HHV-6.

Multiple alignments of glycoprotein H homologs of alpha-, beta, and gammaherpesviruses have indicated the greatest diversity of sequence is in the N-terminal region of the proteins. There is a high degree of homology regarding the location of cysteine residues and N-linked glycosylation sites in the carboxyl-terminus. Out of the 11 cysteine residues in gH of FHV-1, 9 are found at colinear positions in gHs of

Table 1. **Homology analyses of gH polypeptides from alpha-, beta-, and gammaherpesviruses.** The GAP program from the genetics package (UWGCG) were used to compare the polypeptides. The values reported indicate the percentage similarities/percentage identities.

Table 1

| | PHV-1 | EHV-4 | EHV-1 | BHV-1 | VZV | PRV | HSV-1 | HCMV | EBV | HVS | HHV-6 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PHV-1 | 100/100 | 56.5/34.9 | 55.9/35.5 | 53.2/31.8 | 50.6/30.4 | 50.0/28.0 | 45.5/24.6 | 44.2/19.8 | 43.8/18.2 | 43.6/18.5 | 42.6/17.4 |
| EHV-4 | | 100/100 | 91.1/85.7 | 54.0/33.5 | 54.0/34.8 | 55.2/31.6 | 48.1/27.8 | 44.7/20.0 | 47.6/22.9 | 47.4/20.4 | 45.4/22.3 |
| EHV-1 | | | 100/100 | 53.8/32.8 | 55.7/34.4 | 53.3/31.3 | 47.6/28.0 | 45.4/22.2 | 46.8/22.6 | 45.8/19.8 | 43.0/21.7 |
| BHV-1 | | | | 100/100 | 53.3/31.1 | 58.4/38.7 | 50.8/27.9 | 40.8/20.1 | 46.5/23.5 | 42.4/17.5 | 42.6/18.6 |
| VZV | | | | | 100/100 | 52.8/31.2 | 47.5/26.8 | 43.6/21.0 | 44.7/21.4 | 44.0/20.3 | 42.5/18.6 |
| PRV | | | | | | 100/100 | 49.8/27.5 | 44.6/20.6 | 43.6/19.0 | 42.5/17.5 | 40.1/19.2 |
| HSV-1 | | | | | | | 100/100 | 41.8/18.8 | 43.9/19.6 | 44.6/19.6 | 42.0/18.5 |
| HCMV | | | | | | | | 100/100 | 45.0/21.2 | 44.7/19.2 | 51.1/25.8 |
| EBV | | | | | | | | | 100/100 | 50.9/26.7 | 43.9/20.7 |
| HVS | | | | | | | | | | 100/100 | 47.3/20.3 |
| HHV-6 | | | | | | | | | | | 100/100 |

Figure 3. **Multiple alignments of two highly conserved regions of glycoprotein H polypeptides for herpesviruses.** Highly conserved residues (>5 residues aligned) are in bold. Totally conserved residues are denoted with asterisks at the bottom of the alignments. The position of the homologous regions on the nascent chain of individual polypeptides is given at the beginning and end of each sequence.

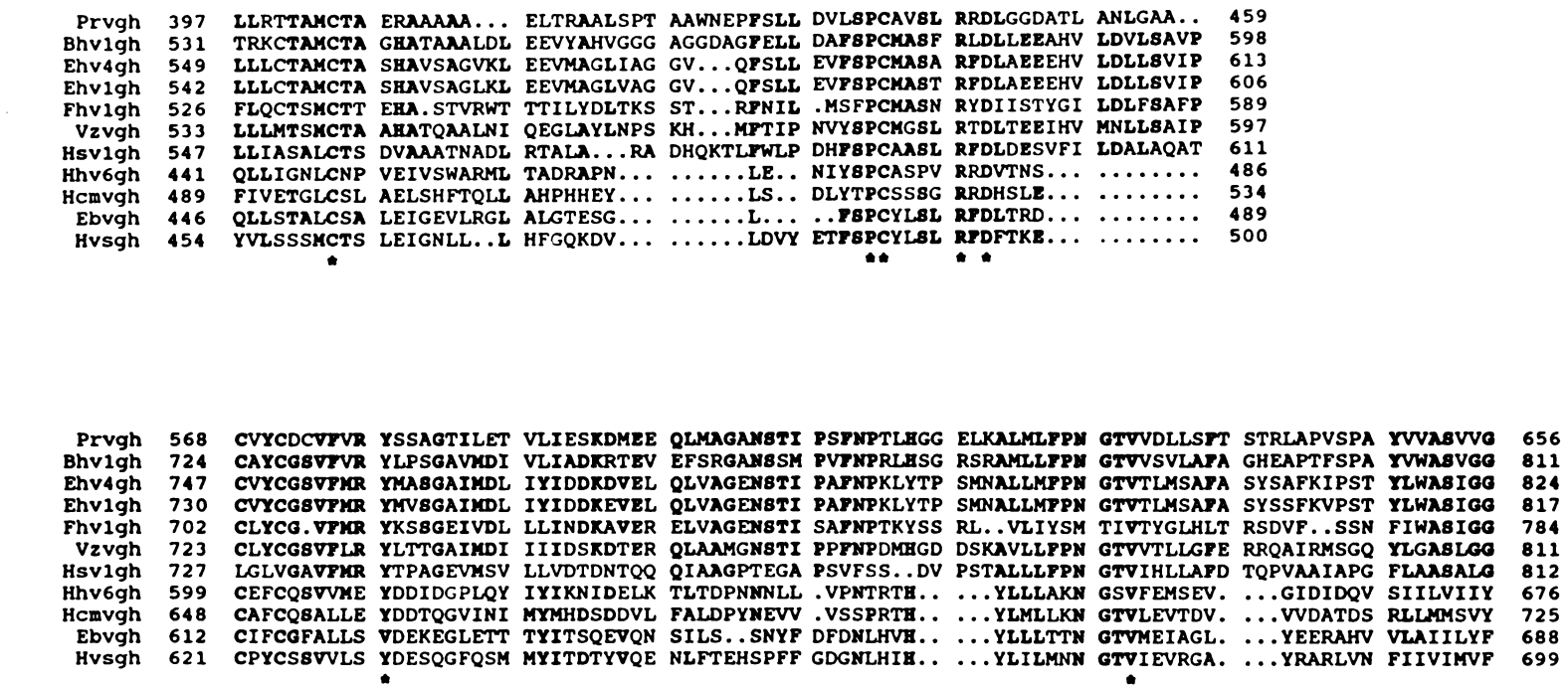


Figure 3

BHV-1, EHV-1, and EHV-4. Although two regions (Figure 3) of amino acid homology, SPC and NGTV, are generally conserved in the gHs identified thus far, FHV-1 does not contain the glycosylation site, NGTV. However, FHV-1 gH does contain the three amino acids stretch, SPC.

To identify transcripts originating from the FHV-1 gH gene, a northern blot (Figure 4) containing total cytoplasmic RNA prepared from FHV-1-infected cells was hybridized with a probe specific for the 3'end of the gH coding region. A major transcript of 2.7 and a minor transcript of 4.0 Kb were detected in RNA isolated late in infection. Based on the nucleotide sequence, the 2.7 Kb transcript is most likely the mRNA encoding gH. No hybridization to uninfected cellular RNA could be demonstrated. On northern blots hybridized with a probe specific for the thymidine kinase gene, two transcripts, 1.5 and 4.0 Kb were detected. Similarly, three transcripts (4.0, 2.7 and 1.5 Kb) were detected on northern blots with probes specific for both the gH and TK genes.

