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THE COMPLETE AND ANNOTATED GENOMIC SEQUENCE OF FELINE HERPESVIRUS 1 (FHV-1) AND AN INFECTIOUS BAC CLONE: A PLATFORM FOR STUDIES OF TARGETED MUTANTS BY RECOMBINEERING

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ABSTRACT

THE COMPLETE AND ANNOTATED GENOMIC SEQUENCE OF FELINE HERPESVIRUS 1 (FHV-1) AND AN INFECTIOUS BAC CLONE: A PLATFORM FOR STUDIES OF TARGETED MUTANTS BY RECOMBINEERING

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Infection with feline herpesvirus-1 (FHV-1) is a major cause of upper respiratory and ocular disease in Felidae. For the first time, the complete genomic sequence of FHV-1 was determined and annotated. Complete genomic sequences were derived from both purified virion DNA and an infectious and virulent FHV-1 BAC clone. The FHV-1 genome is 135,797 bp in size with an overall G+C content of 45%. A total of 78 open reading frames were predicted, encoding 74 distinct proteins. The gene arrangement is collinear with the majority of other sequenced varicelloviruses. A bacterial artificial chromosome (BAC) clone of FHV-1 that contains the entire FHV-1 genome and has the BAC vector inserted at the 5'-end of the U_L was constructed and characterized *in vitro* and *in vivo*. The virus regenerated from the BAC was very similar to the parental C-27 strain *in vitro* in terms of plaque morphology and growth characteristics, and highly virulent in cats in a preliminary *in vivo* study.

Using the latest recombination-mediated genetic engineering (recombineering) techniques, FHV-1 mutants lacking the entire open reading frame encoding glycoprotein C (gC) or E (gE) were constructed based on the FHV-1 BAC clone. The gC^- FHV-1 mutant virus produced primary plaques that enlarged slowly and had relatively few secondary plaques compared to the wild type virus. Analysis on growth

kinetics showed that the gC^- mutant had a growth titer reduced by approximately 6,000 – 9,000 folds compared to the wild-type virus titer, and the titer of intracellular virus was 3.5 – 10 folds higher than the extracellular virus titer. Taken together, the results show that the gC^- FHV-1 mutant is more cell-associated compared to the wild-type virus. Thus, FHV-1 gC, like is the case in other varicelloviruses, plays a significant role in initial attachment/penetration, replication, and egress of FHV-1. In contrast, the gE^- FHV-1 mutant had single-step growth kinetics that were indistinguishable from those of the FHV1ΔBAC, and grew to a titer that was approximately 10-fold lower than that of the FHV1ΔBAC in the multi-step growth kinetics. These results suggest that virus egress, and most likely cell-to-cell spread as shown in other alphaherpesviruses, were affected by gE deletion, while virus entry and replication were not.

A gC^-gE^- and an US3 protein kinase $(PK)^-$ mutant of FHV-1 were also generated, and generation of a gE^-PK^- mutant are currently in progress. Both the gC^- gE^- and the PK^- mutants demonstrated reduced growth rates in CRFK cells. The actual effect of these deletions on growth properties needs to be further characterized.

The infectious FHV-1 BAC clone and the complete and annotated FHV-1 genomic sequence form a very suitable starting platform for mutagenesis aiming at functional studies of viral genes or vaccine development.

To my family

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LIST OF ABBREVIATIONS

BAC	bacterial artificial chromosome
BHV-1	bovine herpesvirus type 1 (*bovine herpesvirus 1*)
BHV-5	bovine herpesvirus type 5 (*bovine herpesvirus 5*)
CRFK	Crandell-Reese feline kidney cells
DIVA	differentiation between infected and vaccinated animals
EHV-1	equine herpesvirus type 1 (*equid herpesvirus 1*)
EHV-4	equine herpesvirus type 4 (*equid herpesvirus 4*)
ELISA	enzyme-linked immunosorbent assay
EMEM	Eagle's minimum essential medium
EGFP	enhanced green fluorescence protein
FBS	fetal bovine serum
FCE	feline corneal epithelial cells
FCV	feline calicivirus
FCWF-4	felis catus whole fetus cells
FHV-1	feline herpesvirus type 1 (*felid herpesvirus 1*)
gB	glycoprotein B
gC	glycoprotein C
gD	glycoprotein D
gE	glycoprotein E
gG	glycoprotein G
gH	glycoprotein H
gI	glycoprotein I

gJ	glycoprotein J
gK	glycoprotein K
gL	glycoprotein L
gM	glycoprotein M
gN	glycoprotein N
HSV-1	herpes simplex virus type 1 (*human herpesvirus 1*)
HSV-2	herpes simplex virus type 2 (*human herpesvirus 2*)
MDV	Marek's disease virus (*gallid herpesvirus 2*)
ORF	open reading frame
PCR	polymerase chain reaction
PK	protein kinase
PRV	pseudorabies virus (*suid herpesvirus 1*)
Recombineering	recombination-mediated genetic engineering
RT-PCR	reverse transcription-polymerase chain reaction
TK	thymidine kinase
VI	virus isolation
VN	virus neutralization
VZV	varicella-zoster virus (*human herpesvirus 3*)

INTRODUCTION

Feline herpesvirus type 1 (FHV-1) is a major cause of upper respiratory infection in cats worldwide. In recent years, its importance in ocular and chronic respiratory disease has also been increasingly recognized. According to the *2007 U.S. Pet Ownership & Demographics Sourcebook*, there were 81,721,000 cats in the U.S. Various studies have shown that 50-90% of cats have serological evidence of exposure to the virus, more than 80% of infected cats remain latently infected for life, and ~45% of latently infected individuals shed virus throughout life. Consequently, the economic losses associated with FHV-1 infection are significant.

Despite its high prevalence and worldwide distribution, our knowledge of FHV-1 at the molecular level is limited. Further, vaccines currently available protect cats from disease, but not from infection and subsequent latency. There is a need for a safer and more effective vaccine.

The goal of the research presented in this dissertation was to obtain an overview of the molecular composition of FHV-1, develop tools for functional studies on viral genes and virulence factors, and construct potential vaccine candidates.

Chapter 1 provides a literature review of FHV-1.

Chapter 2 introduces the state-of-the-art techniques that were essential for this research, including bacterial artificial chromosome (BAC) cloning and recombination-mediated genetic engineering (recombineering).

Chapter 3 is a manuscript submitted to Virology, describing cloning of the entire FHV-1 genome as a BAC, *in vitro* and *in vivo* characterization of this BAC clone, and sequencing and annotation of the entire FHV-1 genome.

Chapter 4 describes the construction and *in vitro* characterization of FHV-1 mutants lacking glycoprotein C or E based on this FHV-1 BAC clone, also in manuscript format.

Chapter 5 summarizes the generation and preliminary characterization of other deletion mutants of FHV-1, including a gC-gE- mutant, a US3- mutant, and a US3-gE- mutant.

Chapter 6 concludes the dissertation and points out potential directions for future research.

CHAPTER 1

A REVIEW OF FELINE HERPESVIRUS TYPE 1 (FHV-1)

1.1 Classification

Felid herpesvirus 1 (FeHV-1, commonly known as feline rhinotracheitis virus, feline herpesvirus type 1, or FHV-1) is currently classified under the Order *Herpesvirales*, Family *Herpesviridae*, Subfamily *Alphaherpesvirinae*, genus *Varicelloviruses* (Davison et al., 2009). Prototype viruses for the *Alphaherpesvirinae* and *Varicelloviruses* are *human herpesvirus 1* (HHV-1, commonly known as herpes simplex virus type 1, or HSV-1) and *3* (HHV-3, commonly known as varicella zoster virus, or VZV), respectively. Other varicelloviruses of veterinary importance that will be frequently mentioned in this and the following chapters include *bovine herpesvirus 1* (BoHV-1 or BHV-1) and *5* (BoHV-5 or BHV-5), *equid herpesvirus 1* (commonly known as equine herpesvirus type 1, or EHV-1) and *4* (commonly known as equine herpesvirus type 4, or EHV-4), *suid herpesvirus 1* (SuHV-1, commonly known as pseudorabies virus, or PRV). Other alphaherpesviruses of human or veterinary medical importance include *human herpesvirus 2* (HHV-2, commonly known as herpes simplex virus type 2, or HSV-2) and *gallid herpesvirus 2* (GaHV-2, commonly known as Marek's disease virus, or MDV).

1.2 Virus Characteristics

1.2.1 Morphology

FHV-1 particles are 120-180 nm in diameter. Herpesvirus particles consist of four elements: (a) an electron-opaque core containing the double-stranded viral DNA

genome, (b) an icosahedral capsid surrounding the core, (c) a largely unstructured proteinacous layer called the tegument that surrounds the capsid, and (d) an outer lipid bilayer envelope surrounding the tegument layer and exhibiting glycoprotein spikes on its surface (Flint et al., 2004; Murphy et al., 1999; Pellett and Roizman, 2007; Roizman et al., 2007).

1.2.2 Host range and cross-reactivity

Compared to some alphaherpesviruses, e.g., HSV-1 and -2, FHV-1 is considered to have a narrow host range. FHV-1 infects only members of the Family Felidae (Povey, 1979). *In vitro*, FHV-1 also replicates only in cells of feline origin. Crandell-Reese feline kidney (CRFK) cells are the cell line routinely used for propagation of FHV-1. *Felis catus* whole fetus (FCWF-4) cells is another cell line that can be used. In addition, feline alveolar macrophages, alveolar pneumocytes, CD4+ T-lymphoblastoid cells (MYA-1 and FL74 cells), and feline corneal epithelial (FCE) cells, have been shown to be suitable for FHV-1 propagation (Sandmeyer et al., 2005a; Spatz, 1993).

FHV-1, *canid herpesvirus 1* (CaHV-1, commonly known as canine herpesvirus type 1, or CHV-1), and *phocid herpesvirus 1* (PhHV-1, seal herpesvirus) are closely related genetically and antigenetically. Cross-protection between these viruses has been reported (Xuan et al., 1992).

1.2.3 Genome

As a member of the Herpesviridae, the FHV-1 genome consists of a single linear molecule of double-stranded DNA. FHV-1 genomes of two different strains have been

mapped using restriction enzymes. Rota et al. (1986) first constructed a *SalI* map of the genome of C-27 strain and determined that it was ~134 kb in size. Grail et al. (1991) mapped the B927 strain and determined that the genome was ~126 kb in size. Both studies found that the genome organization of FHV-1 is similar to that of other varicelloviruses. The genome consists of two segments of unique sequences called Unique Long (U_L) and Unique Short (U_S). The U_S region is flanked by a pair of identical but inverted sequences, known as Internal Repeat Short (IR_S) and Terminal Repeat Short (TR_S) (Figure 3.1).

1.2.4 Genes

One characteristics of herpesviruses is that they carry a large array of enzymes involved in nucleic acid metabolism (e.g., thymidine kinase, thymidylate synthetase, dUTPase, ribonucleotide reductase), DNA synthesis (e.g., DNA polymerase, helicase, primase), and processing of proteins (e.g., protein kinases), although the exact array of enzymes may vary from one herpesvirus to another (Pellett and Roizman, 2007). Alphaherpesvirus genomes typically posess 65-80 open reading frames (Alba et al., 2001). FHV-1 has been shown to contain 23 virion-associated proteins (Fargeaud et al., 1984). Eight glycoproteins have been identified, designated as gB, gC, gD, gE, gG, gH, gI, and gL. There are only limited studies on the genes encoded by FHV-1. Most of them are focused on envelope glycoproteins, most likely because they are predicted to play a role in inducing host immunity, thus, have potential for vaccine development. Studies on the functions of the first seven glycoproteins were reviewed by Maeda et al.

(1998). It is clear from this review that much more work is needed to define their actual functions and roles in viral pathogenesis and immunity.

gB

HSV gB contains two or three transmembrane segments and is essential for entry by fusion of the envelope with the plasma membrane. Virions lacking gB egress the cell but are not infectious. The gB protein binds heparan sulfate (Roizman et al., 2007). Using heparin-affinity column chromatography, Maeda et al. (1997a) showed that FHV-1 gB weakly binds to heparin.

gC

There have been very limited studies on gC of FHV-1. Willemse et al. (1994) first determined a partial sequence of gC in 1994. They also found that the adjacent UL45 gene can be co-transcribed with gC. The complete sequence of FHV-1 gC was later determined by Maeda et al. (1997b). Based on the amino acid sequence deduced from the nucleotide sequence, they predicted that gC is a membrane glycoprotein containing a characteristic N-terminal hydrophobic signal sequence, nine potential N-linked glycosylation sites, and C-terminal transmembrane and cytoplasmic domains. Maeda et al. (1997a) further demonstrated that gC is the major heparin-binding glycoprotein involved in the initial step in virus adsorption to cells as observed in gCs of other herpesviruses. In addition, they found that gC can agglutinate murine red blood cells, and that infection of FHV-1 is inhibited by addition of soluble heparin in cells cultures. Some field isolates of FHV-1 have been found to have genetic rearrangements in the N-

terminal region of gC, however, the function of gC did not seem to be affected (Hamano et al., 2004).

gC homologues have been extensively studied in several alphaherpesviruses. gC homologues are non-essential for herpesvirus replication *in vitro*, but they mediate several important biological functions. First of all, gC is involved in the initial step of viral attachment by interacting with heparan sulfate on the cell surface, as demonstrated in HSV-1, PRV, BHV-1, and EHV-1 (Herold et al., 1991; Mettenleiter et al., 1990; Okazaki et al., 1991; Osterrieder, 1999). gC deficient mutants attach to cells with reduced efficiency (Osterrieder, 1999). Secondly, gCs of HSV-1 and -2 can bind the complement component C3b (Frink et al., 1983; Lubinski et al., 1999). Binding of this complement factor may protect herpesvirus-infected cells from complement-mediated lysis (Fries et al., 1986). Viruses lacking complement-binding domains are less virulent than wild-type virus (Frink et al., 1983; Herold et al., 1991; Lubinski et al., 1999). The gC of FHV-1 has been shown to be the dominant heparin-binding glycoprotein that mediates the initial stage of viral adsorption, as observed in other herpesviruses (Maeda et al., 1998). However, it remains to be determined whether FHV-1 gC protects virus-infected cells from complement-mediated lysis.

gD

HSV gD is a multifunctional protein with the following properties: it interacts with three cellular receptors for entry – HveA, nectin1, and a modified heparin sulfate and, hence, defines viral tropism. On receptor binding, an ensuing change in conformation exposes profusion domains that enable fusogenic glycoprotein gB, gH,

and gL to complete fusion of the envelope with the plasma membrane. The N-terminal domain may be replaced with ligands for entry via other receptors. gD protects the cell from apoptosis induced by ΔgD mutants. Antiapoptotic activity is mediated by the mannose-phosphate receptor. ΔgD mutants grown in cells ectopically expressing gD produce virions that exit the cell, but are not infectious (Brunetti et al., 1998; Cocchi et al., 1998; Geraghty et al., 1998; Reynolds et al., 2001; Roizman et al., 2007).

gE and gI

In our laboratory, it has previously been shown that gE and gI are virulence factors (Kruger et al., 1996; Sussman et al., 1995). Unlike most commercial vaccines, an experimental FHV-1 vaccine with deletions in gE and gI was safe and efficacious via the oronasal route, and was able to highly reduce field virus latency loads in cats that were first vaccinated then exposed one month later to a high dose of field virus. Both glycoproteins have been shown to be nonessential for viral replication *in vitro* (Jacobs, 1994; Sussman et al., 1995; Willemse et al., 1996).

gG

Glycoprotein G (gG) homologues have been described in several alphaherpesviruses as a minor non-essential glycoprotein (Baranowski et al., 1996). Based on the viral species, gG has been reported either as a structural or a non-structural protein. The protein encoded by FHV-1 gG gene exists as two different forms, a membrane-anchored form and a secreted form. The latter is generated by proteolytic cleavage of the former (Drummer et al., 1998). A recent study by Costes et al.

demonstrated that FHV-1 gG belongs to a newly discovered viral chemokine-binding protein (vCKBP) family, binding with high affinity to a broad spectrum of chemokines. Both the secreted form and the membrane-anchored form of gG expressed at the surface of virus-infected cells can bind chemokines. In addition, this study also showed that the expression of a secreted vCKBP activity is a general property of field strains (Costes et al., 2006). Although gG is not essential for virus growth, gG mutants of several alphaherpesviruses have attenuated virulence and, thus, suggested as vaccine candidates. These include PRV (Demmin et al., 2001), avian infectious laryngotracheitis virus (Devlin et al., 2007), EHV-1 and EHV-4 (Huang et al., 2005).