Figure 4. **Northern blot analyses of transcripts detected with gH and TK-specific hybridization probes.** Cytoplasmic RNA was isolated from FHV-1 infected Crandell-Reese feline kidney cells at 10 hrs postinfection. The RNA was fractionated in agarose/formaldehyde gels and electroblotted onto Nytran. Individual blots were hybridized with $^{32}$P-labeled probes as depicted in Figure 1,D. The blots in lanes 1, 2, 3 and 4 were hybridized with radiolabeled fragments (A) 2.5 Kb EcoRI-EcoRV, (B) 2.3 Kb EcoRV-EcoRV (gH-specific), (C) 1.4 Kb EcoRV-EcoRV (TK/gH-specific) and (D) 0.4 Kb EcoRV-EcoRV (TK-specific), respectively.

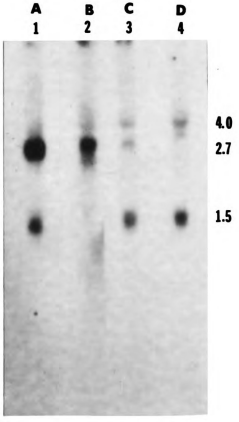



Figure 4

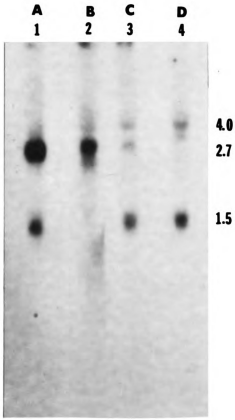



Figure 4

## DISCUSSION

The gene encoding glycoprotein H is the sixth glycoprotein gene localized within the genome of FHV-1. The map location of the FHV-1 gH gene is consistent with the general colinearity of herpesvirus genomes (Davison and Wilkie, 1983). Transcript mapping of the FHV-1 gH mRNA revealed several features. From northern analyses, two transcripts (4.0 and 2.7 Kb) were detected with a gH-specific probe. A TK-specific probe also detected two transcripts of 4.0 and 1.5 Kb. The 2.7 Kb transcript is likely to represent the RNA coding for gH due to the size of the gH ORF (2439 bp) and size range of the mRNA encoding gH of related herpesviruses (2.3-2.7 Kb) (Klupp and Mettenleiter, 1991). It is proposed that the 4.0 Kb transcript detected with both the gH- and TK-specific probes might constitute a bicistronic RNA originating at the TK promoter and terminating at the 3' end of the gH gene. This hybridization pattern, consistent with the occurrence of two overlapping transcripts, has been reported for other herpesvirus glycoprotein genes (Holland et al., 1984; Wagner, 1985; Bell et al., 1990) The occurrence of overlapping transcripts, resulting when the promoter for one mRNA is located within the interior of an upstream mRNA, is suspected to occur for other glycoprotein genes of FHV-1 including ICP18.5/gB, protein kinase/gG, and gD/gI. In these cases, however, terminal mRNA processing signals were absent immediately 3' to the first gene. These signals were clearly

present following the FHV-1 TK ORF. Whether the 4.0 Kb mRNA is the product of accidental transcriptional readthrough and whether it is functional in translating both genes remains to be established. The predicted amino acid sequence of the FHV-1 gene has characteristics of the gHs reported for equine herpesvirus types 1 and 4, bovine herpesvirus-1 and pseudorabies. A high degree of homology could be demonstrated between gHs of FHV-1 and varicella-zoster virus. These similarities between FHV-1 and VZV's glycoproteins were not limited to their gH homologs. Glycoproteins B, I and E of FHV-1 also show surprising relatedness to homologs found in VZV (an alpha-2 herpesvirus), actually greater than that demonstrated with homologs of the herpes simplex viruses (alpha-1 herpesviruses).

Despite a wide variation in base composition among the genomes of the different herpesviruses, which also occurs in their gH genes, alignment of the gH amino acid sequences has demonstrated a pattern of conserved regions or blocks which is likely to have functional significance. Even within divergent sequences near the amino and carboxyl termini all of the eleven gH homologs analyzed have strong hydrophobic domains in the same locations relative to the conserved regions. Four cysteine residues at similar positions relative to the putative transmembrane domain and within conserved local sequences are characteristically conserved in all gHs. However, only two cysteine residues are conserved in this region in HSV-1. Numerous regions of conserved amino acids

could be demonstrated between gHs of herpesviruses within a specific subfamily. This strong conservation between the gHs, second only to the homology found among the gB homologs (Fuller et al., 1989), implies some degree of conservation of the secondary and tertiary structure of these proteins. The tertiary structure of gH is likely to be important in terms of recent reports that (i) recombinant gH of HSV-1 is retained on the nuclear membrane of expressing cells and not present on cytoplasmic membranes, (ii) recombinant gH (HSV-1) is folded incorrectly and not recognized by characterized monoclonal antibodies which recognize conformational epitopes, and (iii) glycoprotein H of HSV-1 forms a complex with gL and this complex is essential for normal folding and surface expression of gH.

Although no immunological or biochemical studies of gH of FHV-1 are presented in this paper, a molecular weight for FHV-1 gH of 107.3 Kd can been calculated for the predicted translation product. This is based upon the fact that gH contains 793 residues and eight glycosylation sites, with each glycan having a MW of 2.5 Kd (Klenk and Rott, 1980). Two FHV-1 glycoproteins of 107-103 Kb are the likely candidates for gH. It is suspected that the 107 Kd protein is gH, since the uncleaved-gB(FHV-1) is likely the 103 Kd glycoprotein. Although it is not known whether the TK/gH transfected cells express glycoprotein H, a 4.0 Kb transcript was detected on northern blots containing RNA isolated from these cells (Data not shown). The 2.7 Kb transcript, thought to encode gH was

not detected on these blots.

The identification and sequence analysis of the FHV-1 gH gene will form the basis for the assessment of the gH protein as a potential vaccine antigen through, for example, the use of poxvirus-vectors or synthetic peptides. In addition, sequence data on conserved genes such as those of the gH family described here are of value in determining evolutionary relationship among the herpesviruses.

# Chapter 5

## Expression of Glycoproteins B and D of Feline Herpesvirus Type 1 in Vaccinia and Raccoon Poxviruses

Stephen Spatz

**ABSTRACT**

The genome of feline herpesvirus -1, the major cause of viral upper respiratory disease in cats, contains several genes encoding HSV-1 homologs of glycoprotein B, D, H, G, I and E. Research involving HSV-1, PRV, EHV-1 and other alpha-herpesviruses has indicated that both glycoproteins B and D are important immunogens, eliciting high titers of virus neutralizing antibodies and cell-mediated immunity. Animals vaccinated with adeno- or poxviruses expressing these glycoproteins have been reported to be protected against the establishment of latency by the virulent challenge strain. To improve on current modified live viral vaccines against feline rhinotracheitis, we have amplified the gB and gD genes of FHV-1 using PCR, and cloned the amplified products into a donor plasmid containing the right and left termini of the vaccinia thymidine kinase gene. Rescue of these constructs into the genome of either vaccinia or raccoon poxvirus generated recombinants that reacted with rabbit anti-FHV-1 serum in an indirect fluorescent antibody test. High titers of virus neutralizing antibodies were generated in rabbits inoculated with vaccinia recombinants expressing either FHV-1 gD or gB. Western blot analyses with potassium tartrate-purified virions and antisera against the vaccinia recombinants have indicated the presence of a 60 Kd (gB) and a 50 Kd (gD) polypeptide. Presented in this report are the construction of the recombinants and preliminary immunological studies.

## INTRODUCTION

Feline rhinotracheitis is a common viral infection in cats, occurring worldwide. The cause is an alphaherpesvirus designated feline herpesvirus-1 (FHV-1). Clinical signs are mostly upper respiratory in nature (Povey, 1979; Maes et al., 1984). Serological studies have indicated that 50-70% of adult domestic cats have detectable antibodies to this virus (Tham et al., 1987). The pathobiology of the virus has been reviewed by Povery (1979). Like other herpesviruses, FHV-1 establishes a latent infection in ganglia. In the case of FHV-1 these latent infections are very easily reactivated. The prevalence and seriousness of the disease is largely controlled by the use of licensed modified-live virus (MLV) vaccines. Although clinical disease is less severe and of shorter duration in vaccinated cats, vaccinates can still develop clinical signs when exposed to challenge virus. Another shortcoming of the existing vaccines is that they do not prevent reinfection. The result of this is that vaccinated, asymptomatic cats that are exposed to virulent virus will become latently infected with the virulent virus. Reactivation and subsequent shedding of this virulent virus results in the perpetuation of the disease, especially in multiple cat households.

Recent advances in molecular biology have been applied to developing new strategies to vaccinate cats against feline viral rhinotracheitis. Using affinity chromatography with FHV-1 specific monoclonal antibodies against the glycoproteins,

Limcumpao et al., (1991) isolated three glycoproteins and ascertained their relative immunogenicity in mice. All glycoproteins (143/108 Kd, 113 Kd and 60 Kd) induced detectable levels of neutralizing antibodies. Although no challenge studies in cats were conducted by this group, Benoit et al., (1983) was able to induce a high level of protection in cats vaccinated with a hydrosoluble fraction of the FHV-1 virus particle.

We have previously identified the genes encoding the major immunogens of FHV-1: gB, gD, gE, gH, gG, gI and gE, all of which are generally conserved in alphaherpeviruses. Studies involving immunogenicity and induction of protective immunity of the individual glycoproteins of HSV-1 and PRV have indicated that glycoprotein D recombinants (i) induced the highest neutralizing antibodies titers, (ii) increased the rate of HSV-1 clearance and (iii) provided good protection against latency. Glycoprotein B also stimulated good neutralizing antibody titers and as good a protection from the establishment of latency. The rate of virus clearance in animals vaccinated with gB/vaccinia recombinants was, however, not as great as after gD/vaccinia immunization. Based upon these previously reported results, we have expressed the genes encoding FHV-1 gB and gD in vaccinia and the related orthopoxvirus Raccoon Poxvirus (RPV) (Moss and Flexner, 1987; Knight et al., 1992). Selection of raccoon poxvirus as a vector was based upon a report that high titers of neutralizing antibodies were generated in cats infected with

raccoon poxvirus. Furthermore, no adverse reactions were observed in the vaccinated cats (Scott, 1988). Recently, the usefulness of raccoon poxvirus as a vaccine vector was demonstrated by the generation of RPV recombinants expressing the nucleocapsid and G glycoprotein of rabies (Esposito, et al., 1988; Lodmel et al., 1991). Raccoon, skunks, and mice immunized with the either recombinant were protected when challenged with lethal raccoon rabies street virus (STV) (Fekdau et al., 1991). Oral rabies immunization of free-ranging raccoons with these recombinants has recently been approved for release on the barrier islands of South Carolina (Hable et al., 1992; Linhart et al., 1991).

The FHV-1 gB/gD raccoon poxvirus recombinants described in this paper are expected to be more immunogenic than MLV vaccines. In addition they should elicit better protection against reinfection and subsequent latency establishment. Poxvirus recombinants will also offer additional savings in storage and shipment costs of FVR vaccine, due to the higher stability of these recombinants over MLV vaccines.

## MATERIAL AND METHODS

### Cells and viruses

Crandell Reese feline kidney (CRFK) cells cultured in Eagle's Minimum Essential Medium (MEM), supplemented with antibiotics (100 Units/ml Penicillin and 100 ug/ml Streptomycin) and 10% fetal bovine serum (FBS) were used to propagate FHV-1, strain C-27. Rat-2 and human 143B cells, both thymidine kinase negative (TK-), were grown in the same medium. Vaccinia virus strain Wyeth, raccoon poxvirus and recombinant viruses derived from both were propagated initially on 143B cells and plaque purified on Rat-2 cells in the presence of 25 ug/ml of 5-bromo-2'deoxyuridine (BUdR).

### PCR-amplification and Plasmid Construction

The complete coding sequences of FHV-1 glycoproteins B and D were amplified using flanking oligonucleotides specific for the 5' and 3' ends of each gene. Oligonucleotides were synthesized using a 380B automated DNA synthesizer (Applied Biosystems) with a three column upgrade.

The gene encoding glycoprotein B was amplified using two primers, 5' TAC CTC GAG TCA TGT CCA CTC GTG GCG ATC 3' and 5' GGT CTC GAG GGT TAG ACA AGA TTT G 3'. Each primer contained an XhoI recognition sequence, which facilitated the cloning of the amplified 2.8 Kb product. A 3.3 Kb SstI fragment containing the complete gB gene was excised from agarose gels and used as the amplification template. The template (100ng)

was boiled for 2 minutes and 50 pmoles of each primer was allowed to anneal to the template until the temperature was 50°C. The conditions for 37 cycles of amplification were as follows: 2 minutes at 53° C, 5 minutes at 72°C and 1 minute at 95°C. One unit of pfu polymerase (Stratagene) was used in a buffer containing 20 mM Tris-HCl (pH 8.2), 10 mM KCl, 6.0 mM $(NH_4)_2SO_4$, 2.0 mM $MgCl_2$. 0.1% Triton X-100 and 10 ug/ml BSA.

To amplify the gene encoding gD of FHV-1, two oligonucleotides, 5' CAT CTC GAG TAA TGA TGA CAC GTC TAC A 3'and 5' TGT GAA TTC AAG GAT GGT GAG TTG TA 3' were used. The later oligonucleotide contained an XhoI recognition site, while the former contained an EcoRI recognition site. Incorporation of these two restriction sites into the amplified PCR-product facilitated directional cloning. The PCR buffer was identical to the one mentioned above, while the PCR conditions differed slightly; 1 minute at 60°C, 2 minutes at 72°C and 1 minute at 95°C for 37 cycles with one unit of pfu polymerase (Stratagene).

Both gB and gD PCR-amplified products were digested with the appropriate restriction endonucleases (XhoI for the gB gene and XhoI/EcoRI for the gD gene) and cloned into pKG19 (a gift from Dr. Paul Rota).

**Transfections and Selection**

Recombinant plasmid DNA was purified from transformed DH5 alpha cells using alkaline lysis (Ausubel et al., 1988). These DNA were further purified by centrifugation in cesium chloride

ethidium bromide gradients. The purified plasmid DNA was then used to transfect human 143B TK- cells using the lipofectin method (BRL). One hour prior to transfection, the cells grown to 75% confluency in 35 mm plates, were washed with 2X OptiMEM I medium without serum. The cells were then infected with either vaccinia or raccoon poxvirus using a m.o.i. of <1.0. Lipid/DNA complexes were created by mixing 25 ul of $H_2O$, 25 ul of lipofectin (approx. 30 ug) and 50 ul of recombinant plasmid DNA containing 20 ug. This mixture was incubated for 15 minutes at room temperature before addition to the infected cells. After absorption at 37°C for 4-6 hours in a 4% $CO_2$ atmosphere, the cells were fed with MEM containing 10% FCS and incubation was continued for 48 hours. Transfected cells were then pelleted using low-speed centrifugation and resuspended in 1.0 ml of Mandel's solution. Three cycles of freeze/thawing with vortexing between each cycle were used to release the cell-associated virions. Serial 10-fold dilutions of the viral supernatants were made and Rat-2 cells were infected for 1 hour at 37°C. Following this, 3.0 ml of 1% LMP agarose (45°C) containing 1X MEM and 25 ug/ml BUdR were overlaid on the cells. After an incubation at 37°C for 48 hours, 3.0 ml of 0.5% neutral red in 1X PBS) was added and the cells were stained for < 3.0 hours. Visible plaques were picked and resuspended in 500 ul of 1X Mandel's solution. Three cycles of freeze/thawing were used to release cell-associated virions. Recombinants were plaque purified 3 times, always in the presence of BUdR.