1.3 Replication

All alphaherpesviruses are believed to follow a similar replication pattern as HSV-1, which has been extensively studied. The replication cycle is summarized below (Flint et al., 2004; Pellett and Roizman, 2007; Roizman et al., 2007).

1.3.1 Attachement and Entry

The replication cycle begins with virion attachment to the cell surface via binding of gC and gB to the heparin sulfate or chondroitin sulfate proteoglycans on the cell surface. Following the initial attachment, gD initiates fusion of viral envelope and cell membrane, mediated by gB, gD, gH, and gL. As a result of membrane fusion, viral nucleocapsid is released into the cytoplasm. It attaches to microtubules and is transported to the nucleus. Also released into the cytoplasm are the tegument proteins.

1.3.2 Gene Expression and DNA Replication

The expression of three classes of genes (immediate-early, early, and late) in a temporal order is a characteristic of alphaherpesvirus replication. After viral DNA is released into the nucleus via the nuclear pore, VP16 interacts with host transcription proteins to initiate the transcription of immediate-early genes by host RNA polymerase II. Immediate-early gene mRNAs are transported to the cytoplasm and translated. The immediate-early proteins are transported back to the nucleus, activate transcription of early genes, and down-regulate transcription of immediate-early genes. Early gene mRNAs are also transported to the cytoplasm and translated. Early proteins are primarily involved in DNA replication and nucleotide metabolism. Subsequently, viral DNA synthesis is initiated from viral origins of replication. The replication of genomic DNA is often referred to as a rolling circle model, in which DNA replication and recombination produce long, concatemeric DNA, from a circularized template. The concatemeric DNA serves as a template for late gene expression. Late mRNAs are transported to the cytoplasm and translated. Late proteins are primarily virion structural proteins and additional proteins needed for virus assembly and particle egress. Envelope glycoproteins are also late proteins that are made on and inserted into membranes of the rough endoplasmic reticulum. The precursor glycoproteins are transported to the Golgi apparatus for glycosylation, further modification and processing. Mature glycoproteins are transported to the plasma membrane of the infected cell.

1.3.3 Virion Assembly and Egress

Newly replicated viral DNA is packaged into nucleocapsids in the nucleus. DNA-containing nucleocapsids, surrounded by some tegument proteins, bud from the inner nuclear membrane into the perinuclear lumen, acquiring a preliminary envelope that contains precursor viral membrane proteins. This process is known as primary envelopment. Immature enveloped virions then fuse with the outer nuclear membrane from within, and release the nucleocapsid into the cytoplasm. The nucleocapsids are transported to and bud into the late Golgi-endosome compartment, acquiring an envelope containing mature viral envelope proteins and the complete tegument layer. The process is known as secondary envelopment. The enveloped virus particle then buds into a vesicle that is transported to the plasma membrane for release by exocytosis.

1.4 Latency

Lifelong latency following the acute phase of the disease is a hallmark of herpesvirus infections. During the latent stage, the viral genome persists in neural tissues but infectious virus is not produced. The trigeminal ganglion is considered a primary site of latency for FHV-1, although recent studies implied other tissues as potential sites (Jacobi, 2008). When the latent state is established, viral DNA can circularize and persist in the nucleus as an episome. Lytic gene expression is repressed, while the latency-associated transcript (LAT) is expressed, which yields several RNA species by splicing. These multiple species are collectively referred to as LATs. Low-level or sporadic transcription of immediate-early and early genes can occur, but is not

sufficient to initiate a productive infection. No infectious virions can be detected in the ganglia during latent infection. The LAT RNA is spliced, and a stable intron in the form of a lariat, called the 2-kb LAT, is produced in the nucleus. The spliced LAT mRNA is transported to the cytoplasm where several small ORFs may be translated into proteins. The function of LAT RNAs and the production of LAT proteins are still controversial. Different biological stresses, or the administration of corticosteroids, can induce the necessary biochemical stimuli in latently infected cells that lead to renewed production of progeny virions. Infectious virus is carried by anterograde axonal transport to peripheral tissues, usually to cells at or near the site of initial infection, and is a potential source of viral transmission (Pellett and Roizman, 2007; Roizman et al., 2007). Depending on several factors, including the status of host immune system, the reactivation may be asymptomatic or lead to a recurrent disease, which can vary considerably in severity. Severe keratitis can also result from reactivation. The role of reactivation in the epidemiology is directly related to the frequency by which it takes place. Some herpesviruses, including FHV-1, reactivate much more easily than others from the latent state, both under natural and experimental conditions. The ease by which latent FHV-1 is reactivated is an important element in the justification of FHV-1 infection of cats as a natural host model to study the molecular pathogenesis of herpesvirus latency and approaches to prevent it.

1.5 Clinical Manifestations and Pathogenesis

Following entry via the oronasal route, FHV-1 replicates extensively in the mucosae of the upper respiratory tract. Pathologic examination reveals necrosis of epithelia of the nasal cavity, pharynx, epiglottis, tonsils, larynx, and trachea. In extreme cases and in young kittens, there can be an extensive rhinotracheitis and an associated bronchopneumonia. Clinically, FHV-1 infection manifests itself as a sudden onset of sneezing, coughing, serous nasal and ocular discharge which can progress to mucopurulent discharges, frothy salivation, dyspnea, anorexia, weight loss, and fever after an incubation period of 24-48 hours. Conjunctivitis and keratitis are also common. Occasionally there may be ulcers on the tongue. Infection in cats over six months of age is likely to result in mild or subclinical infection. The mortality can reach 50% in kittens, since the virus tends to generalize in this age group. Exposure of pregnant queens can lead to abortion, but infection with FHV-1 is not a common cause of abortion in cats. The main reason is that viremia is low, because the natural temperature sensitivity of FHV-1 favors replication in the upper respiratory tract, which is below body temperature. In addition, there is no evidence that the virus crosses the placenta and fatally infects fetuses, and virus has not been isolated from placentas or aborted fetuses; abortion, when it occurs, is thought to be secondary to fever and toxemia. Neurological disorders are rare but have been observed clinically.

In recent years, the importance of FHV-1 in ocular disease has been increasingly recognized. FHV-1 is the most common cause of conjunctivitis in cats (Nasisse, 1990). Epithelial keratitis commonly occurs during acute infection in young cats and resolves spontaneously in most cases (Nasisse, 1995). In adult cats, reactivation of latent virus

can result in corneal ulceration, accompanied by a varying degree of conjunctivitis (Stiles, 2000). Since herpetic stromal keratitis caused by HSV-1 is the leading cause of infectious blindness in the industrialized countries, ocular infection of FHV-1 in cats is considered a very good natural host model.

1.6 Diagnosis

Clinically, the acute disease of FHV-1 infection is very similar to that caused by feline calicivirus (FCV). Profuse frothy salivation and corneal ulcers suggest feline herpesvirus infection, whereas ulcers of the tongue, palate, and pharynx are encountered more frequently in calicivirus infections.

In our laboratory, commonly performed diagnostics for FHV-1 infection includes virus isolation (VI), polymerase chain reaction (PCR) assays, virus neutralization (VN) tests, and direct fluorescent antibody (FA) staining.

VI detect infectious virus and has been the gold standard for the diagnosis of alphaherpesvirus infections (Maggs, 2005). It is performed by grinding tissue samples or elute the virus from swabs in Borvarnick's buffer, passing through 0.45 μm filters, and incubate on CRFK monolayers in a 24-well plate or 25-cm^2 flask. The cell culture is observed for the development of characteristic cytopathic effect caused by FHV-1.

Compared to VI, PCR is more sensitive and efficient. Multiple PCR assays have been described for use in the detection of FHV-1 DNA (Jacobi, 2008). The TaqMan based real-time PCR assay described by Vogtlin et al. (2002) targeting the gB gene is routinely used in our laboratory for diagnosis of FHV-1 infection. It can be converted

into a quantitative assay by incorporating a standard curve, consisting of a serial dilution of FHV-1 DNA whose genomic copy number had been determined. Our result showed that the detection limit of this assay is approximately 10 copies of FHV-1 genome (unpublished data). A quantitative SYBR-Green based real-time PCR assay targeting gE and gI genes was also developed in our laboratory. The detection limit for this assay is also approximately 10 copies (unpublished data). An internal control can be spiked in the reaction to determine whether a negative reaction was caused by inhibitory factors in the sample (unpublished data).

Virus neutralizing antibody titers are determined by VN tests, which are commonly used to detect prior infection of FHV-1 in cats. However, the presence of neutralizing antibody in serum does not necessarily correlate with clinical disease (Dawson et al., 1998).

Feline herpesvirus-specific proteins on conjunctival or corneal smears or biopsies can be detected by direct FA staining. Polyclonal fluorescein conjugated antibody binds to FHV-1 epitopes on the cell surface and can be visualized by fluorescent microscopy.

1.7 Treatment and Control

1.7.1 Supportive Treatment

The European Advisory Board on Cat Diseases (ABCD) recently published a guideline for management of this disease (Thiry et al., 2009). Like many viral infections, supportive treatment is advised. To prevent secondary bacterial infections, broad-

spectrum antibiotics that achieve good penetration into the respiratory tract should be given in all acute cases. Food intake is extremely important. Food should be highly palatable and flavorful, since many cats will not eat because of loss of their sense of smell or the presence of ulcers in the oral cavity. Appetite stimulants (eg, cyproheptadine) may be used. If the cat does not eat for more than 3 days, a feeding tube should be placed. In cats with severe clinical signs, restoration of fluids, electrolytes and acid-base balance is required, preferably by intravenous administration. Nasal decongestants and mucolytic drugs have been suggested to help clear airways. Nebulization with saline can be used to ease dehydration of the airways. Eye drops or ointments can be administered several times a day.

1.7.2 Antiviral Therapy

Nucleoside analogue antivirals have been widely used to treat HSV and VZV infections. Generally speaking, in infected cells, these nucleoside analogues are converted into triphosphates by viral thymidine kinase and other host enzymes, and competitively inhibit viral DNA polymerase, preventing DNA chain elongation (Snoeck, 2000). As a result, viral DNA synthesis, and hence viral replication, are disrupted. However, use of these agents against FHV-1 infection has been largely limited to topical administration. First generation nucleoside analogues, including acyclovir and its pro-drug valacyclovir, have little efficacy against FHV-1 *in vitro* and moderate effect *in vivo*. More importantly, they produce serious side effects in cats, including myelosuppression, hepatotoxicity, and nephrotoxicity, when administered systemically at therapeutic levels (Nasisse et al., 1997; Owens et al., 1996). According to the

European ABCD's guideline (Thiry et al., 2009), trifluridine is the topical treatment of choice in cats with ocular FHV-1 manifestations. Acyclovir, ganciclovir, and idoxuridine are also suggested for topical use in the guideline. Other drugs that have been proposed for the treatment of FHV-1 ocular infections include bromovinyldeoxyuridine, cidofovir, famciclovir, HPMA (N-[2-hydroxypropyl] methacrylamide), penciclovir, ribavirin, valaciclovir, vidarabine, foscarnet and lactoferrin. It was noted that, except for acyclovir, there is a lack of controlled *in vivo* efficacy study for these agents in the literature (Thiry et al., 2009). The efficacy of topical application of cidofovir on primary ocular FHV-1 infection was recently demonstrated (Fontenelle et al., 2008). Famciclovir, which is converted into penciclovir triphosphate by viral thymidine kinase in alphaherpesvirus-infected cells, was recently shown to be safe and efficacious when administered orally in treating ocular signs, cutaneous disease, and rhinosinusitis induced by FHV-1 infection in 10 cats (Malik et al., 2009). Although it was not a controlled study, this is probably the first antiviral shown to be safe for systemic administration in cats.

L-lysine is an antagonist of arginine, the latter has been shown to be essential for human herpes simplex virus and FHV-1 proteosynthesis (Maggs et al., 2003). Treatment with L-lysine therefore decreases viral replication and has been shown to have some inhibitory effect against both human herpesvirus and FHV-1 infection (Maggs et al., 2000; Maggs et al., 2003; Stiles et al., 2002). Oral supplementation with L-lysine reduces the severity of experimentally induced FHV-1 conjunctivitis (Stiles et al., 2002) and ocular virus shedding associated with reactivation of latent infection (Maggs et al., 2003). It was suggested for use early in acute disease or as a means of

reducing the severity of disease and virus shedding at times of stress (Gaskell et al., 2007). Feline interferon (IFN) ω and human IFN-α can be given systemically along with L-lysine to reduce clinical disease (Thiry et al., 2009).

1.8 Immunity and Vaccination

Primary FHV-1 infection induces both humoral and cellular immune responses. It is likely that virus neutralizing antibodies recognize incoming virus during the acute phase of infection, and contributes to antibody-dependent cellular cytotoxicity and antibody-induced complement lysis (Wardley et al., 1976). The immunity induced by natural FHV-1 infection protects cats from the disease, but not from infection. In fact, the protection becomes incomplete six months after the primary infection (Gaskell and Povey, 1977; Walton and Gillespie, 1970), and mild clinical signs have been observed following re-infection as soon as 150 days after the primary infection (Thiry et al., 2009). Virus neutralizing antibody titers are generally low and in some cases undetectable after primary infection, although after further exposure to virus, they tend to rise to more moderate levels and thereafter remain reasonably stable (Gaskell et al., 2007; Gaskell and Povey, 1979). Since FHV-1 is a pathogen of the respiratory tract, mucosal immune responses are important (Lappin et al., 2006).

During the first weeks of life, maternally derived antibodies protect kittens against disease, although the exact time of protection varies considerably among individuals. Generally speaking, maternally derived antibodies persist for 2 to 10 weeks, with mean titers falling below detectable levels (< 1:2) by nine weeks of age (Gaskell and Povey,

1982). More recent studies found that about 25% of the kittens have an antibody level of < 1:4 at only 6 weeks of age. It should also be noted that kittens with low levels of maternally derived antibodies are not necessarily protected from subclinical infection and latency (Gaskell and Povey, 1982), and that these kittens may respond to early vaccination (Dawson et al., 2001). On the other hand, in some individuals, maternally derived antibodies may still be at interfering levels at 12–14 weeks of age (Dawson et al., 2001; Gaskell et al., 2007). The European ABCD recommends two vaccine injections, at 9 and 12 weeks of age, followed by yearly boosters (Thiry et al, 2009). The American Association of Feline Practitioners Feline Vaccine Advisory Panel advices that the primary series of vaccination should begin as early as 6 weeks of age, then one dose every 3 to 4 weeks until 16 weeks of age, followed by 2 doses 3 to 4 weeks apart. A single dose is given 1 year following the last dose of the primary series, then one dose every 3 years (Richards et al., 2006).

FHV-1 vaccines protect cats against disease but not against infection and, as a consequence, latency. An approximately 90% reduction in clinical scores to experimental challenge has been achieved with vaccination, and virus excretion is reduced (Gaskell et al., 2007). There is complete cross-protection among FHV-1 strains, as they all belong to the same single serotype. All current commercial FHV-1 vaccines are multivalent, most commonly include FCV and feline panleukopenia virus components, and are collectively called feline viral rhinotracheitis, calicivirus, and panleukopenia (FVRCP) vaccines. Of the three vaccine components, the protection induced against FHV-1 is generally the least effective (Lappin et al., 2002; Scott and Geissinger, 1999).