**Immunofluorescence**

Indirect immunofluorescence tests were carried out on transfected cells cytocentrifuged onto glass slides. Cells were fixed with cold absolute methanol for 15 minutes and then blocked with 5% low-fat milk powder in 1X PBS for 1 hour. The cells were incubated with a 1/100 dilution of rabbit anti-FHV-1 diluted in 1X PBS. After an hour incubation at room temperature, the cells were washed twice with 1X PBS for 15 minutes each. A goat anti Rabbit FITC conjugate was diluted in 1X PBS containing 0.1% Evan's Blue and applied to the cells for 30 minutes. The cells were then washed and fluorescence was observed using a Zeiss UV microscope. Photographs were taken with Kodak Ektachrome daylight 1,000 ASA film.

**Production of Anti-vaccinia Recombinant and Anti-FHV-1 Sera**

Female New Zealand white rabbits were injected intraperitoneally with $10^7$ PFU of vaccinia recombinants (VVgB and VVgD) in 500 ul of 1X PBS. Serum was collected 14 days after inoculation and analyzed on immunoblots containing wild-type vaccinia. The rabbits were then boosted with $10^7$ PFU of the respective recombinant and bled 2 weeks later. Similarly, rabbits were injected (i.p.) with $10^6$ $TCID_{50}$ of FHV-1 (C-27) and boosted three weeks later.

**Western Blot Analyses**

FHV-1 virions from infected CRFK cells were purified by rate zonal centrifugation through 10-40% potassium tartrate

gradients (Talens and Zee, 1976). Purified virions were resuspended in 1X PBS and separated by SDS-PAGE. Immunoblotting was done according to procedures described by Ausubel et al., 1988, using 5.0% low fat milk powder as a blocking agent. Alkaline phosphatase-labeled anti rabbit conjugates, along with the chromogens BCIP and NBT, were used to visualize the bands.

**Virus Neutralization Assay**

Antisera, each specific for the vaccinia recombinants (VVgB and VVgD), were assayed for the presence of virus-neutralizing antibodies by a microneutralization assay. Briefly, heat inactivated (56°C, 30') sera were used to make a two-fold dilution series. Approximately 100 $TCID_{50}$ of FHV-1 (C-27) was added to each dilution. The virus-serum mixtures were incubated for 1 hour at 37°C. CRFK cells (15,000) were added to each well and the plates were incubated at 37°C in an atmosphere of 5% $CO_2$ in air. The VN titers were expressed as the reciprocal of the highest serum dilution resulting in complete inhibition in CPE.

## RESULTS

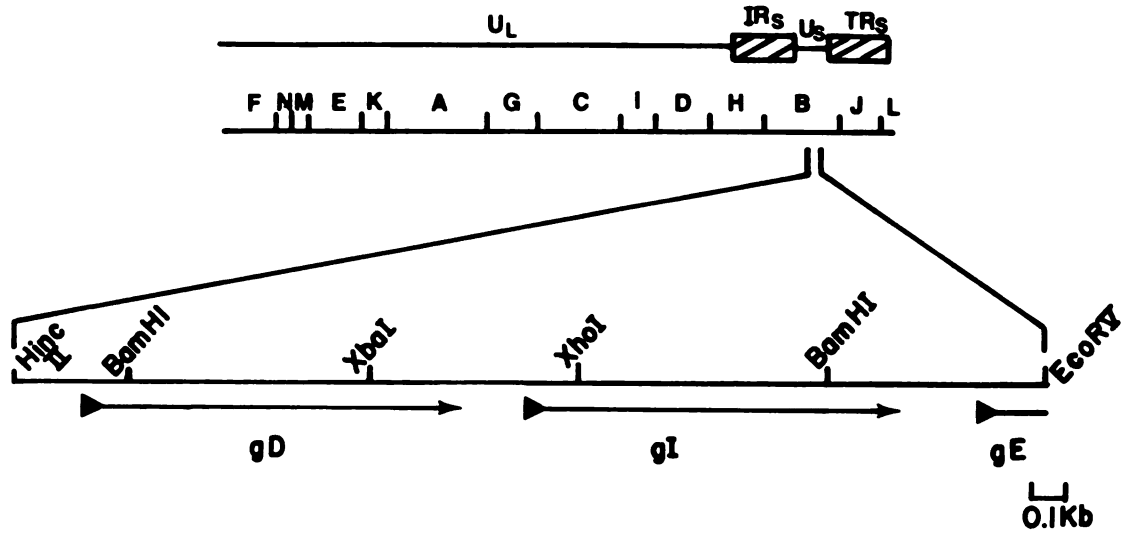
### Construction of recombinants vaccinia and raccoon poxviruses expressing glycoprotein B and D of FHV-1

The genomic location of the genes encoding glycoproteins B and D of FHV-1 is illustrated in Figure 1. The gene encoding FHV-1 gB is located within a 3.3 Kb SstI subfragment of the larger SalI G. The SalI B (14.5 Kb) fragment from the unique short region encodes glycoprotein D. The gene is confined to a 1.5 Kb HincII-XhoI subfragment. These subfragments were purified and used as the templates for the amplification of both genes. Amplification of the gB and gD genes resulted in a 2.8 and 1.1 Kb PCR-product, respectively (Figure 2). Restriction analyses of the gD PCR-product is presented in Figure 3. Both amplified products were cloned into the vaccinia-thymidine kinase donor plasmid (pKG19) as depicted in Figure 4. Restriction endonuclease analysis (Figures 5 and 6) of the recombinants (pKGgD and pKGgB), respectively, verified the authenticity of the cloned gD gene (pKGgD) and indicated the two possible orientations of the cloned gB product.

Recombinant donor plasmids were transfected into vaccinia or raccoon poxvirus infected Human 143B TK- cells. The number of BUdR resistant recombinants obtained was higher for vaccinia than the slower growing raccoon poxvirus. Actually, due to the slow growth of recombinants in the human cells and the fact that plaquing morphology was difficult to determine, RPV-recombinants were plaqued on Rat-2 TK- cells. Compared to

Figure 1. **The genomic organization of the genes encoding glycoproteins B and D of FHV-1 (C-27).** The genome of FHV-1, containing two unique regions ($U_L$ and $U_S$) with inverted repeats bracketing the $U_S$ region (Rota et al., 1986) is presented along with the genomic positions of the genes encoding gD and gB.

(A) A detailed restriction map of the 2.9 Kb HincII-EcoRV fragment from the $U_S$ region of the genome. This region contains the genes encoding gD, gI and part of gE. (B) A restriction map of the 3.3 Kb SacI fragment containing the genes encoding gB. This fragment maps within the $U_L$ region of the FHV-1 genome.


UL
IRS
US
TRS
F NM E K A G C I D H B J L
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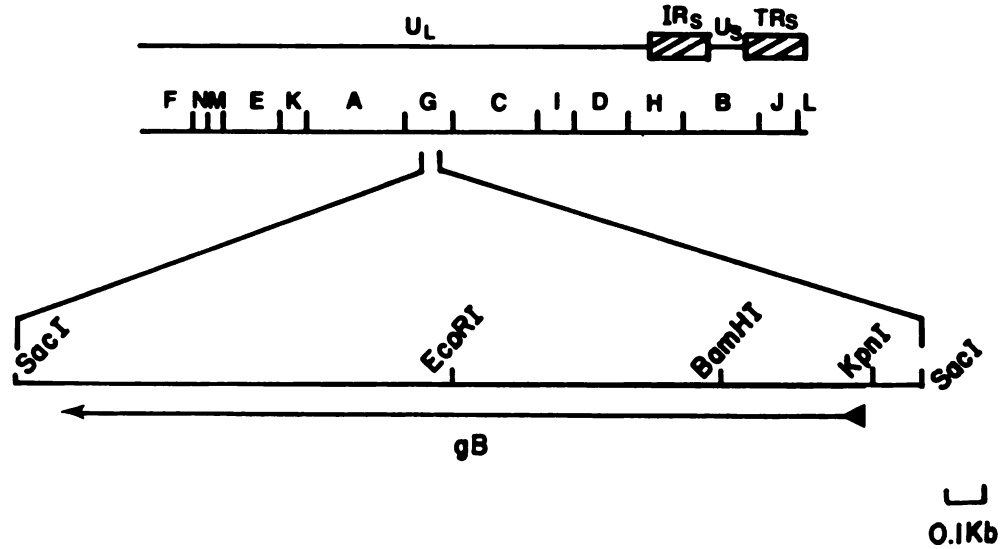
BamHI
XbaI
XhoI
BamHI
EcoRI
gD
gI
gE
0.1Kb


UL
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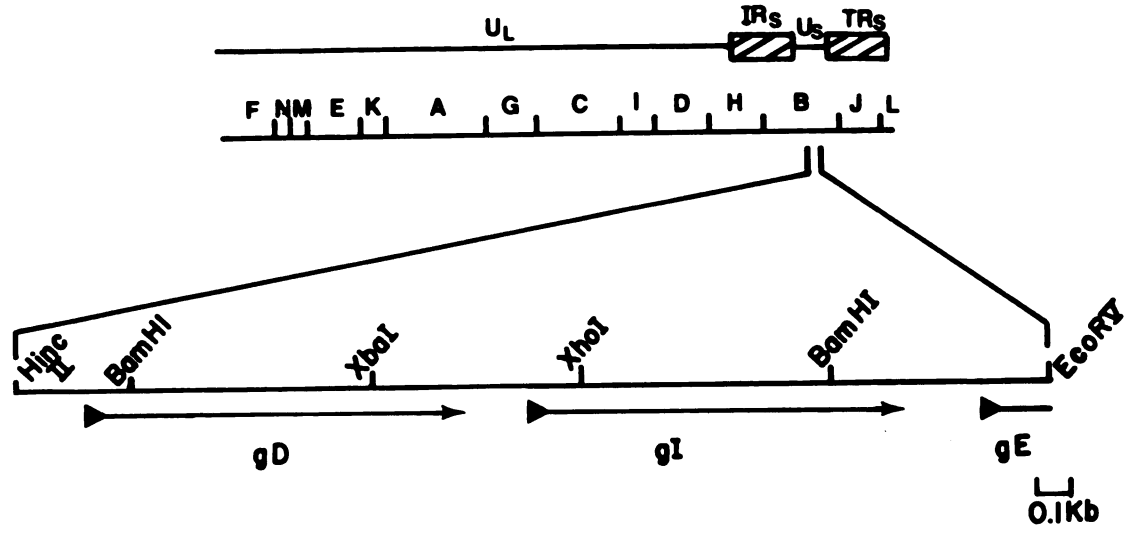
SacI
EcoRI
BamHI
KpnI
SacI
gB
0.1Kb

Figure 1


UL
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XhoI
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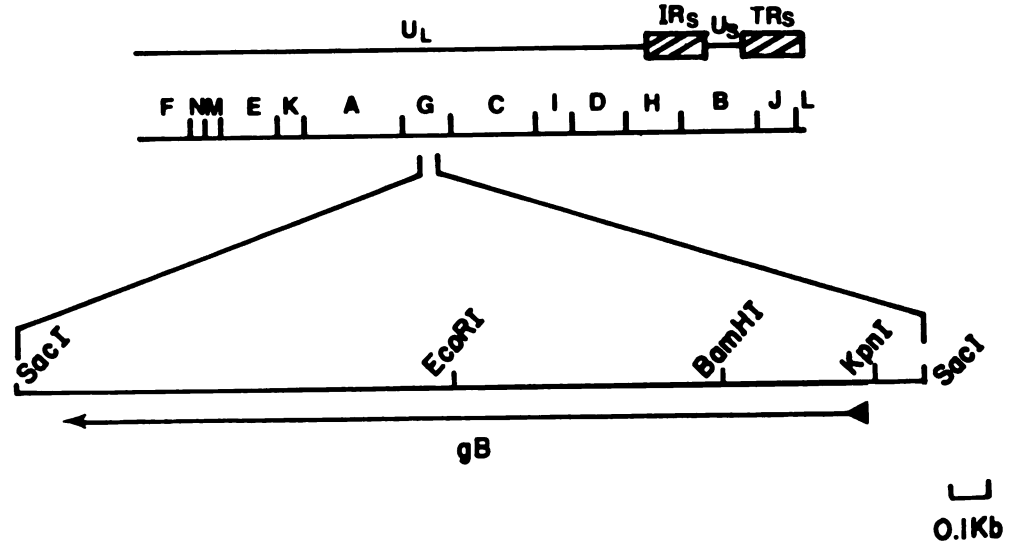
EcoRI
gD
gI
gE
0.1Kb


UL
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SacI
EcoRI
BamHI
KpnI
SacI
gB
0.1Kb

Figure 1

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lane

B).

Figure 2. **Visualization of the PCR-amplified gD and gB products.** Presented are a photographs of an EtBr-stained agarose gel containing electrophoretically separated fragments from the amplification of 1.14 Kb gD (A) and 2.8 Kb gB (B) lanes 1-3. Molecular weight standards are in lanes 4 (A and B).

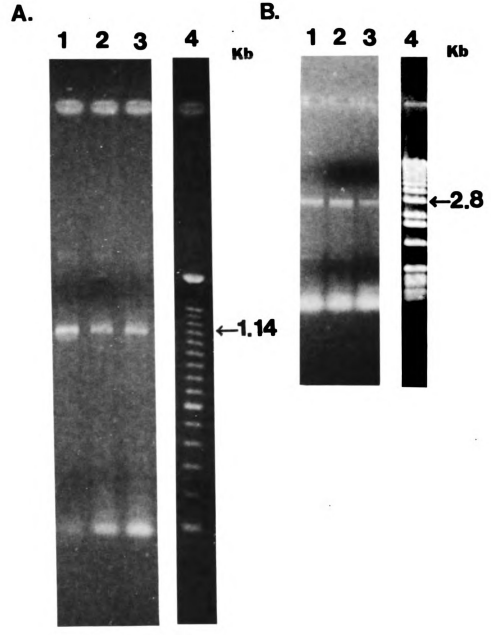


Figure 2

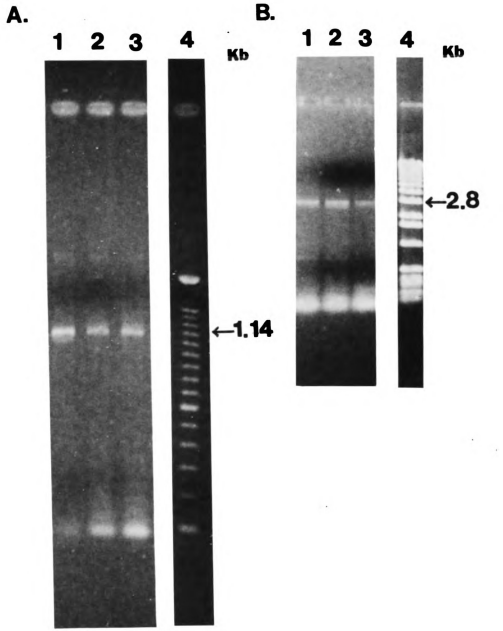


Figure 2

Figure 3. **Restriction analysis of the gD PCR-product.** (A) Computer-predicted restriction maps based on nucleotide sequencing of the gD gene. (B) Visualization of an agarose gel containing restriction endonuclease digested PCR-products. Prior to electrophoresis, these products were digested with the following enzymes: BamHI (lane A), EcoRI (lane B), ClaI (lane C), SstI (lane D) and KpnI (lane E).