A number of modified live and killed FVRCP vaccines for parenteral administration are available in the United States (Lappin et al., 2006; Richards et al., 2006). Modified-live vaccines are routinely used, but they have residual virulence and may induce clinical signs if administered incorrectly (e.g. by accidental aerosolisation or spillage on the skin) (Kruger et al., 1996). Killed virus vaccines are generally preferred for use in pregnant queens (and only if absolutely necessary) and in FeLV- or FIV-infected cats (Richards et al., 2006).

In addition to vaccines labeled for systemic immunization, an intranasal multivalent vaccine including an FHV-1 component is commercially available. Testing under experimental conditions showed that this vaccine (Feline UltraNasal FVRC or FVRCP Vaccine, Heska Corporation, Loveland, CO) was safe and induced protection against the clinical signs of field virus exposure within a week post-vaccination (Lappin et al., 2006). With systemically administered vaccine this would take 2-3 weeks (Lappin et al., 2009). However, it is unclear whether the new vaccine reduces the length and level of field virus shedding. It is also unclear whether the new vaccine prevents latent infection.

Several FHV-1 deletion and/or insertion mutants have been constructed as potential vaccine candidates, some of which have incorporated other genes including the FCV capsid gene (Cole et al., 1990; Mishima et al., 2001; Mishima et al., 2002; Wardley et al., 1992; Yokoyama, 1995, 1996a, 1996b, 1998). A gE-gI deletion mutant was previously developed in our laboratory (Kruger et al., 1996; Sussman et al., 1997; Sussman et al., 1995). Williamse et al. (1996) developed a gI insertional mutant, which also appeared to affect the transcription pattern and expression of the upstream gene gD.

Williamse et al. (1994) also developed an FHV-1 mutant by inserting β-galactosidase marker gene into UL45, adjacent to the gC gene. Yokoyama et al. (1996c, 1998) developed a TK- recombinant FHV-1 vaccine. In general, these deletion/insertional mutants are less virulent for cats and offer good protection against disease, especially via the oronasal route. One reason that none has so far been marketed is probably because the protection offered is not superior to conventionally attenuated vaccines (Gaskell et al., 2007).

1.9 *In vitro* models of FHV-1 infection

FHV-1 infection in cats is an excellent natural model for herpes simplex virus infection in humans, especially for ocular diseases induced by HSV-1 and -2. Other herpesvirus infections in large animals, including EHV-1, BHV-1, and PRV, have also been used extensively to study common aspects of herpesvirus biology. However, animal experiments in these species are expensive, labor-intensive, and sometimes of ethical concerns.

In recent years, several *in vitro* systems have been developed as substitutes for *in vivo* animal experiments.

Tracheal organ cultures have been used in several species to study physiology and infectious diseases, having the advantage over cell cultures of more closely mimicking the natural situation (Cook et al., 1976; Dhinakar Raj and Jones, 1996). This technique has only been used in one comparative study involving FHV-1 and FCV, which showed that viral titers peaked later in tracheal cultures than in cell monolayers (Milek et al.,

1976). Leeming et al. (2006) established feline tracheal organ cultures as an *in vitro* system for the examination of functional and morphological effects of FHV-1 on the respiratory epithelium. Based upon daily assessment of cilia movement and tissue morphology, the respiratory epithelium remains viable in culture for at least 120 hours, allowing sufficient viral replication and development of lesions. Therefore, aspects of FHV-1 infection in respiratory epithelium can now be studied in a model that closely mimics natural infection in airways.

Recently, *ex vivo* cultures of nasal respiratory, nasopharygeal, and tracheal mucosa from equines have been established, the explants were maintained *in vitro* for up to 96 hours on fine-meshed gauze at an air-liquid interface, mimicking the air-liquid interface found in the respiratory tract of the living animal (Vandekerckhove et al., 2009). *Ex vivo* cultures of porcine nasal mucosa have also been established and maintained for up to 60 hours post-sampling (Glorieux et al., 2007). Further, a quantitative analysis system was established to study the kinetics of horizontal and vertical spread of PRV, including crossing of the basement membrane, in the porcine nasal mucosa explant system. This system was subsequently used to examine different historical PRV strains isolated between the 1960s and 2000, and found them behave differently in the respiratory nasal mucosa (Glorieux et al., 2009).

Ocular infection of FHV-1 in cats is an excellent natural model for ocular diseases induced by HSV-1 and -2. Conjunctival and corneal epithelial tissues are the primary targets for FHV-1 in ocular infections (Bistner et al., 1971; Nasisse et al., 1989). A primary culture of FCE cells has been established and maintained in heavily-supplied medium for 6 passages, and CPE of FHV-1 in these cells has been characterized

(Sandmeyer et al., 2005a). FCE cells have also been used to evaluate the effects of interferon-alpha and cidofovir on cell viability and replication of FHV-1 (Sandmeyer et al., 2005b; Sandmeyer et al., 2005c).

1.10 Conclusions

In summary, FHV-1 has long been causing a widespread and very important respiratory disease in cats. Ocular lesions are also an important aspect of FHV-1 infections, like is the case for HSV-1 in humans. Expanding our limited knowledge of FHV-1, particularly in the regards of gene contents and functions, molecular pathogenesis, mechanism of latent infection, and host-virus interactions, will not only benefit cats, but also contribute to the shared aspects of herpesvirus biology. Furthermore, FHV-1 infection in cats has potential as a natural host system for the study of immunity against herpesvirus infections, as well as the study of antivirals and new therapeutic approaches to treatment of herpesvirus infections.

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CHAPTER 2

BACTERIAL ARTIFICIAL CHROMOSOME (BAC) AND RECOMBINATION-MEDIATED GENETIC ENGINEERING (RECOMBINEERING)

2.1 BAC Cloning

Traditionally, functional studies of viral genes have been achieved by creating mutant alleles and screening for phenotypic alterations, an approach known as reverse genetics. Because the natural mutation rate in DNA viruses such as herpesviruses is low, experimental generation of viral mutants is necessary. However, the fact that the frequency of the experimentally induced mutation must be controllable, in order to prevent accumulation of multiple mutations, makes the process inefficient. In addition, to map these mutations, time-consuming and labor-intensive procedures are required (Schaffer, 1975; Schaffer et al., 1984).

Advances in molecular cloning technologies facilitated functional studies at the level of isolated genes. Viral genes and genomic fragments can be cloned, then manipulated *in vitro* and reintroduced into the viral genome. Nevertheless, due to the limitations in the capacity of vectors, this method allowed only step-by-step analysis for large and complex genomes. In the late 1980s herpesvirus genomes were subcloned as a set of overlapping cosmid clones in *Escherichia coli*. After co-transfecting cells with the cosmid set, infectious virus can be reconstituted by multiple homologous recombinations via the overlapping sequences (van Zijl et al., 1988). Mutations can be introduced into one of the fragments. Because recombination procedures in cells cannot be controlled, mutagenesis is often associated with unwanted and illegitimate mutations and extensive post-mutagenesis analysis of the isolated mutants is therefore required. Null mutants of an essential gene can only be propagated in cell lines that provide the gene product in trans (DeLuca and Schaffer, 1985). In addition, the successful use of these strategies is determined by the replication efficiency of the virus under study. The

plentiful selection of mutants available for herpes simplex viruses is in contrast to the paucity of mutants of other herpesviruses that remain cell associated, that replicate slowly or that have the propensity to enter the lytic cycle only under specific conditions.

Yeast artificial chromosomes (YACs) were the first cloning vectors for large genomic DNA fragments. Unfortunately, YACs also caused difficulties, such as frequent spontaneous rearrangements and insert instability, and there can be substantial contamination of purified YACs with yeast DNA (Ramsay, 1994; Schalkwyk et al., 1995).

Bacterial artificial chromosomes (BACs) are single copy F-factor-based plasmid vectors that can stably hold 300 kb or more of foreign DNA (Shizuya et al., 1992). BACs have several advantages over the other methods. First of all, BACs are much more stable than other vectors, because the strict control of the F-factor replicon maintains a single copy of the BAC per bacterial cell. This reduces the risk of otherwise frequent recombination events via repetitive DNA elements present in the DNA inserts (Kimman et al., 1994; Shizuya et al., 1992). The capacity and stability of BACs enables the cloning of an entire herpesvirus genome into a single plasmid. Secondly, for subsequent functional genetics study, once a viral genome is cloned into a BAC, it can be manipulated within *E. coli*. Utilizing prokaryotic recombinases, such as recA, recE, recT (Horsburgh et al., 1999; Link et al., 1997; Narayanan et al., 1999) or the mini-lambda system (Costantino and Court, 2003; Court et al., 2003), site-specific mutations can be introduced, theoretically anywhere in the viral genome. All mutagenesis steps can be controlled and analyzed in *E. coli*, and the manipulated viral genome can be stably maintained in the *E. coli*. This is in contrast to the other methods, where the

recombination takes place in eukaryotic cells and the analyses can only start after the virus has been reconstituted and isolated. Unwanted additional changes that may have occurred in the viral genome, such as deletions, rearrangements or illegitimate recombinations frequently can only be observed after considerable expense of time and effort. Finally, it is safer to work with herpesviruses when the viral genome is maintained as a BAC. These properties have made BACs the vectors of choice for the cloning of eukaryotic genome libraries, and have attracted herpesvirologists as well. Several herpesvirus genomes of medical and veterinary importance have been cloned in BAC since the first infectious BAC of herpesvirus reported in 1997 (Messerle et al., 1997), including herpes simplex virus (Saeki et al., 1998; Stavropoulos and Strathdee, 1998), Epstein-Barr virus (Delecluse et al., 1998), human cytomegalovirus (Borst et al., 1999; Marchini et al., 2001; Yu et al., 2002), pseudorabies virus (Smith and Enquist, 1999), equine herpesvirus 1 (Rudolph et al., 2002), and Marek's disease virus (Niikura et al., 2006; Schumacher et al., 2000).

2.2 Recombineering

Recombineering is a powerful method for fast and efficient manipulation of the BAC. (See Figure 2 for overview.) It allows DNA cloned in *E. coli* to be modified via lambda (λ) Red-mediated homologous recombination, obviating the need for restriction enzymes and DNA ligases. Specific bacterial strains have been constructed for this purpose (Lee et al., 2001; Warming et al., 2005; Yu et al., 2000). A defective λ prophage (mini- λ) is inserted into their bacterial genome and encodes three genes that

make recombineering possible: *exo*, *bet* and *gam*. Exo is a 5'-3' exonuclease that creates single-stranded overhangs on linear DNA introduced into the bacteria. Bet protects these overhangs and assists in the subsequent recombination process. Gam prevents degradation of linear DNA by inhibiting *E. coli* RecBCD protein. *Exo*, *bet*, and *gam* are transcribed from the λPL promoter. This promoter is repressed by the temperature-sensitive repressor cI857 at 32°C and derepressed (the repressor is inactive) at 42°C. When bacteria containing mini-λ prophage are kept at 32°C, no recombination proteins are produced. However, after 15 minutes of heat shock at 42°C, sufficient amounts of recombination proteins are produced. Linear DNA (PCR product, oligonucleotide, etc.) with sufficient homology in the 5' and 3' ends to a target DNA molecule already present in the bacteria (plasmid, BAC, or the bacterial genome itself) can be introduced into heat-shocked and electrocompetent bacteria using electroporation. The introduced DNA will now be modified by Exo and Bet and undergo homologous recombination with the target molecule. In our case, once a viral genome is cloned into a BAC, it can be easily and efficiently manipulated within *E. coli*. Utilizing recombineering techniques, site-specific mutations can be introduced anywhere in the viral genome, provided that the genomic sequence is known. All mutagenesis steps can be strictly controlled and analyzed in *E. coli*, and the manipulated viral genome can be stably maintained in the *E. coli*. This is in contrast to the other methods, where the recombination takes place in eukaryotic cells and the analyses can only start after the virus has been reconstituted and isolated. Unwanted additional changes that may have occurred in the viral genome, such as deletions, rearrangements or illegitimate recombinations frequently can only be observed after considerable expense of time and effort.

In 2006, Tischer et al. published a technique called two-step Red-mediated recombination, which further improves the efficiency of recombineering. This technique is suitable for site-specific deletion, insertion, and point mutation. An overview of this technique is provided in Figure 1 of Chapter 4.

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CHAPTER 3

COMPLETE GENOMIC SEQUENCE AND AN INFECTIOUS BAC CLONE OF FELINE HERPESVIRUS-1 (FHV-1)

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Complete Genomic Sequence and an Infectious BAC Clone of Feline Herpesvirus-1 (FHV-1)

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ABSTRACT

Infection with feline herpesvirus-1 (FHV-1) is a major cause of upper respiratory and ocular disease in Felidae. We report the first complete genomic sequence of FHV-1, as well as the construction and characterization of a bacterial artificial chromosome (BAC) clone of FHV-1, which contains the entire FHV-1 genome and has the BAC vector inserted at the left-hand side (5'-end) of the U_L region. Complete genomic sequences were derived from both the FHV-1 BAC clone and purified virion DNA. The FHV-1 genome is 135,797 bp in size with an overall G+C content of 45%. A total of 78 open reading frames were predicted, encoding 74 distinct proteins. The gene arrangement is collinear with that of most sequenced varicelloviruses. The virus regenerated from the BAC was very similar to the parental C-27 strain *in vitro* in terms of plaque morphology and growth characteristics, and highly virulent in cats in a preliminary *in vivo* study.

Keywords: Feline herpesvirus; FHV-1; genomic sequence; infectious BAC clone

INTRODUCTION

Feline herpesvirus-1 (FHV-1) is a significant viral pathogen of Felidae, first isolated in 1957 by Crandell and Maurer (1958). FHV-1 accounts for approximately 50% of all diagnosed viral upper respiratory infections in cats and is also a significant cause of ocular lesions (Nasisse, 1990). The pathobiology of FHV-1 has been reviewed (Gaskell, 2007; Maggs, 2005; Stiles, 2003). Briefly, following entry via the oronasal route, FHV-1 replicates extensively in the mucosae of the upper respiratory tract resulting in high fever, depression, anorexia, sneezing, conjunctivitis, keratitis, and ocular and nasal discharge. The acute phase of the disease is followed by life-long latency, a hallmark of herpesvirus infections. During the latent stage, viral DNA appears to persist mainly in sensory ganglia. Different biological stresses, or administration of corticosteroids, can induce the necessary biochemical stimuli in latently infected cells that lead to renewed production of infectious virus, which can travel to the periphery and is a potential source of viral transmission. In addition to the primary disease, FHV-1 is also a contributor to feline chronic rhinosinusitis (Johnson and Maggs, 2005). Despite its high morbidity and worldwide distribution, currently available vaccines cannot totally protect cats from field virus infection and, as a consequence, from field virus latency (Gaskell and Povey, 1979; Tham and Studdert, 1987). In addition, some commercial vaccines are avirulent when administered subcutaneously, but virulent when administered oronasally (Kruger et al., 1996; Povey, 1979). Since reactivation of latent FHV-1 is common, vaccinated cats may act as

asymptomatic carriers and contribute to the spread of virulent virus (Gaskell and Povey, 1982).