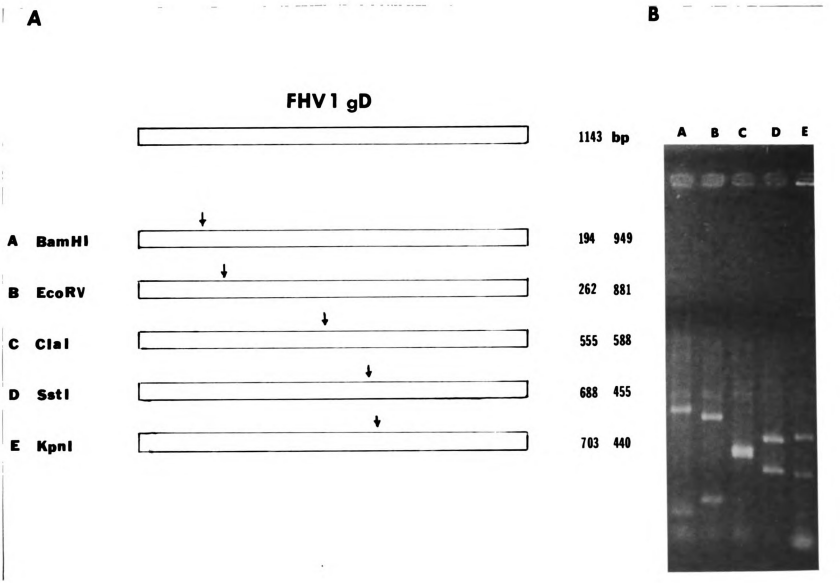
A
B
FHV 1 gD
1143 bp
A BamHI
194 949
B EcoRV
262 881
C ClaI
555 588
D SstI
688 455
E KpnI
703 440
A B C D E

Figure 3

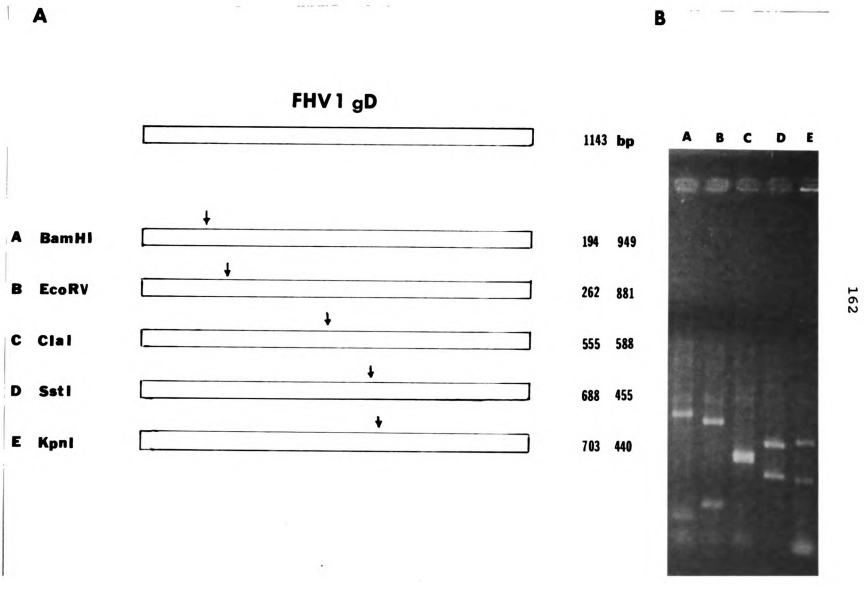
A
B
FHV1 gD
1143 bp
A BamHI
194 949
B EcoRV
262 881
C ClaI
555 588
D SstI
688 455
E KpnI
703 440
A B C D E

Figure 3

Figure 4. **Constructs of the recombinant plasmids pKGgD and pKGgB.** Recombinants were generated via cloning restriction digested PCR-amplified products into the donor plasmid pKG19. Relative restriction sites are indicated along with the molecular size of each construct.

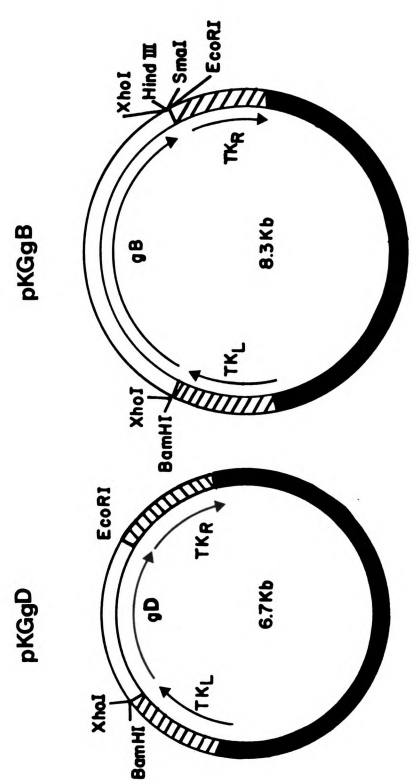
pKGgB
XhoI
Hind III
SmaI
EcoRI
TK_R
gB
8.3Kb
TK_L
XhoI
BamHI
pKGgD
EcoRI
TK_R
gD
6.7Kb
TK_L
XhoI
BamHI

Figure 4

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Figure 5. **Restriction analysis of the recombinant plasmid, pKGgD.** Visualization of an EtBr-stained agarose gel containing digested recombinant plasmid, pKGgD. Prior to electrophoresis, the recombinant DNA was digested with restriction endonucleases XhoI and EcoRI (lanes 1-6; MW standard, lane 7).

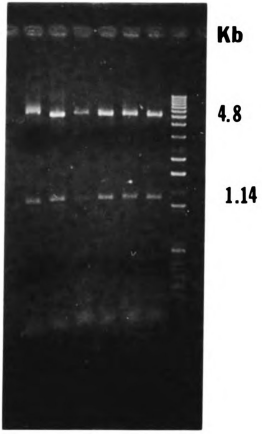



Figure 5

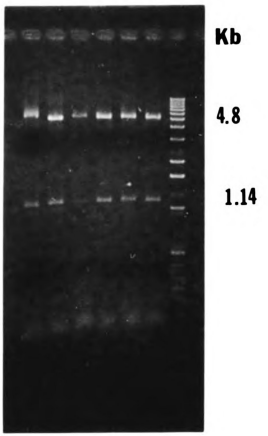



Figure 5

Figure 6. **Analyses of the recombinant donor plasmids, pKGgB and pKGrgB.** Restriction endonuclease analyses of the PCR-amplified FHV-1 gB gene cloned into the donor plasmid pKG19. Recombinant plasmid (A) pKGgB, contained an insert in the correct orientation with respect to the Vac $P_{7.5}$ promoter. The control plasmid (B) pKGrgB, contained an inverted insert. Computer-generated restriction maps of the recombinant plasmids are shown with a photograph of an EtBr-stained agarose gel containing the results of various restriction digestions.

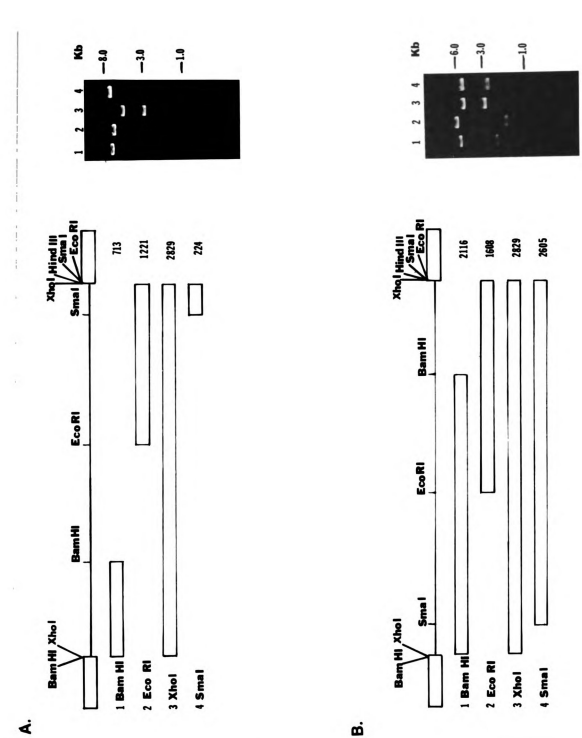
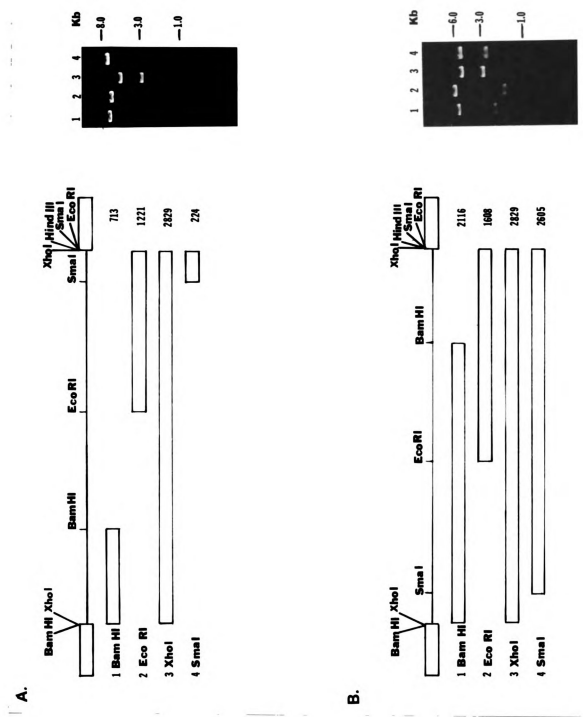

A.
BamHI XhoI
BamHI
EcoRI
XhoI HindIII SmaI EcoRI
SmaI
1 Bam HI
2 Eco RI
3 XhoI
4 SmaI
713
1221
2829
224
1 2 3 4 Kb
8.0
3.0
1.0
B.
BamHI XhoI
SmaI
EcoRI
BamHI
XhoI HindIII SmaI EcoRI
1 Bam HI
2 Eco RI
3 XhoI
4 SmaI
2116
1608
2829
2605
1 2 3 4 Kb
6.0
3.0
1.0

Figure 6

A.


A.
Bam HI Xho I
Bam HI
Eco RI
Xho I Hind III Sma I Eco RI
Sma I
1 Bam HI
2 Eco RI
3 Xho I
4 Sma I
713
1221
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224
Kb
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1 2 3 4
B.
Bam HI Xho I
Sma I
Eco RI
Bam HI
Xho I Hind III Sma I Eco RI
1 Bam HI
2 Eco RI
3 Xho I
4 Sma I
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6.0
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1 2 3 4

Figure 6

to the human 143B cells, the Rat-2 cells formed a better cell monolayer and RPV formed plaques in these cells within 4-5 days.

**Expression of glycoproteins B and D in transfected Rat-2 cells**

Transfected cells were analyzed for the expression of FHV-1 gB and gD using an indirect fluorescent antibody assay. As shown in Figure 7, cytoplasmic staining was observed only in transfected cells previously infected with either vaccinia or raccoon poxviruses. Fluorescence was not observed in the controls. Cells infected with wild type vaccinia and raccoon poxvirus served as one form of negative control. The other control consisted of cells transfected with vaccinia or raccoon poxvirus donor plasmids containing an inverted gB gene with respect to the vaccinia 7.5 promoter.

Western blot analyses (Figure 8) were done with rabbit antisera against VVgB and VVgD and potassium tartrate-purified FHV-1 virions. Major immunodominant bands of 60 Kd (cleaved form of gB) and 50 Kd (gD) were detected at a 1/400 dilution of the polyclonal sera. FHV-1 proteins did not react with an antiserum specific for WT- vaccinia virus (Data not shown). Rabbits immunized with VVgB or VVgD had VN titers of 64 and 1024, respectively in a microneutralization assay.

Figure 7. **Indirect fluorescent antibody assay.** Visualization by immunofluorescence of gB and gD synthesized in vaccinia or raccoon poxviruses infected Rat-2 cells. Photographs (A-C) represent cells infected with Vaccinia virus and then transfected with DNA from plasmids; pKGgB (panel A), pKGgD (panel B) and pKGrgB (panel C). Photographs (D-F) represent cells infected with Raccoon poxvirus and then transfected with pKGgB (panel D), pKGgD (panel E),and pKGrgB (panel F). Fixed cells were treated with a rabbit anti-FHV-1 antibody, followed by a fluorescein isothiocyanate conjugated goat anti-rabbit antibody. The final magnification was 250X.

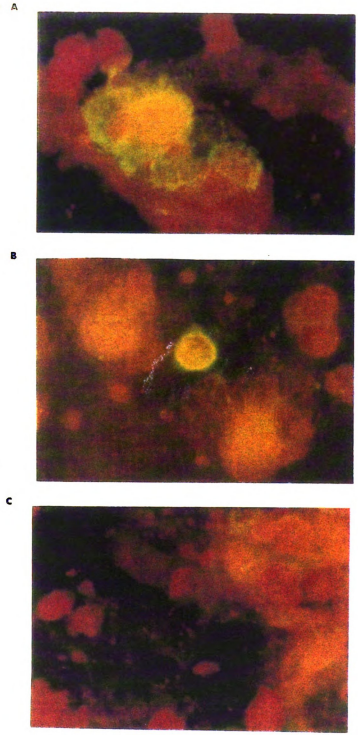


Figure 7

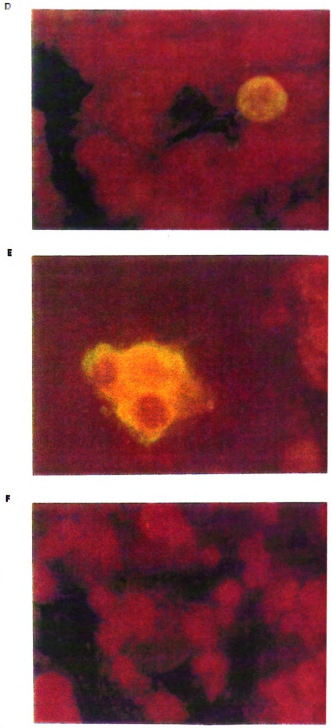


Figure 7 (Cont)

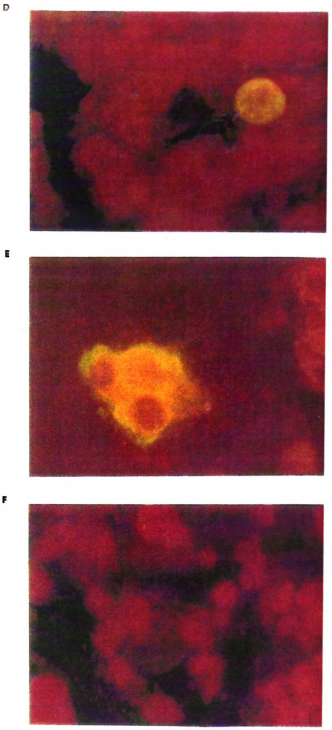


Figure 7 (Cont)

Figure 8. **Western blot analyses of FHV-1 polypeptides with rabbit antisera against VVgB and VVgD.** Denatured purified virions were separated using SDS-PAGE and electrophoretically transferred to Nytran. Rabbit anti-VVgB and anti-VVgD sera were used to probe blots A and B, respectively. A rabbit anti-FHV-1 sera was used to probe blot C. Mouse anti-rabbit alkaline phosphatase labelled conjugates were used as the secondary antibody.