Our knowledge of FHV-1 at the molecular level is still limited. FHV-1 is a member of *Herpesviridae* family, *Alphaherpesvirinae* subfamily, genus *Varicellovirus* (Davison et al., 2009). All FHV-1 isolates appear to be relatively similar, and belong to one serotype. The FHV-1 genome, mapped by restriction endonuclease digestion techniques, is approximately 134 kb, and has a typical type D structure similar to other varicelloviruses (Figure 3.1). The double-stranded DNA genome consists of two segments of unique sequences, known as Unique Long (U_L) and Unique Short (U_S). The U_S region is flanked by a pair of identical but inverted repeat sequences, known as Terminal Repeat Short (TR_S) and Inverted Repeat Short (IR_S) (Grail et al., 1991; Rota et al., 1986). Alphaherpesviruses usually encode 65-80 open reading frames (ORFs) (Alba et al., 2001). FHV-1 has been shown to contain 23 virion-associated proteins (Fargeaud et al., 1984). Eight glycoproteins have previously been identified and designated as gB, gC, gD, gE, gG, gH, gI and gL. Knowledge about the functions of these glycoproteins has been reviewed by Maeda et al. (1998) though it is clear that much more work is needed to define their actual functions and roles in viral pathogenesis and immunity. In recent years, many herpesvirus genomes of human and veterinary medical importance have been completely sequenced and annotated, among them herpes simplex virus types 1 (HSV-1) (McGeoch et al., 1988) and 2 (HSV-2) (Dolan et al., 1998), varicella-zoster virus (VZV) (Davison and Scott, 1986), bovine herpesvirus types 1 (BHV-1) (Schwyzer and Ackermann, 1996) and 5 (BHV-5) (Delhon et al., 2003), equine herpesvirus types 1 (EHV-1) (Telford et al., 1992) and 4 (EHV-4) (Telford et al., 1998), Marek's disease

virus (MDV) (Lee et al., 2000; Tulman et al., 2000), and pseudorabies virus (PRV) (Klupp et al., 2004). Prior to this report, only portions of the FHV-1 genomic sequence were available.

Bacterial artificial chromosomes (BACs) are single copy F-factor-based plasmid vectors that can stably hold 300 kb or more of foreign DNA (Shizuya et al., 1992). BACs have advantages over other vectors including higher cloning capacity, greater stability in *E. coli*, and the efficiency of manipulation. Because of these advantages, BACs have been widely used for cloning of entire herpesvirus genomes, and the resulting clones have been shown to be infectious. Herpesviruses that have been cloned as BACs include HSV-1 (Saeki et al., 1998; Stavropoulos and Strathdee, 1998), Epstein-Barr virus (Delecluse et al., 1998), human cytomegalovirus (Borst et al., 1999; Marchini et al., 2001; Yu et al., 2002), PRV (Smith and Enquist, 1999), EHV-1 (Hansen et al., 2006; Rudolph et al., 2002), MDV (Schumacher et al., 2000; Niikura et al., 2006), canine herpesvirus (Arii et al., 2006; Strive et al., 2006), and FHV-1 (Costes et al., 2006).

In this report, we present the first complete and annotated genomic sequence of FHV-1, as well as a BAC clone that contains the entire FHV-1 genome, and is infectious both *in vitro* and *in vivo*.

RESULTS

Cloning of the FHV-1 genome as a BAC

The C-27 strain FHV-1 genome was cloned as a BAC. *US4*, which encodes gG, was selected as the target site for the BAC cassette insertion because it has been shown to be non-essential for virus growth in several alphaherpesviruses (Balan et al., 1994; Baranowski et al., 1996) including FHV-1 (Costes et al., 2006). Analyses later revealed that the BAC vector was in fact inserted at the genomic termini, or between U_L and TR_S in the circular DNA (Figure 3.1). In addition, an unknown sequence of ~2.7 kb in size was present immediately upstream of the BAC vector.

Restriction pattern analysis was used to examine the overall integrity of the FHV-1 genome in the BAC clone. The *SalI* digestion pattern of the BAC clone was very similar to that of the parent strain (Figure 3.2).

In addition to the restriction pattern analysis, PCR assays were used to examine the insertion of the BAC vector. Using primers targeting different BAC vector components (Table 3.1), including the enhanced green fluorescence protein (EGFP) gene (RM0901 and RM0944, or RM0945 and RM0911), the chloramphenicol resistance gene (RM0955 and RM0956), and the bacterial origin of replication (RM0957 and RM0958), PCR assays confirmed that the BAC vector was inserted into the FHV-1 genome (data not shown). However, PCR primers targeted to the expected junction between the BAC vector and the viral sequence (RM0910 and RM0959) failed, and primers RM0988 and RM0989 amplified the full length of an intact *US4*. These results

suggested that the BAC was inserted into the FHV-1 genome, but not into *US4* as intended.

Sequencing of the FHV-1 genome

Using "454" next generation sequencing technology, complete genome sequences were generated *de novo* for both the viral genomic DNA and BAC clone, and then compared to one another. The sequence assembly of the BAC clone represented 97.62% of the entire FHV-1 genome with an average read depth of 33x. The assembly of the viral DNA represented 97.52% of the entire genome with an average read depth of 25x.

To complete the sequences, gaps were filled by primer walking, using both pure viral DNA and BAC DNA as template. Three major gaps were present in both assemblies. Gap no. 1 represents the junction of U_L and IR_S, gap no. 2 was located inside both IR_S and TR_S, and gap no. 3 contained the genomic termini, or the junction of TR_S and U_L when the genome is circularized. Based on the *Sal*I restriction pattern, the estimated sizes for gaps 1, 2, and 3 were 1.0 kb, 0.6 kb, and 1.0 kb, respectively. Attempts to close these gaps by primer walking were partially successful. Gaps 1 and 3 were completely closed, and the sequence lengths were as predicted. Gap 2 could not be fully sequenced. Primer walking results revealed that this region contains repetitive units of very high G+C content (77%). Use of additional sequencing chemistry (i.e., dGTP BigDye Terminator) failed to sequence through this gap. The remaining ~314 bp lacking sequence was tentatively filled with the same repetitive unit found at both ends of the gap. No ORFs were found inside or spanning these repeats.

When sequences derived from the viral genome and BAC were compared to each other, they were completely identical, except for the insertion of the BAC vector between U_L and TR_S in the BAC clone, an addition of a 2.7-kb fragment (Contig 3) in the BAC clone, and three aforementioned gaps whose sequence could only be determined from the BAC clone. A BLASTN search against the non-redundant nucleotide collection database nr/nt (Altschul et al., 1997) found that Contig 3 did not resemble any known herpesvirus sequence. A BLAST search in the WGS Contigs database of the cat genome project (http://www.ncbi.nlm.nih.gov/projects/genome/seq/BlastGen/BlastGen.cgi?pid=10703) revealed that a 219-bp portion near the right hand side (5'-end) of Contig 3 is highly similar to a genomic region of cat DNA. PCR primers RM1025 and RM1026 (Table 3.1) targeting sequences near the left hand side (3'-end) of Contig 3 successfully amplified the same 225-bp sequence from the DNA of uninfected Crandell-Reese feline kidney (CRFK) cells (data not shown). Therefore, it is very likely that Contig 3 is a part of the cat genome, presumably acquired by the virus during the recombination process in CRFK cells.

The complete FHV-1 genomic sequence was compared to all FHV-1 DNA sequences in GenBank that contained at least one complete gene. The results are presented in Table 3.2. The DNA sequences were highly similar across strains. The differences observed were mostly limited to single bases. It was noted that the copy number of repetitive sequences can vary between strains, or even within the same strain.

The complete DNA sequences of the FHV-1 genome and the FHV-1 BAC clone were deposited in GenBank under accessions **FJ478159** and **GU250525**, respectively.

Structure of the FHV-1 Genome

The FHV-1 genome is 135,797 bp in length. The overall G+C content of FHV-1 genome is 45%. The genome consists of a 106,369 bp U_L and a 8,424 bp U_S region, with the former being flanked by an inverted 33-bp sequence, and the latter being flanked by IR_S and TR_S elements of 10,469 bp each.

ORF finding, gene content, and gene arrangement

To identify all proteins encoded in the genome, we employed two methods. First, ORFs encoding proteins of $\geq$ 60 amino acids with a methionine start codon were evaluated for coding potential by searching for homologs in other alphaherpesviruses. Homology searches were conducted using BLASTX on the non-redundant protein sequence database nr (Altschul et al., 1997). Other criteria used included compact gene arrangements on both strands with little gene overlap. To find novel genes in the FHV-1 genome *ab initio* and as a second approach to verify the annotation, the sequence was submitted to GeneMarkS (Besemer et al., 2001). GeneMarkS predicted 10 fewer genes, namely *UL3*, *UL11*, *UL24*, *UL26.5*, *UL43*, *UL53*, *US2*, *US6*, *US8.5*, and *US9*. However, these genes are consistently present among varicelloviruses (Tables 3.3 and 3.4), and were easily identified using BLASTX, except for *US2*. Despite the low degree of sequence similarity, *US2* was also predicted based on relative position similar to other varicelloviruses.

Table 3.3 lists all FHV-1 ORFs predicted in the genomic sequence and summarizes the characteristics of the predicted gene products. The arrangement of these

ORFs in the FHV-1 genome is shown in Figure 3.3. Seventy-eight ORFs were predicted in the FHV-1 genome, encoding 74 different proteins, as genes encoding ICP4, US1, and US10 proteins are found twice, once in the IR_S and once in the TR_S, and *UL15* consists of two ORFs. Seventy-two ORFs are present as single copies with 64 located in the U_L, seven located entirely in the U_S, and one that initiates in the U_S and ends in the TR_S. Three ORFs are located entirely in the repeat region, each present once in IR_S and once in TR_S. All ORF start locations were assumed to be the first possible ATG. The name of each protein was given based on its homology to HSV-1 and VZV genes. The predicted properties and functions assigned to each predicted gene were based on those assigned to other varicelloviruses by Refseq entries in the NCBI database. In addition to the ORFs listed in Table 3.3, 53 ORFs with a coding capacity of more than 60 amino acids were identified: 25 were found on the top strand and 28 were on the bottom strand. However, searches for cellular or viral homologs of these ORFs failed to find any significant match, and none of these ORFs were considered as strong candidates for new genes.

Table 3.3 also lists the amino acid sequence similarity of each protein to their counterparts in the other sequenced varicelloviruses. All FHV-1 gene products showed some degree of homology to the gene products of the other varicelloviruses. Not surprisingly, genes involved in nucleotide metabolism, DNA replication and packaging were among the most conserved. Glycoprotein genes were less conserved, although gB did show a high degree of similarity to gB of the other varicelloviruses. FHV-1 proteins were most similar to homologues of EHV-1 and EHV-4, averaging 49.4% and 49.2% animo acid identity, respectively. Phylogenetic relationships between FHV-1 and other

varicelloviruses were examined using single glycoprotein and concatenated amino acid sequence alignments (McGeoch et al., 2000). The results are presented in Figure 3.4 and showed that FHV-1 is most closely related to EHV-1 and EHV-4.

The arrangement of FHV-1 genes is collinear with VZV, BHV-1, BHV-5, EHV-1 and EHV-4. The gene content is highly similar to those of varicelloviruses, especially EHV-1 and -4, with a few exceptions (Table 3.4). FHV-1 lacks the homologs of *V67* and *US5*, while both are present in EHV-1 and -4. *V67* was found in EHV-1, EHV-4, BHV-1, BHV-5, and VZV, but is absent in PRV. *US5*, encoding gJ, was found only in EHV-1 and -4. None of the genes identified were unique to FHV-1.

Polyadenylation signals and splice sites

The PolyADQ program (Tabaska and Zhang, 1999) was used to search for all potential polyadenylation signals in the FHV-1 genome. The search found 323 sites; each was given a score between 0 and 1. In the PRV study (Klupp et al., 2004), experimental results were used to determine a cut off score. Since there were very few, if any, data for the mapping of FHV-1 transcripts, every signal was located in the complete genomic sequence initially. Many signals did not associate with any upstream gene, while very often multiple signals were found for a given ORF or a set of coterminal transcripts. The polyadenylation signals for each gene were annotated based on the assumption that each transcript either ends near the termination codon or is a member of a 3'-coterminal family. The ones that are closest to the stop codon and have the highest scores were annotated.

UL15 is made up of two exons and is well conserved among herpesviruses. The region between and including the two exons of *UL15* were submitted to the Neural Network Splice Site Prediction program conditioned for human splice site recognition (Reese et al., 1997). The predicted donor site with the highest score (0.98) located 39 bp upstream from the end of the first ORF of UL15. The predicted acceptor site with the highest score (0.99) located between the ORF of *UL17* and the second ORF of *UL15*. The spliced mRNA would encode a protein of 734 amino acids.

Origins of replication and tandem repeats

The origins of replication of herpesviruses are characterized by the presence of an AT-rich palindromic sequence. EHV-1, EHV-4, and PRV have at least 3 origins of DNA replication: one copy of OriL found in U_L, between *UL21* and *UL22*, and two copies of OriS found in IR_S and TR_S (Klupp et al., 2004; Telford et al., 1992; Telford et al., 1998). In contrast, VZV lacks an OriL (Davison and Scott, 1986). In the FHV-1 genome, the OriS palindrome was found in IR_S and TR_S. However, no sequence that resembles an OriL was found in the region between *UL21* and *UL22*. Comparison of the DNA sequences in this region between the BAC clone and wild type C-27 strain (data not shown) has ruled out the possibility that a similar palindrome might have been mis-assembled or deleted during cloning.

Using the Tandem Repeats Finder program (Benson, 1999), eight different types of tandem repeat elements at 16 locations in the FHV-1 genome were identified (Table 3.5). One tandem repeat element was found in U_L, one in U_S, and six in IR_S/TR_S. Four tandem repeat elements were located near genomic termini. In addition, the Tandem

Repeats Finder found an imperfect 78-mer repeat in the ORF of *UL44*. The two repeat units have a 91% match with each other.

Reconstitution of virus particles from BAC clone and excision of the BAC vector

Virus particles were reconstituted from the BAC by transfecting CRFK cells with BAC DNA. The reconstituted virus, with the BAC vector in its genome, produced fluorescent plaques in CRFK monolayers (data not shown). To reduce possible effects the BAC vector might have on viral growth characteristics and virulence, CRFK cells were co-transfected with BAC DNA and pcDNA-Cre. pcDNA-Cre expresses Cre protein, which specifically recognizes the loxP sites flanking the BAC cassette and EGFP gene, excises them, and re-ligates the DNA leaving a single loxP site (Figure 3.1). After plaque purification, no fluorescent plaques were found. The excision of BAC vector was also verified by PCR and sequence analysis (data not shown). The BAC-excised FHV-1 BAC clone, FHV1ΔBAC, was used for subsequent *in vitro* and *in vivo* characterizations.

In vitro characterization of FHV1ΔBAC

To define the *in vitro* growth characteristics of the BAC-derived virus, plaque morphologies and multiple-step growth curves for the C-27 strain and FHV1ΔBAC were compared. Plaques produced by the C-27 strain and FHV1ΔBAC virus are morphologically undistinguishable from each other. The average size of 100 plaques was determined for each virus strain. The mean plaque diameter of the FHV1ΔBAC virus was 101% of the C-27 parent strain and not significantly different ($p = 0.740$).

Multi-step growth curves were constructed for both the FHV1ΔBAC virus and the C-27 parent strain (Figure 3.5). At 48 and 72 hours p.i., the FHV1ΔBAC virus grew to titers 0.5 - 1 logs lower than those of the parent strain. Analysis of the growth curves by ANOVA indicated that the differences at 48 hours ($p = 0.003$) and 72 hours p.i. ($p = 0.013$) were statistically significant.

Preliminary *in vivo* characterization

In order to investigate possible attenuation resulting from BAC cloning, a preliminary challenge experiment was carried out using four specific-pathogen-free (SPF) cats. Two cats were inoculated with the FHV1ΔBAC virus, one cat with the C-27 strain (positive control), and another cat was inoculated with cell culture medium (negative control). The results are summarized in Table 3.6 and Figure 3.6. The negative control cat did not show any clinical signs throughout the study. The cats inoculated with the C-27 strain and FHV1ΔBAC virus all developed similar clinical signs. The cumulative clinical scores of the FHV1ΔBAC-infected cats and the cat inoculated with the parent strain were comparable. Virus shedding was detected by virus isolation in the cats inoculated with the C-27 strain and the FHV1ΔBAC virus on days 3, 6, and 9 p.i. Virus neutralization tests demonstrated that the positive control cat and the FHV1ΔBAC virus-infected cats all showed seroconversion on day 14 p.i. The neutralizing antibody titers of the FHV1ΔBAC virus-infected cats and the cat inoculated with the parent strain were very similar.