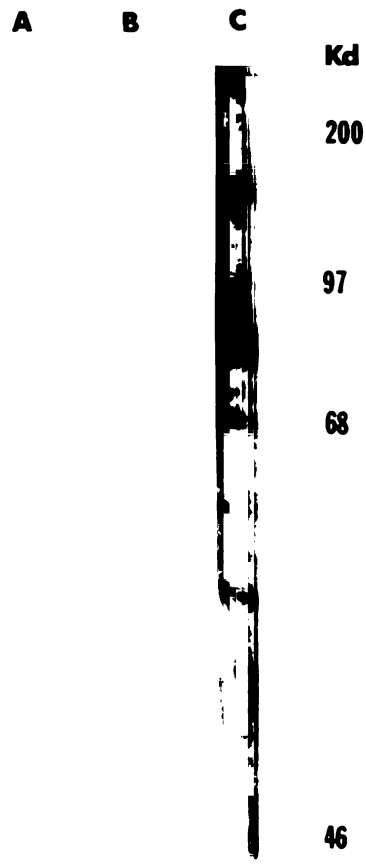


Figure 8

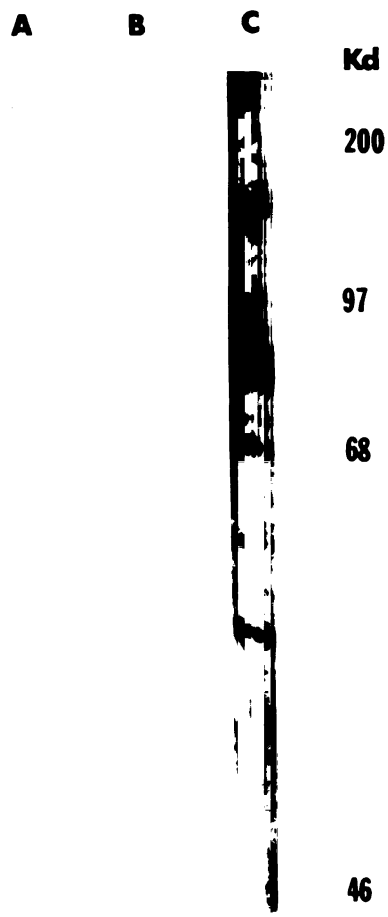


Figure 8

## DISCUSSION

Feline herpesvirus-1 is an important viral pathogen of cats. Current vaccines can protect against clinical disease, but not against infection and latency. It is clear from work with other herpesviruses that viral glycoproteins are the obvious candidates for inclusion in newer vaccines. The development of poxvirus as a eukaryotic expression vector capable of adequate expression of a variety of herpesvirus genes has resulted in the construction of live poxvirus recombinants that are capable of protecting immunized animals against infection with HSV-1, HSV-2, PRV, EHV-1 and MDV (Cantin et al., 1987; Paoletti et al., 1984; Marchioli et al., 1987; Yanagida et al., 1992; Britt et al., 1990; Bell et al., 1990; Blacklaws et al., 1990)

Extensive research involving glycoproteins B and D of HSV-1 and their respective homologs in animal herpesviruses has demonstrated the importance of these glycoproteins in both humoral and cell-mediated immunity. A recombinant vaccinia virus expressing glycoprotein D of HSV-1 was the first genetically engineered vaccine which could prevent the development of latency in mice by virulent challenge (Cantin et al., 1987)

In this report, we describe the construction of four recombinant poxviruses expressing either gB or gD of FHV-1. The genes encoding these glycoproteins were rescued via homologous recombination into the genomes of both vaccinia and

raccoon poxviruses. Donor plasmids used were derived via cloning of gB- and gD-PCR products into the donor plasmid pKG19. Transfected Rat-2 (TK-) cells, previously infected with either vaccinia or RPV, expressed the appropriate glycoprotein as detected by immunofluorescence with rabbit anti-FHV-1 sera. The fact that donor plasmids containing an inverted gB gene could be rescued into the genomes of these two poxviruses demonstrated the success of the BUdR selection.

Immunogenicity of recombinant gB and gD polypeptides was demonstrated by the response of immunized rabbits whose sera reacted specifically to proteins of KT- purified FHV-1. Using western blot analysis, two polypeptides (60 and 50 Kd) from purified virions were detected using antisera from rabbits inoculated with either VVgB or VVgD, respectively. Previously, we have reported that two endoproteolytic cleavage sites were predicted from the DNA sequence of FHV-1 gB, producing two lower MW forms of 62 and 58 Kd as detected by western and immunoprecipitation assays with anti-HSV-1gB sera. Although the uncleaved FHV-1 gB can be detected with this crossreactive antisera, only the cleaved form (a doublet of 60 Kd) could be detected with antisera against recombinant VVgB. The uncleaved FHV-1 gB polypeptides could only be detected on overexposed western blots.

This is the first report of a FHV-1 polypeptide of 50 Kd homologous to glycoprotein D of HSV-1. The diffuse nature of the gD band may indicate incorporation of partially trimmed glycoprotein D in the virion. This heterogeneity in the

glycan moieties of gD may have little influence on the protein's immunogenicity, since gD of HSV-1 expressed in prokaryotic or baculovirus expression vectors elicits high titers of virus neutralizing antibodies.

A significant complement-independent neutralizing antibody response to virulent FHV-1 was demonstrated in rabbits immunized with either vaccinia recombinant (VVgB or VVgD). High titers of VN-antibodies to FHV-1 glycoproteins have also been reported in animals immunized with affinity-purified FHV-1 glycoproteins (Limcumpoa et al.., 1991). Likewise, there is a correlation between the onset of virus-neutralizing antibody response and the detection of glycoprotein-specific immunoprecipitins. Cats naturally exposed to FHV-1 (C-27) develop VN antibodies against FHV-1 glycoproteins by two weeks postinfection (Bergener and Maes, 1988). The successful generation of these poxvirus recombinants expressing gB and gD (FHV-1) will aid in the assessment of these glycoproteins in the induction of humoral and cell-mediated immunity in immunized animals. Protection studies with SPF cats inoculated with either RPV recombinant or a cocktail of both are needed to address the immune response to these glycoproteins in curtailing the replication of virulent challenge virus and therefore latency establishment. This would be very significant since current vaccines cannot prevent reinfection. Moreover, latent FHV-1 in carriers is very easily reactivated, thus a continuous source of virulent virus is available to infect susceptible cats.

## SUMMARY

Feline herpesvirus is the major cause of viral rhinotracheitis in cats worldwide. Because of this, the genes encoding major immunogens of FHV-1, glycoproteins B, D, H, G, I and E, have been identified and will be of significant value in the assessment of the immune response to these glycoproteins. The generation of vaccinia and raccoon poxvirus recombinants containing gB and gD is a practical application of the data generated from nucleic acid sequencing of the FHV-1 genome. Antisera generated against the vaccinia gD recombinant is currently being used for diagnostic purposes. The antisera generated against the poxvirus recombinants along with monoclonal antibodies recently generated by Drs. Maes and Deheck will be of value in future characterization of these glycoproteins. Future cat studies are planned to investigate the protective nature of the raccoon poxvirus recombinants. Other applications of the research data presented in this thesis involve the generation of "engineered" modified live viral vaccines against feline rhinotracheitis. Many of the glycoprotein genes identified encode glycoproteins that are suspected to be nonessential for viral replication. Glycoproteins G, I and E of HSV-1 and homologs found in PRV and EHV-1 have been reported to fit into this categorization. More importantly, g1 (gE) of PRV has been reported to be involved in neurovirulence and neuroinvasiveness. It would be of great value to generate FHV-1 recombinants containing

deletions in this gene. In order to accomplish this, a suitable marker gene (i.e. beta-galactosidase) can be rescued into the gene encoding gE of FHV-1. Assessment of these gE deletion mutants of FHV-1 in kittens may provide useful information on the role of this suspected neurovirulence factor. Infection of the CNS is often seen in naturally or experimentally infected kittens. This results is rarely observed in adult cats.

The transcriptional analysis of RNA originating from these six glycoprotein genes is far from complete. The main conclusion from the northern analysis is that co-terminal transcripts have been detected for the majority of the six gp genes. $S_1$-nuclease and primer extension experiments, as well as a functional assay for the transcripts, are needed for a more thorough investigation into the transcriptional pattern of these genes.

Finally, an evolutionary lineage between alpha-herpesviruses was generated illustrating the relationship of FHV-1 and viruses of the genus Varicellovirinae.

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