DISCUSSION

We report here the first BAC clone containing the entire C-27 strain FHV-1 genome that exhibits very high virulence in preliminary *in vivo* inoculation experiments. The restriction pattern of this BAC clone was nearly identical to that of the parent C-27 strain with all exceptions being related to the insertion of the BAC vector or circularization of the genome (Figure 3.2). The size of the genome, derived from both the sequence assembly and the restriction pattern analysis, is consistent with the literature (Rota et al., 1986).

An unexpected 2.7-kb cat DNA was inserted along with the BAC at the genomic termini. A possible explanation is the fact that herpesviruses can acquire parts of host genomes, through an unknown mechanism, during virus replication (Niikura et al., 2006).

The BAC clone-derived FHV-1 virus was characterized *in vitro* by observing its plaque morphology and comparative growth curve analysis. Plaques produced by the BAC clone-derived virus were identical in size to those produced by the parent C-27 strain. The comparative growth curve analysis also showed that the BAC clone-derived virus can grow to $> 10^7$ $TCID_{50}$/ml, which was 0.5-1.0 logs lower than its parent strain (Figure 3.5). Although the BAC vector was excised from the genome through Cre-loxP recombination, parts of the recombination arms, as well as the cellular sequence still remained. The presence of these sequences, totaling 3.5 kb, might have subtle influences on virus growth.

In a limited *in vivo* study, the BAC clone-derived virus showed very similar pathogenicity in the infected cats. The delayed onset of clinical signs and the longer persistence could be related to the slightly slower replication of the BAC-derived virus, as shown in the *in vitro* characterization. However, since the number of cats used was very small, it could also be just a difference in susceptibility between individuals. Although the number was very small, the fact that both of the cats developed symptoms similar to the wild-type strain suggested that the BAC-derived virus is infectious, and its virulence appears to be similar to that of the parent strain. Moreover, in previous studies, it was shown that the symptoms induced by FHV-1 in experimentally infected cats are quite consistent (Kruger et al., 1996; Sussman et al., 1995). The fact that this BAC-derived virus behaves very similarly to its parent strain both *in vitro* and *in vivo* makes it an excellent starting platform for defining the function of individual viral genes, especially those mediating the virulence of FHV-1.

We also present the first complete and annotated sequence of the FHV-1 genome, and a complete overview of the molecular composition of FHV-1. Prior to this study, sequencing efforts were focused on smaller fragments of the genome or individual genes from various strains. A full list of FHV-1 genes completely sequenced prior to this report is included in Table 3.2. Envelope glycoprotein genes drew the most interest; 8 out of 11 had been completely sequenced.

The sequence presented in this report was obtained by sequencing both the FHV-1 DNA genome and the FHV-1 BAC clone using automated high-throughput pyrosequencing. This system is rapid, provides high read depth, does not require any subcloning, and is more economical than traditional Sanger sequencing. One

disadvantage of this method is its short read length. Compared to 500-1000 base read lengths of Sanger sequencing, the read lengths of pyrosequencing averaged 100 bases. Recently, however, the read length has been improved to ~500 bases. The short read length poses difficulties for assembly of repetitive sequences, such as the "a" sequences at the genomic termini. The "a" sequences, which consists complex tandem repeats, could not be assembled, and had to be resolved by primer walking. Another inherent problem of pyrosequencing is the difficulty in determining the number of incorporated nucleotides in homopolymeric regions of 5 or more nucleotides (Ronaghi and Elahi, 2002). Therefore, despite the high redundancy, there is a slight possibility that the lengths of such repeats in this genomic sequence are not entirely accurate.

The physical structure of the FHV-1 genome was first mapped in 1986 when Rota et al. reported a *Sal*I restriction map of the C-27 strain (Rota et al., 1986). In 1991, Grail et al. mapped another strain of FHV-1, B927 (Grail et al., 1991). The complete genomic sequence showed that the gene arrangement in the FHV-1 genome is collinear with that of many varicelloviruses, including BHV-1, BHV-5, EHV-1, EHV-4, and VZV. The shuffling of gene blocks found in the PRV genome did not appear in the FHV-1 genome of this strain. A search for *Sal*I sites in our genomic sequence revealed that the *Sal*I map of C-27 is very similar to that of the B927 strain, with only a few differences. The region in the B927 strain where there is a 16.5-kb *Sal*I fragment contains a 13.5- and a 13.6-kb fragment in the C-27 strain. This could explain why the predicted length of the B927 genome was ~9 kb shorter than our sequence. The second and fourth fragments in the C-27 strain seem to have exchanged their locations in the B927 strain, implying a possible re-arrangement of the genes, as seen in the PRV genome (Klupp et al., 2004).

Most of the FHV-1 ORFs were identified by homology search except for *US2* and *US26.5*. Due to its low similarity to the counterparts in the other varicelloviruses, *US2* was annotated based on its relative position in the genome, which was similar to the other varicelloviruses. *UL26.5* is in the same reading frame as *UL26*, but use alternative start codons. It was also annotated based on its relative position. The *ab initio* gene finding program did not identify new genes unique to FHV-1.

A few alphaherpesviruses, including HSV-1, HSV-2, BHV-1, and PRV, have evolved genomes with a relatively high G+C content. In these genomes, the third codon position is particularly biased towards G or C, since it is the most flexible concerning the amino acid encoded. In PRV, all functional ORFs could be easily identified by screening for ORFs with a high G+C content on the third nucleotide position of codons (Klupp et al., 2004). The FHV-1 genome has not acquired this characteristic. The average G+C percentage of FHV-1 genome was 45%, one of the lowest among sequenced varicelloviruses. Therefore, this method was not applicable for FHV-1.

The number of tandem repeats found in the FHV-1 genome is far lower than in other varicelloviruses. Due to the short read length of the pyrosequencing, it is possible that multiple copies of the same repetitive unit were assembled into far fewer copies. However, many of the reiterated elements in varicellovirus genomes are shorter than 50 bp, which means in the sequence assembly there should still be at least two copies, if they are present, hence detectable by the Tandem Repeats Finder program. Therefore, it is more likely that the FHV-1 genome does not have as many tandem repeat elements. In other alphaherpesvirus genomes, tandem repeats are frequently found between two polyadenylation signals from convergent transcripts, or within 1 kb from the genomic

termini. Based on the location of the repeats, it was hypothesized that they play possible roles in insulation against accidental read-through by RNA polymerase into the oppositely transcribed gene, as well as in the process of genome circulization (Klupp et al., 2004).

In conclusion, the FHV-1 BAC clone we report contains the entire FHV-1 genome, the BAC clone-derived virus grows to slightly lower titers *in vitro* compared to the C-27 parent strain, and its plaque morphology is not significantly altered. Preliminary indications are that its *in vivo* virulence is also similar to that of the C-27 parent strain. Recent advances in recombineering (recombination-mediated genetic engineering) techniques have made it easier to introduce site-specific mutations into a BAC clone (Tischer et al., 2006). In addition, random libraries of herpesvirus BAC mutants can be generated by the use of transposon-mediated insertion mutagenesis (Brune et al., 1999). These characteristics, along with the availability of the complete genome sequence, will make the BAC clone an excellent starting platform for future mutagenesis-based functional studies of viral genes and ultimately for future vaccine developments.

MATERIALS AND METHODS

Cells and Viruses

CRFK cells (ATCC, Manassas, VA) were cultured in Eagle's Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS) and 10 μg/ml of

ciprofloxacin. The FHV-1 prototype strain C-27 (ATCC, Manassas, VA) was propagated in the CRFK cells and used as wild-type virus throughout this study. The purified FHV-1 virions were prepared by pelleting the supernatant through a 20% potassium tartrate cushion at 24,000 x g for 2 hours at 4°C.

Plasmids and Vectors

The 0.6-kb upstream homologous region, which includes the 3'-end of *US3* gene, the intergenic region of *US3* and *US4*, and the 5'-end of *US4* (gG) gene, was amplified from purified FHV-1 genomic DNA by PCR using the Extended High Fidelity PCR System (Roche Applied Science, Indianapolis, IN) with the following primers: RM0874, which contains a *Mlu*I site at the 5'-end; and RM0875, which contains a *Swa*I site, a *Bsi*WI site, and a loxP site at the 5'-end (Table 3.1). The PCR product was cloned into a pCRII vector (Invitrogen, Carlsbad, CA), resulting in pCRII-UgG.

The 1.0-kb downstream homologous region, which includes the intergenic region of *US4* (gG) and *US6* (gD), and the 5'-end of *US6* (gD) gene, was amplified from purified FHV-1 genomic DNA by PCR using the Extended High Fidelity PCR System with the following primers: RM0876, which contains a *Hind*III site, a *Sal*I site, and a loxP site at the 5'-end; and RM0877, which contains a *Mlu*I site at the 5'-end (Table 3.1). The PCR product was also cloned into a pCRII vector, resulting in pCRII-DgG.

pCRII-EGFPm2 was constructed as follows. The vector pEGFP-N1 (Clontech Laboratories, Mountain View, CA) was digested by *Xho*I and *Sal*I and re-ligated, eliminating *Xho*I, *Sac*I, *Hind*III, and *Sal*I sites. The 1.6-kb region containing the modified EGFP expression cassette, which includes the CMV immediate early promoter,

the EGFP ORF, and SV40 early mRNA polyadenylation signal, was amplified from the modified pEGFP-N1 by PCR using Extended High Fidelity PCR System with the following primers: RM0872, which contains a *Bsi*WI site at the 5'-end; and RM0873, which contains a *Sac*I and *Sal*I site at the 5'-end (Table 3.1). The PCR product was cloned into a pCRII vector, resulting in pCRII-EGFPm2.

pGEM3Zf-DUgG2, which contains two homologous regions and two loxP sites, was constructed by ligation of the following fragments: the *Mlu*I-*Swa*I fragment of pCRII-UgG; the *Hind*III-*Mlu*I fragment of pCRII-DgG; and the pGEM-3Zf vector (Promega, Madison, WI), digested with *Hind*III and *Sma*I.

pGEM3Zf-DUgGEGFP2, which contains two homologous regions, two loxP sites, and a EGFP expression cassette, was constructed as follows: pCRII-EGFPm2 was digested with *Bsi*WI and *Sac*I, and the fragment containing EGFP expression cassette was ligated with pGEM3Zf-DugG2, also digested with *Bsi*WI and *Sac*I.

To target the BAC cassette insertion site to *US4*, the BAC vector pBAC04, which contains the BAC cassette, two (upstream and downstream) homologous recombination arms between two loxP sites and an EGFP expression cassette, was constructed as follows. The BAC vector pBeloBAC11 (Invitrogen, Carlsbad, CA) was digested with *Sal*I and dephosphorylated; the 6.4-kb fragment was purified and ligated with the *Sal*I fragment of pGEM3Zf-DUgGEGFP2.

To construct pcDNA-Cre, a plasmid that expresses Cre recombinase, the entire ORF of Cre was excised by *Kpn*I and *Sma*I digestions from pGIKS-Cre (ATCC, Manassas, VA) and ligated with pcDNA3.1 vector (Invitrogen, Carlsbad, CA), digested with *Kpn*I and *Eco*RV.

All the plasmid constructs were verified by both restriction digestion and sequencing. pBAC04 was transformed into the *E. coli* strain DH10B (Invitrogen, Carlsbad, CA). All the other constructs were transformed into the *E. coli* strains TOP10 or DH5α (both from Invitrogen). The transformants were plated on selective agar that contained 75 μg/ml of ampicillin or 34 μg/ml of chloramphenicol.

DNA Extraction

Pure viral genomic DNA was prepared as follows: Purified virions were incubated in lysis buffer (0.1 M Tris-Cl, pH8.0, 1 mM EDTA, 1% SDS) with 50 μg/ml proteinase K at 37°C. DNA was subsequently extracted with phenol-chloroform-isoamylalcohol (25:24:1), and precipitated with 100% ethanol. Care was used to avoid shearing the DNA. High copy number plasmids were extracted using the Plasmid Mini Kit (Qiagen, Valencia, CA). Small-scale BAC DNA purifications were carried out using the alkaline lysis method (Sambrook and Russell, 2001). Large-scale and high-purity BAC DNA purifications were carried out using the Large Construct Kit (Qiagen, Valencia, CA) following the manufacturer's instructions.

BAC Cloning

The strategy used for cloning FHV-1 genome as a BAC exploits spontaneous homologous recombinations in the transfected CRFK cells, and was similar to those used in previous reports (Costes et al., 2006; Niikura et al., 2006). To reduce possible sequence alterations during *in vitro* passage in cell culture, low-passage C-27 strain virus (P4) was used for BAC cloning. Purified FHV-1 genomic DNA and pBAC04

were co-transfected into CRFK cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The supernatant of co-transfected cells was collected for plaque assay and plaques were examined for EGFP expression. One virus clone that consistently produced fluorescent plaques was obtained. After two times of plaque purification, CRFK cells were inoculated with this virus clone, and the circular form replication intermediate of the virus genome was extracted from the cells using the method of Hirt (1967) and transformed into *E. coli* DH10B cells. Colonies growing on the chloramphenicol plate were examined for the presence of FHV-1 genome and BAC vector by restriction pattern analysis and PCR. A clone that had a *Sal*I pattern representative of most bacterial clones and most similar to that of the C-27 strain was used for all subsequent studies.

Reconstitution of infectious virus from BAC

The CRFK cells were co-transfected with 1 μg of the BAC DNA and 1 μg of pcDNA-Cre using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Sequencing, sequence assembly and gap closure

The majority of the sequence was determined by shotgun sequencing, using the high-throughput pyrosequencing instrument Genome Sequencer 20 (Roche Applied Science, Indianapolis, IN) at the Genomics Core of Michigan State University's Research Technology Support Facility (RTSF). The reads were assembled using the Newbler assembly program (Roche Applied Science, Indianapolis, IN) at the

Bioinformatics Core of RTSF. Gaps were closed by primer walking. Gap closure was carried out using BigDye Terminator 3.0 and dGTP BigDye Terminator chemistries and Gene Analyzer 3100 (Applied Biosystems, Foster City, CA).

ORF finding and sequence analysis

To identify the genes encoded in the FHV-1 genome, all potential ORFs with a minimum length of 60 codons and a methionine as start codon were analyzed for homology to known proteins using BLASTX against non-redundant protein database (Altschul et al., 1997). To find novel genes in the FHV-1 genome *ab initio*, and as a second approach to verify the annotation, the sequence was submitted to GeneMarkS (Besemer et al., 2001). To search for polyadenylation signals, the FHV-1 genome sequence was submitted to PolyADQ, a eukaryotic polyadenylation signal search engine (Tabaska and Zhang, 1999). The Neural Network Splice Site Prediction program conditioned for human splice site recognition (Reese et al., 1997) was used to identify possible splice donor and acceptor sites of UL15. The Tandem Repeats Finder program (Benson, 1999) was used to search for tandem repeats. Phylogenetic analyses were carried out using the MEGA4 software (Tamura et al., 2007).

Plaque Morphology

One hundred fifty $TCID_{50}$ units of the virus were inoculated on CRFK monolayers. After one-hour adsorption, the diluted viruses were removed, and fresh growth medium added. The cells were then incubated at 37°C in an atmosphere of 5% CO_2. After 40 hours of incubation, 100 plaques produced by each virus were

photographed and the diameters determined using the QCapture software (QImaging, Surrey, BC, Canada). The measurements were analyzed by ANOVA.

Multiple-step Growth Curve

The wild type virus and the BAC clone were inoculated on CRFK monolayers in triplicate at an m.o.i. of 0.01. After one-hour adsorption, the diluted viruses were removed and the cells overlaid with fresh medium. The first supernatant samples were collected at this time as 0 hour p.i. The monolayers were then incubated at 37°C in an atmosphere of 5% CO_2. Supernatants were collected at 0, 6, 24, 48, and 72 hours p.i. and stored at -80°C until titration. The mean titers of each time point were analyzed by ANOVA.

Cats

Four 12-week old, female SPF cats were used (Liberty Research, Waverly, NY). Cats were housed in individual cages in rooms with controlled temperature, humidity, and lighting. They were fed a combination of dry and moist diets. Each group of cats was housed in a separate Biocontainment Level-2 room. All cats were acclimated for 13 days before virus exposure. Two cats were inoculated oronasally with 2×10^5 $TCID_{50}$ of the FHV1ΔBAC. The other two cats were either inoculated oronasally with 2×10^5 $TCID_{50}$ of the C-27 strain wild type virus (positive control) or Eagle's Minimum Essential Medium (negative control). The titers were re-checked prior to inoculation. Clinical signs induced by inoculation of the viruses were scored as described in the USDA Supplemental Assay Method 311 (U.S. Department of Agriculture, Animal and

Plant Health Inspection Service, National Animal Veterinary Services Laboratory, 1985; Table 3.1). Oral swabs were collected from each cat at days 0, 3, 6, 9, 14, and 21 p.i. for virus isolation. Serum samples were collected from each cat at days 0, 14, and 21 p.i. for virus neutralization testing. The cat study was reviewed and approved by the Institutional Animal Care and Use Committee at Michigan State University.

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TABLES

Table 3.1 List of primers used in this study

Primer	Sequence (5' – 3')
BAC Construction	
RM0872	CGTACGTAGTTATTAATAGTAATCAATTACGG
RM0873	GTCGAC GAGCTCACGCCTTAAGATACATTGATGAG
RM0874	ACGCGTGAATTCCCAGGCGACCCGAC
RM0875	ATTTAAAT CGTACG*ATAACTTCGTATAATGTATGC* *TATACGAAGTTAT*TATCTCTGGTGATCTTGAAGAG
RM0876	AAGCTT GTCGAC*ATAACTTCGTATAGCATACATTA* *TACGAAGTTAT*TAAACTGATTAAATTTAATTAAAG
RM0877	ACGCGTGAGATAGTTGTACCATAAAATCTG
Sequence verification of the transfer plasmid BAC04	
RM0898	CAATTTCACACAGGAAACAG
RM0899	ACGGATTTTTAAGCACACCA
RM0900	TATTCGAACCATACAACTATC
RM0903	AAGGCGATTAAGTTGGGTA
RM0904	GTTGGGGTCTTTGCTCAGGG
RM0905	AAGAAGTCGTGCTGCTTCATG
RM0906	GGAGTTGTTACGACATTTTGG
RM0907	TTACTATGGGAACATACGTC
PCR Assays	
RM0901	ATACGATTGTATGTCAAAATAC
RM0910	AACGGAGTAACCTCGGTGTG
RM0911	AATGCTTAATGAATTACAACAG
RM0944	GCGTTACTATGGGAACATAC
RM0945	ATCAGCCATACCACATTTGTAG
RM0955	ACCAATTCTCATGTTTGACAGC
RM0956	ATCGGCACGTAAGAGGTTCC
RM0957	TGGACAGAACAACCTAATGAAC
RM0958	ATGACAGATCCATGTGAAGTG
RM0959	TAATCACGAGCTCCATCAAGGC
RM0988	ATTACGTTTATTGGACCCGAG
RM0989	TGAGACTTTAATTAAATTTAATCAG
RM1025	ACTTCTTTTCTACTTGCCCTGG
RM1026	TAACCCCAGCTTAAACAGAAC

Restriction sites are underlined; loxP sites are in italic.

Table 3.1, continued

Primer	Sequence (5' – 3')
Gap closure by primer walking	
RM1023	AATATCACCCCGGTAAACGACAC
RM1024	ACTTCCTGGAATAATGCACATC
RM1027	GTTTTTGTTAATACTTCTGTGGAT
Rm1028	AGTATATATAAACCCTACCTACG
RM1037	ACACCTCTCGCTTCTCCTCTGAC
RM1038	TCAGAGACGAAACTGCTCGCATAG
RM1039	AATAGTTATTTTTATCTCACATGC
RM1040	CCTCCGCGGGTTCTAAAACTTG
RM1041	GAGGACGCGGAGAGTGGATGGTTC
GTS565	CATATTCAAATCTGGCCTAAT
GTS569	AGATTTGAATATTTACTGATGTGA
RM1240	TCCTTATTAGGCCAGATTGAATATG
RM1242	TCTACATATGAATAGTAGATTTGAATATTTACT
RM1243	TATTTACTGATGTGATAACTCGATCC
RM1244	GGGGGAAAAAAATTGAGTTGGAGCTC
RM1245	TGAGTTGGAGCTCAGTCTCCATATACG
RM1246	CATATACGGAGGTGGACAGTGTAG

Restriction sites are underlined; loxP sites are in italic.

Table 3.2 Comparison of the complete FHV-1 genomic sequence with sequences available in GenBank

Accession No.	Strain or isolate	Sequence length (nt)	Encoded genes		Sequence identity (%)	Notes
			with complete cds	with incomplete cds		
D14563	G2620	3,038	UL45	UL46, UL44	99.9	
GM036023	N/A	10,803	UL45, UL44, UL43, UL42, UL41, UL40	UL46, UL39	99.6	
AX018721	N/A	2,956	UL45, UL44	UL46, UL43	99.9	
AR018958, I58415	N/A	1,007	UL45	UL46, UL44	99.9	
D86616	C7301	2,337	UL44	UL45	99.8	
AX244515, BD396132	N/A	1,602	UL44	—	99.9	
AJ006454	B927	5,587	UL40, UL39	UL41, UL38	99.4	
AJ224971	B927	4,848	UL30	UL31, UL29	99.5	
S66371	C-27	3,340	UL27	UL28	99.5	
S49775	C7301	3,240	UL27	UL28	99.8	
AX244511, BD396130	N/A	2,829	UL27	—	99.7	
GM981119	N/A	2,847	UL27	—	99.8	
E12463	N/A	1,619	UL23	UL24, UL22	99.9	
M26660	UCD	1,619	UL23	UL24, UL22	100.0	
S64566	C7301	3,746	UL22	UL23, UL21	100.0	
AB112595	00-035	3,007	UL17	UL16	100.0	
AF022391	C-27	4,918	UL2, UL1, ICP0	UL3	99.4	21-mer repeat: >11 vs 11 copies
DQ452613	Taichung 1	882	UL2	—	99.7	
D30766	C7301	6,175	ICP0	—	100.0	20-mer repeat: 20.1 vs 6.1 copies
AY740677	B927	3,127	US4	US3, US6	99.9	16-mer repeat: 3 vs 12 copies
S72415	C-27	6,208	US4, US6, US7, US8	US3, US8.5	99.6	16-mer repeat: 13 vs 12 copies
D30767	C7301	2,317	US6	US4, US7	100.0	16-mer repeat: 7 vs 12 copies
D42113	G2620	8,276	US6, US7, US8, US8.5, US9, US10, US1	US4	99.9	16-mer repeat: 9 vs 12 copies
AX244521, BD396135	N/A	1,122	US6	—	100.0	
GM981120	N/A	1,125	US6	—	99.7	
A37005, AR281844	N/A	6,154	US7, US8, US8.5, US9, US10, US1	—	99.9	
X98448	B927	1,155	US7	—	99.7	
X98449	B927	1,599	US8	—	100.0	

Table 3.3 Predicted FHV-1 ORFs

Protein	Length (aa)	aa identity (%)						Predicted function or property
		EHV-1	EHV-4	BHV-1	BHV-5	PRV	VZV	
UL56	203	22.9	**23.2**	N/A	N/A	10.5	19.7	membrane protein UL56
V1	144	**27.0**	23.9	N/A	N/A	N/A	15.3	membrane protein V1
CIRC	278	**32.0**	31.7	31.5	31.5	N/A	26.1	myristylated tegument protein CIRC
UL55	209	**47.5**	46.0	N/A	N/A	N/A	33.9	nuclear protein UL55
UL54	438	40.7	41.8	42.1	**42.5**	42.1	30.8	multifunctional expression regulator
UL53	344	44.9	**46.1**	34.0	33.2	34.3	42.6	envelope glycoprotein K
UL52	1,062	**48.1**	47.3	40.5	43.0	43.9	39.9	helicase-primase primase subunit
UL51	242	46.4	**46.6**	38.7	37.8	41.6	38.6	tegument protein UL51
UL50	325	**38.8**	**38.8**	32.8	34.1	36.7	27.7	deoxyuridine triphosphatase
UL49.5	95	40.0	**41.1**	31.5	35.2	35.2	32.2	envelope glycoprotein N
UL49	345	26.2	26.8	26.7	23.6	**27.3**	19.9	tegument protein VP22
UL48	447	**54.6**	54.4	44.0	43.7	46.6	42.6	transactivating tegument protein VP16
UL47	858	**39.3**	38.4	29.5	30.8	31.6	19.5	tegument protein VP13/14
UL46	720	**38.8**	**38.8**	31.0	30.7	28.5	27.9	tegument protein VP11/12
UL45	193	**28.3**	**28.3**	N/A	N/A	N/A	N/A	membrane protein UL45
UL44	534	**32.1**	30.9	29.0	28.2	20.0	28.3	envelope glycoprotein C
UL43	415	**40.3**	39.4	27.3	26.7	22.5	24.1	envelope protein UL43
UL42	395	38.7	**39.8**	30.5	28.7	27.5	24.1	DNA polymerase processivity subunit
UL41	453	**59.6**	59.4	52.4	52.6	56.6	40.8	tegument host shutoff protein
UL40	331	67.0	66.2	65.6	68.3	**70.3**	64.4	ribonucleotide reductase subunit 2
UL39	786	**64.8**	64.7	57.9	58.5	61.2	52.9	ribonucleotide reductase subunit 1
UL38	463	50.6	**50.9**	45.8	44.2	48.2	40.6	capsid triplex subunit 1
UL37	1,027	**43.9**	43.8	34.0	33.5	37.2	32.2	tegument protein UL37
UL36	3,033	42.5	**42.6**	33.9	34.2	34.9	32.3	large tegument protein
UL35	108	46.7	43.9	50.0	49.1	**54.0**	36.8	small capsid protein
UL34	271	**49.8**	48.5	44.4	42.0	44.2	44.1	nuclear egress membrane protein
UL33	123	46.3	45.0	**49.1**	49.5	45.0	39.0	DNA packaging protein UL33
UL32	582	**54.3**	53.8	48.8	49.6	53.4	52.6	DNA packaging protein UL32
UL31	337	**63.4**	62.5	54.1	52.8	61.0	55.3	nuclear egress lamina protein
UL30	1,205	64.9	**65.1**	59.0	59.4	62.7	58.7	DNA polymerase catalytic subunit

Table 3.3, continued

Protein	Length (aa)	aa identity (%)						Predicted function or property
		EHV-1	EHV-4	BHV-1	BHV-5	PRV	VZV	
UL29	1,200	**66.2**	**66.2**	57.7	57.5	59.9	54.4	single-stranded DNA-binding protein
UL28	779	57.8	**59.0**	51.3	51.6	53.8	50.5	DNA packaging terminase subunit 2
UL27	948	58.2	59.1	59.2	59.1	**59.4**	53.2	envelope glycoprotein B
V32	130	**47.2**	43.0	N/A	N/A	N/A	36.9	protein V32
UL26.5	294	**37.3**	36.0	21.4	23.2	16.2	26.0	capsid scaffold protein
UL26	598	**49.1**	47.3	39.6	40.0	42.0	34.4	capsid maturation protease
UL25	623	**56.1**	55.1	52.0	52.0	49.7	49.2	DNA packaging tegument protein UL25
UL24	260	48.6	50.2	38.1	37.6	**55.6**	36.2	nuclear protein UL24
UL23	343	47.4	**48.0**	37.9	36.9	47.3	35.3	thymidine kinase
UL22	821	**33.8**	33.1	30.0	29.5	27.4	29.0	envelope glycoprotein H
UL21	527	**47.5**	46.9	35.2	36.3	40.4	39.0	tegument protein UL21
UL20	229	**45.9**	43.2	31.6	30.7	26.7	33.2	envelope protein UL20
UL19	1,377	**72.8**	72.2	65.9	66.3	68.1	60.0	major capsid protein
UL18	312	**64.1**	**64.1**	59.8	61.1	58.8	55.3	capsid triplex subunit 2
UL17	691	**51.2**	50.8	41.8	41.4	44.4	37.0	DNA packaging tegument protein UL17
UL16	363	**52.1**	51.0	44.0	45.3	39.1	37.3	tegument protein UL16
UL15	734	**71.3**	71.0	63.6	63.5	61.3	61.3	DNA packaging terminase subunit 1
UL14	322	34.5	35.4	32.5	32.7	**42.7**	31.6	tegument protein UL14
UL13	606	**49.2**	49.0	36.8	36.0	31.9	35.5	tegument serine/threonine protein kinase
UL12	544	59.9	**60.4**	41.0	41.2	47.1	37.6	deoxyribonuclease
UL11	76	**36.1**	35.6	33.8	34.7	25.4	**36.1**	myristylated tegument protein
UL10	422	**40.7**	40.5	36.7	37.0	39.6	33.2	envelope glycoprotein M
UL9	860	**58.5**	58.4	48.8	51.5	49.8	46.5	DNA replication origin-binding helicase
UL8	782	**46.0**	45.2	36.8	36.5	36.2	36.6	helicase-primase subunit
UL7	296	**46.1**	44.9	36.9	38.5	40.4	37.3	tegument protein UL7
UL6	717	57.9	58.7	50.9	53.5	**60.1**	48.2	capsid portal protein
UL5	863	**70.7**	70.0	64.9	65.0	60.5	66.1	helicase-primase helicase subunit
UL4	236	**43.6**	42.9	36.4	37.8	32.4	26.7	nuclear protein UL4
UL3.5	181	21.9	21.4	18.3	16.3	18.0	**22.5**	protein V57
UL3	201	67.7	**69.5**	54.5	51.0	44.4	43.0	nuclear protein UL3
UL2	293	49.8	49.8	**50.0**	48.1	46.2	43.4	uracil-DNA glycosylase
UL1	147	**28.8**	**28.8**	25.4	27.3	24.6	27.1	envelope glycoprotein L
ICP0	498	**23.4**	22.0	21.4	20.1	18.9	14.2	ubiquitin E3 ligase ICP0

Table 3.3, continued

Protein	Length (aa)	aa identity (%)						Predicted function or property
		EHV-1	EHV-4	BHV-1	BHV-5	PRV	VZV	
ICP4	1,398	42.7	**43.2**	39.6	40.7	40.2	35.4	transcriptional regulator ICP4
US1	334	**54.8**	53.4	44.3	43.5	33.5	31.2	regulatory protein ICP22
US10	213	**45.0**	44.5	N/A	N/A	N/A	27.1	virion protein US10
US2	102	12.7	11.8	10.8	11.8	**13.7**	N/A	virion protein US2
US3	351	**54.4**	51.6	41.9	41.0	46.1	38.2	serine/threonine protein kinase US3
US4	434	**36.3**	**36.3**	28.7	28.4	27.0	N/A	envelope glycoprotein G
US6	374	24.9	25.1	30.1	**30.2**	26.3	N/A	envelope glycoprotein D
US7	384	35.4	**36.7**	23.6	23.4	22.9	23.3	envelope glycoprotein I
US8	532	**45.9**	45.4	30.8	29.8	22.9	25.2	envelope glycoprotein E
US8A	100	13.0	**14.6**	N/A	N/A	N/A	N/A	membrane protein US8A
US9	152	30.1	35.2	25.9	26.3	**35.8**	26.7	membrane protein US9
US10	213							virion protein US10
US1	334							regulatory protein ICP22
ICP4	1,398							transcriptional regulator ICP4

Table 3.4 Differences in gene composition among sequenced Varicellovirus genomes

Protein	FHV-1	EHV-1	EHV-4	BHV-1	BHV-5	PRV	VZV	Predicted Function or Property
UL56	+	+	+	–	–	+	+	membrane protein UL56
V1	+	+	+	–	–	–	+	membrane protein V1
CIRC	+	+	+	+	+	–	+	myristylated tegument protein CIRC
UL55	+	+	+	–	–	–	+	nuclear protein UL55
VZV ORF13	–	–	–	–	–	–	+	thymidylate synthase
UL45	+	+	+	–	–	–	–	membrane protein UL45
V32	+	+	+	–	–	–	+	protein V32
V67	–	+	+	+	+	–	+	virion protein V67
US10	+	+	+	–	–	–	+	virion protein US10
US4	+	+	+	+	+	+	–	envelope glycoprotein G
US5	–	+	+	–	–	–	–	envelope glycoprotein J
US6	+	+	+	+	+	+	–	envelope glycoprotein D
US8A	+	+	+	–	–	–	–	membrane protein US8A

Table 3.5 Tandem repeats within the FHV-1 genome

Location	Repeat unit size (bp)	Copy no.	Sequence	Total length (bp)	Note
121 – 497	17	22.2	tggagtctaggtgtggg	377	U_L / Gap3
106,506 – 106,736 (135,497 – 135,727)	21	11	ggcctaataaggaaggggagg	231	IR_S / Gap1 (TR_S / Gap3)
106,734 – 106,949 (135,284 – 135,499)	30	7.2	tctggcggtttgtgggttggcatattcaaa	216	IR_S / Gap1 (TR_S / Gap3)
106,963 – 107,066 (135,167 – 135,270)	30	3.5		104	IR_S / Gap1 (TR_S / Gap3)
110,463 – 110,501 (131,732 – 131,770)	18	2.2	tggagcgacgctcactga	39	IR_S / *ICP4* (TR_S / *ICP4*)
111,766 – 111,877 (130,346 – 130,467)	20	6.1	accttcgctcctcccctcgt	122	IR_S / Between *ICP4* and *US1* (TR_S / Between *ICP4* and *US1*)
112,613 – 112,814 (129,419 – 129,620)	53	3.8	aggttggaagccatgttgttccggttgcac atctaatctacatgaaagtggga	202	IR_S / Between *ICP4* and *US1* (TR_S / Between *ICP4* and *US1*)
112,851 – 113,459 (128,774 – 129,382)	26	23.4	gggggatcgaggggggggcagagggga	609	IR_S / Gap2 (TR_S / Gap2)
120,219 – 120,410	16	12	ggggctgtggggacga	192	U_S / Between *US4* and *US6*

Table 3.6 Cumulative clinical score, virus shedding pattern, and virus neutralizing antibody titers of SPF cats inoculated with the C-27 parent strain, the FHV1ΔBAC strain, or mock-inoculated with cell culture medium

Days p.i.	AJJ5 (C-27)		AJC2 (FHV1ΔBAC)		AJC3 (FHV1ΔBAC)		AJD3 (EMEM)	
	VI	VN	VI	VN	VI	VN	VI	VN
0	–	<4	–	<4	–	<4	–	<4
3	+	ND	+	ND	+	ND	–	ND
6	+	ND	+	ND	+	ND	–	ND
9	+	ND	+	ND	+	ND	–	ND
14	–	64	–	32	–	32	–	<4
21	–	64	–	32	–	64	–	<4
Cumulative clinical score	22		22		26		0	

VI: virus isolation; VN: virus neutralization; ND: not determined.

FIGURES AND FIGURE LEGENDS

Figure 3.1 Diagrams showing (A) structure of FHV-1 genome, (B) location of the BAC cassette and cellular sequence insertion, (C) the composition of the BAC cassette, and (D) the FHV-1 BAC clone after excision of the BAC cassette. The diagram is not to scale.

A
B
C
D
UL
IRS
US
TRS
BAC vector
UL56
Cellular sequence
loxP site
Partial recombination arm
EGFP
CmR
OriS
repE
parA
parB
parC
loxP site
Partial recombination arm
loxP site
Partial recombination arms
UL56
Cellular sequence

Figure 3.1

Figure 3.2 Three bacterial clones of FHV-1 BAC (lanes 1-3) and C-27 parent strain genomic DNA (lane 4) were treated overnight with restriction endonuclease *SalI* at 37°C. The DNA fragments were resolved and visualized using a 0.7% agarose gel stained with ethidium bromide. Based on sequence analysis, the linear FHV-1 C-27 strain genome contains the following *SalI* fragments. A: 16,123 bp; B: 14,011 bp; C: 13,648 bp; D: 13,466 bp; E: 11,730 bp; F: 10,121 bp; G: 9,559 bp; H: 9,119 bp; I: 8,564 bp; J: 6,977 bp; K: 6,701 bp; L: 4,951; M: 3,996 bp; N: 2,587; O: 1,724 bp; P: 769 bp; Q: 677 bp. The 414-bp R fragment and two 330-bp S fragments were not visible in this picture. G and M fragments that contained genomic termini (marked by diamonds in lane 4) would have been ligated when the genome circularized, but were instead resolved as three fragments because of the introduction of additional *SalI* sites by the BAC insertion. The first two of these fragments, 9,445 and 9,171 bp in size (arrows), form a triple band with the H fragment. The other fragment was 6,384-bp in size (arrows) The size of the K fragment was found to be variable among bacterial clones (arrowheads). Position of 1 kb DNA ladders are indicated at the left side.

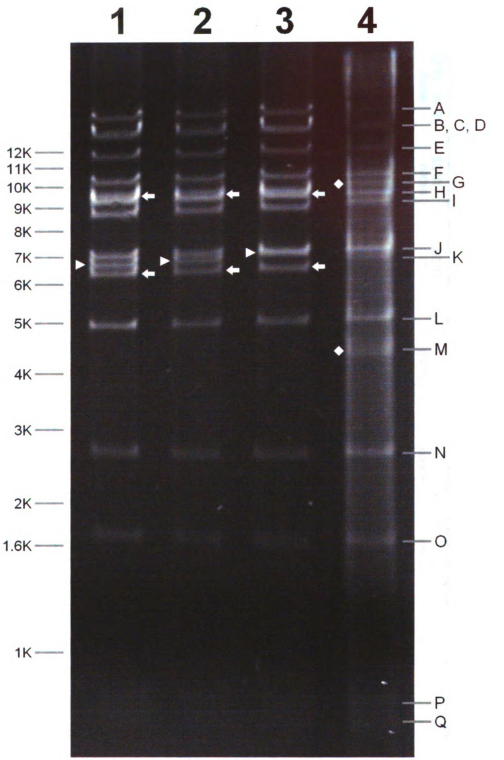

1
2
3
4
12K
11K
10K
9K
8K
7K
6K
5K
4K
3K
2K
1.6K
1K
A
B, C, D
E
F
G
H
I
J
K
L
M
N
O
P
Q

Figure 3.2

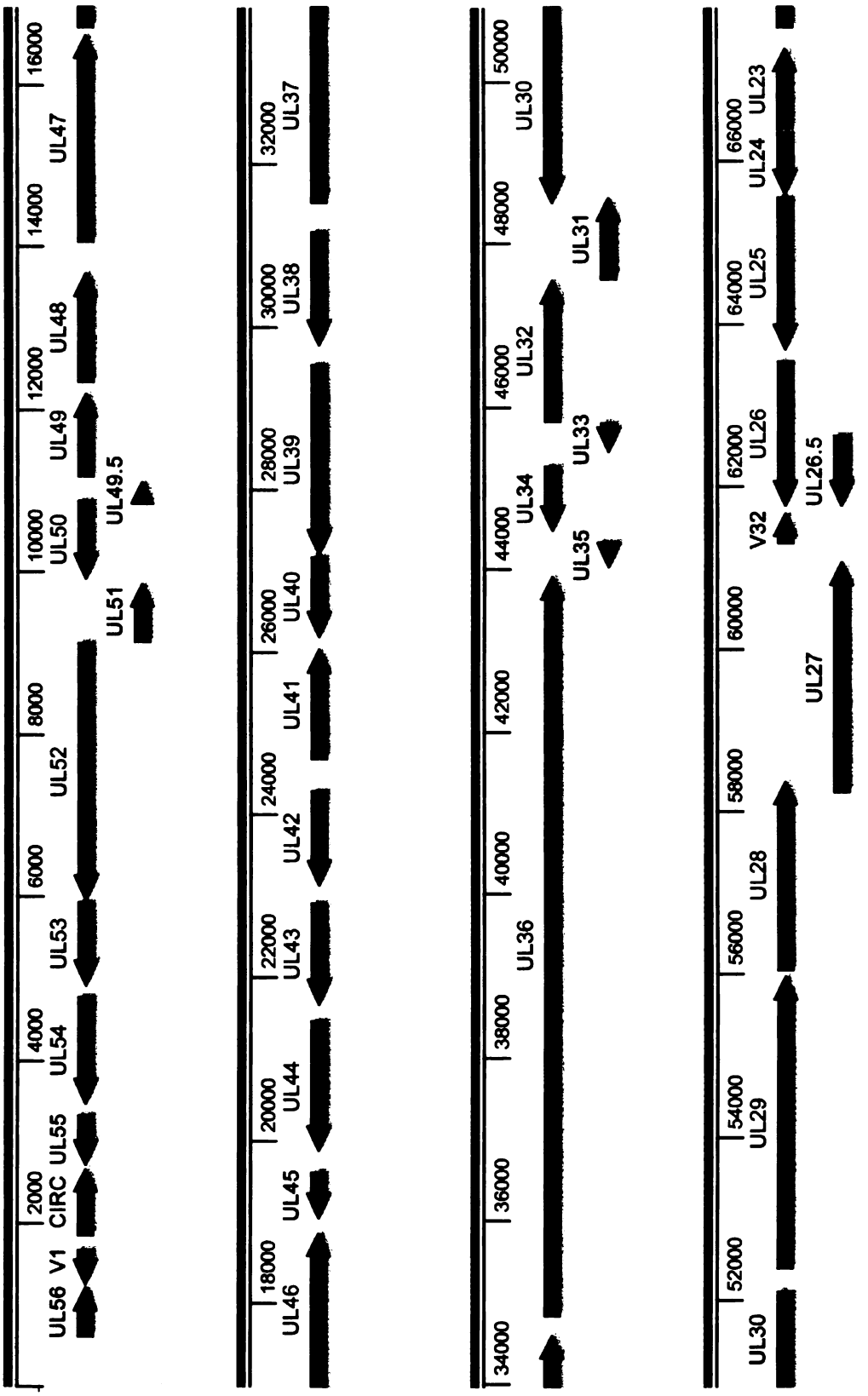


Figure 3.3 Predicted FHV-1 gene arrangement.

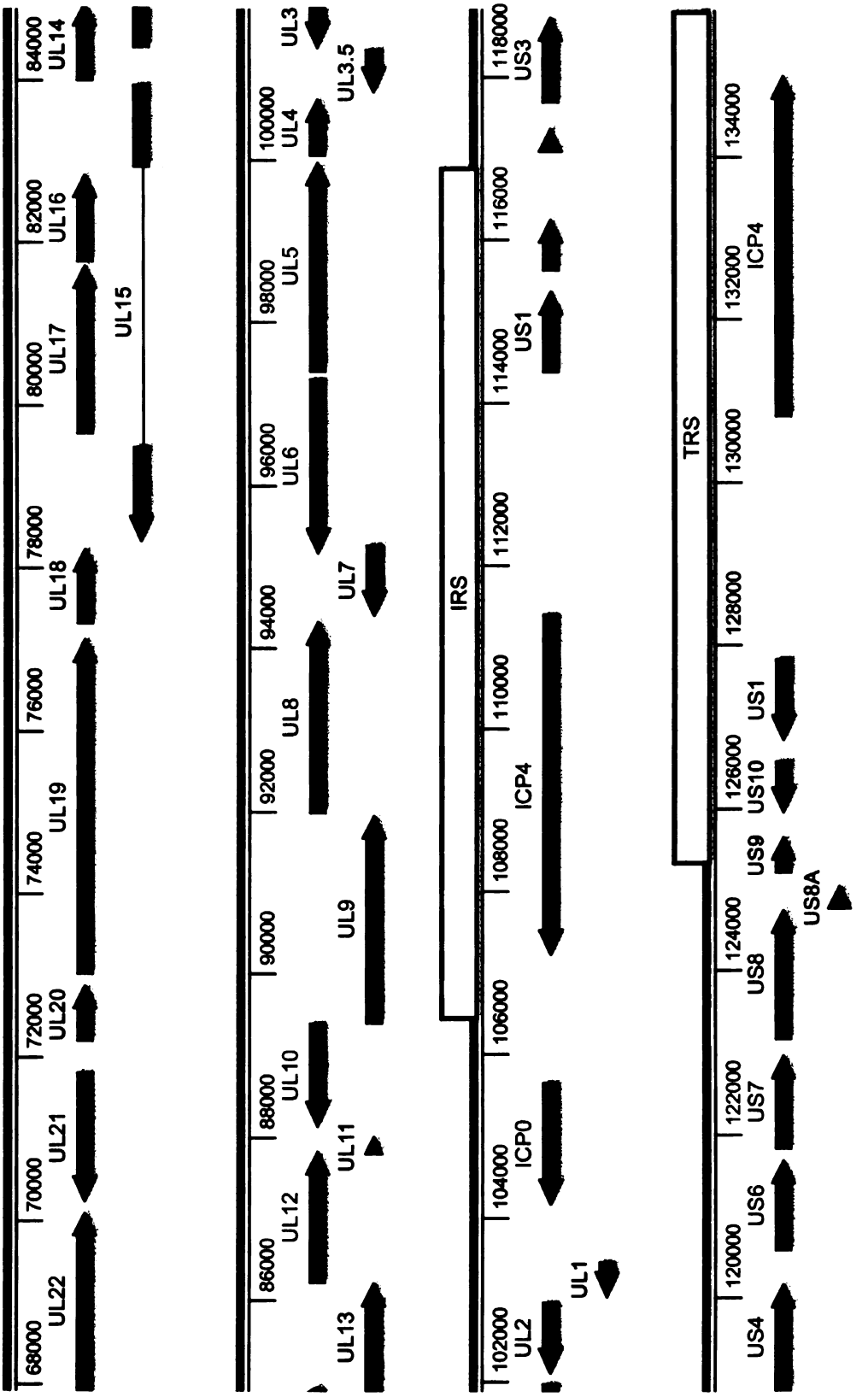


Figure 3.3, continued

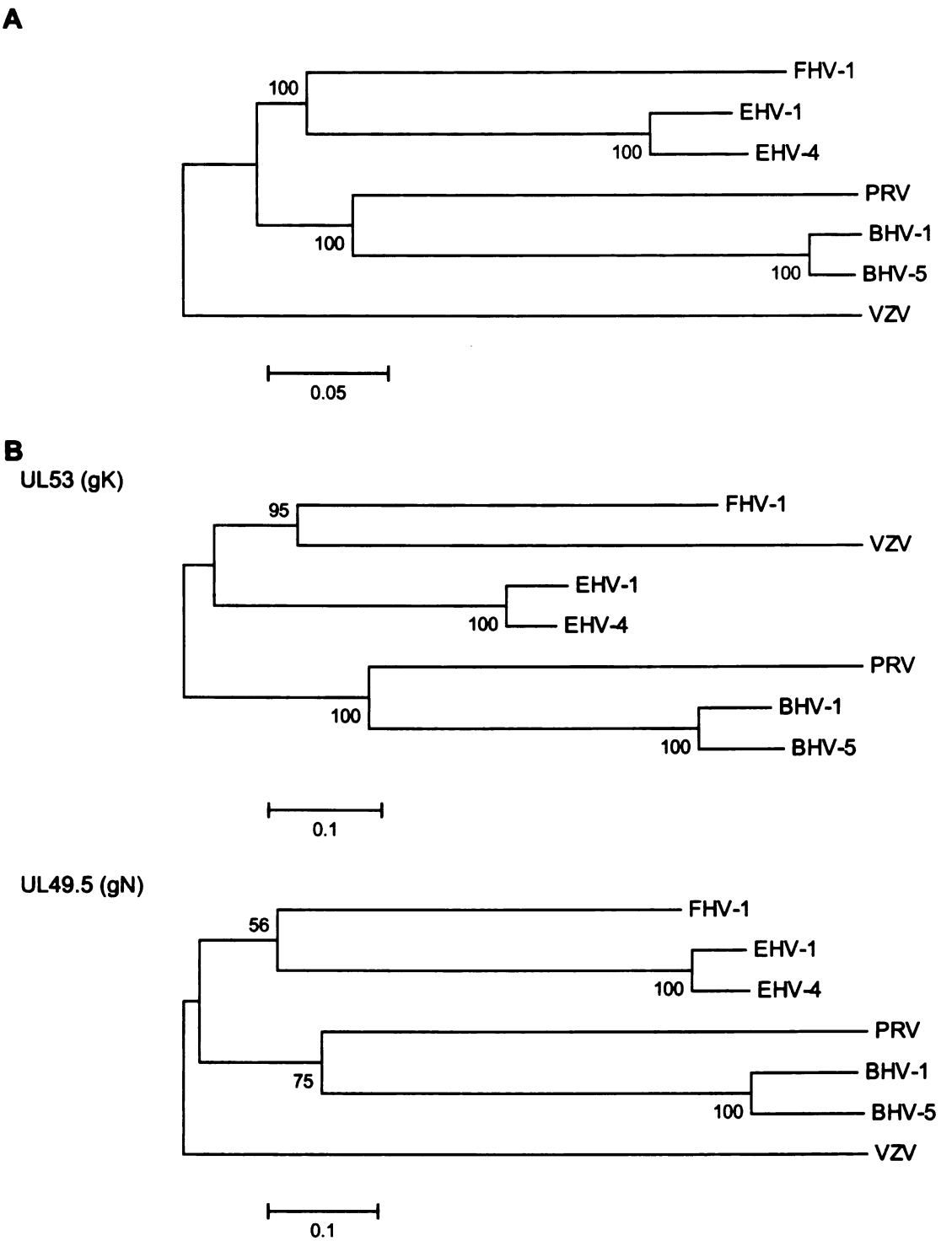


Figure 3.4 Phylogenetic analyses based on concatenated amino acid sequence alignment of conserved genes (A) and individual glycoprotein amino acid sequence alignments (B). Trees were generated using the neighbor-joining method. Bootstrap values (1,000 replicates) are given for each branch. Scale bars represent number of amino acid substitutions per site.

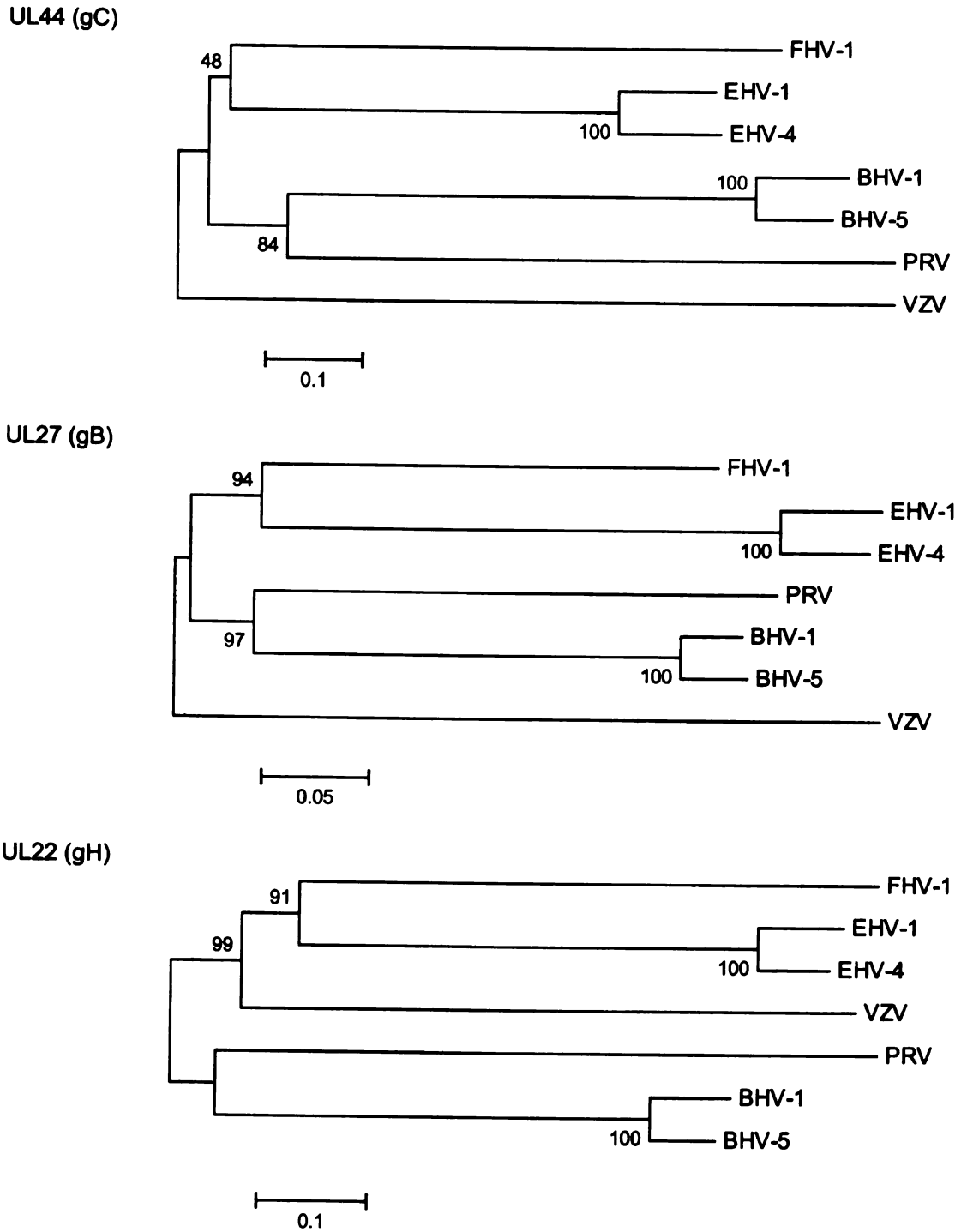


Figure 3.4, continued.

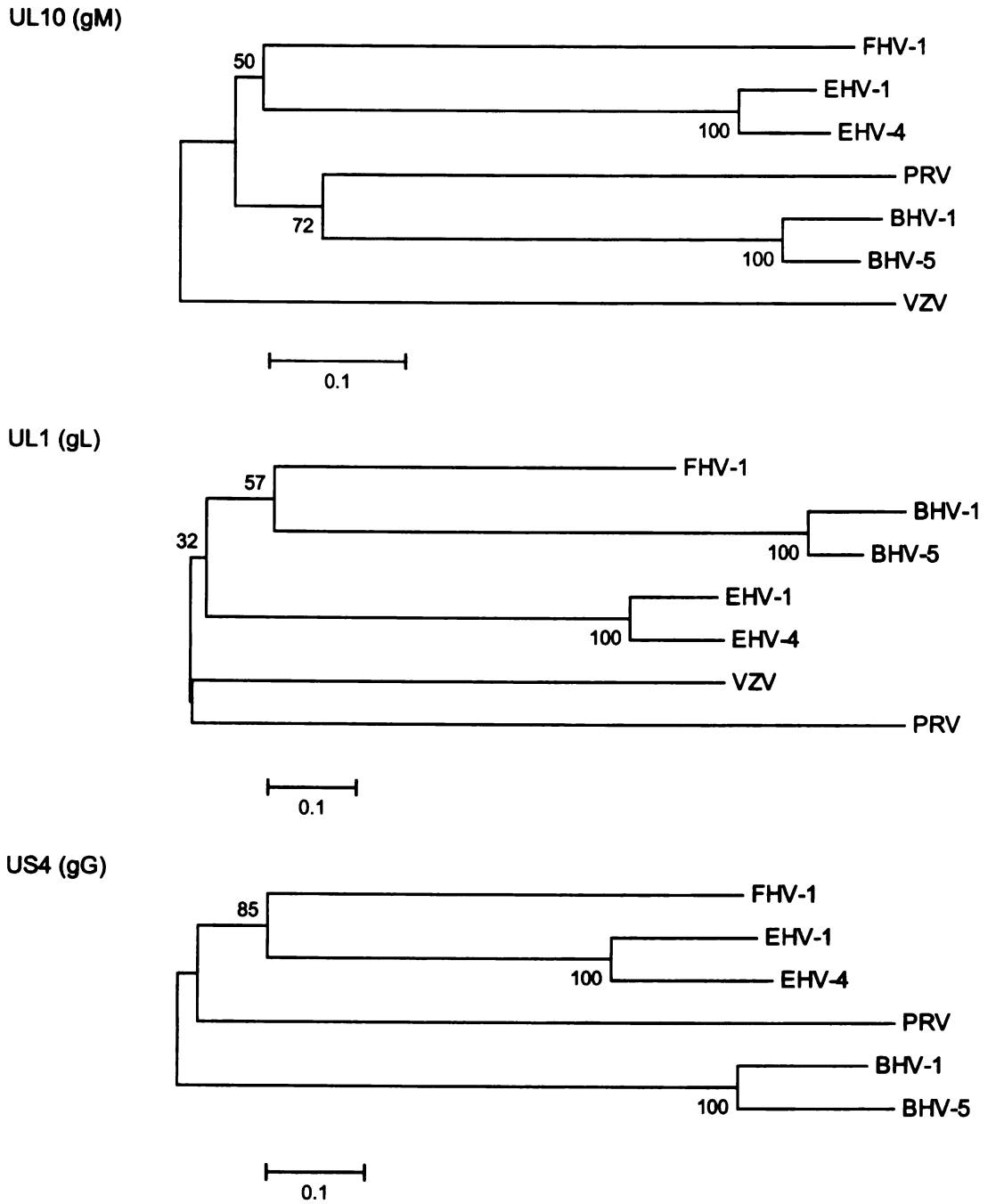
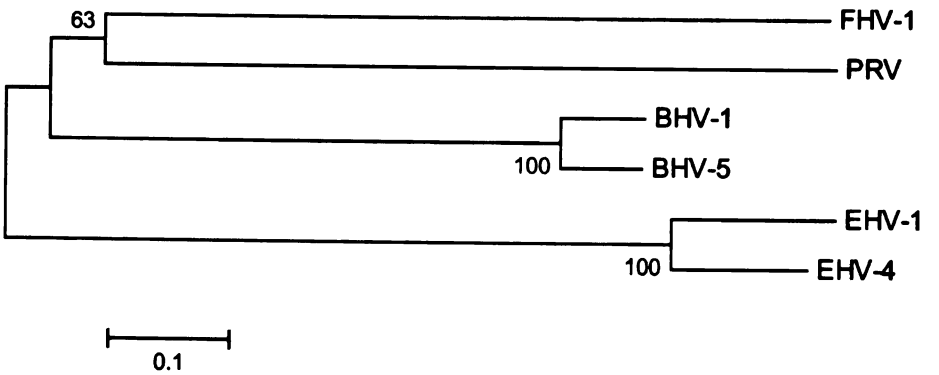
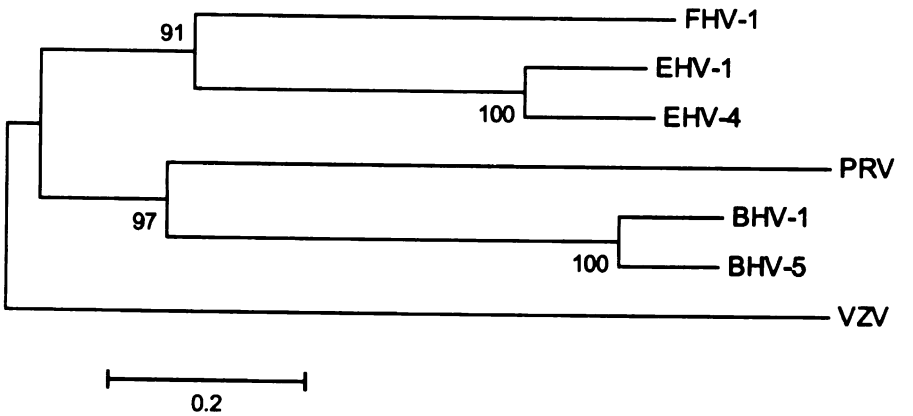


Figure 3.4, continued.

US6 (gD)



US7 (gI)



US8 (gE)

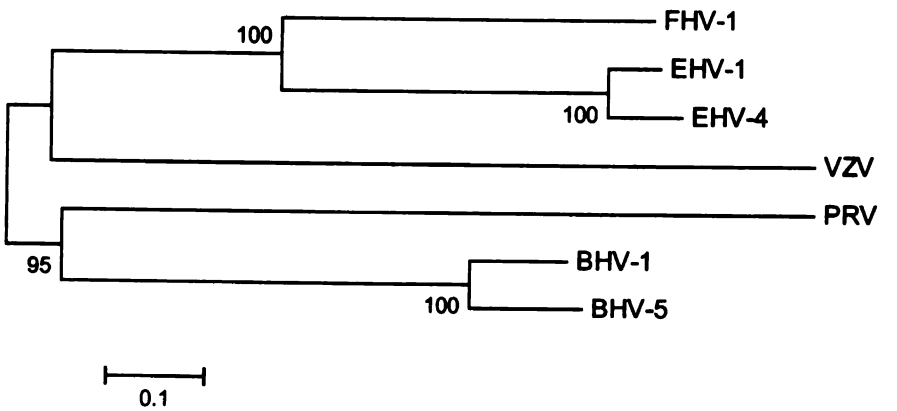


Figure 3.4, continued.

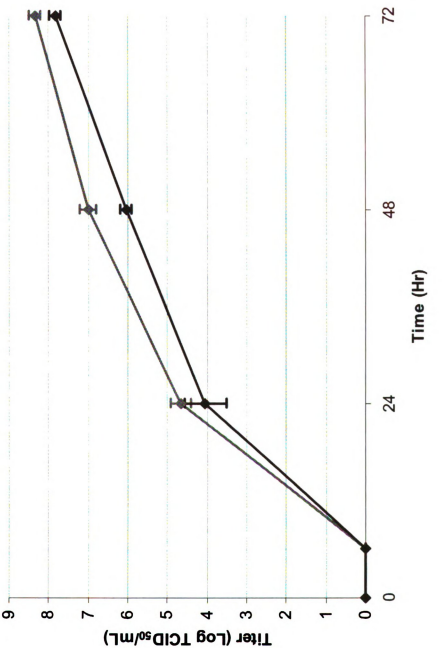


Figure 3.5 Multi-step growth curve of the parent wild type strain C-27 (grey) and the BAC-derived virus (black). The wild type virus and the FHV1ΔBAC were inoculated on CRFK monolayers at an m.o.i. of 0.01, and supernatants collected and titrated at 0, 6, 24, 48, and 72 hours post inoculation (p.i.). Error bars at each data point represent ±1 standard deviation.

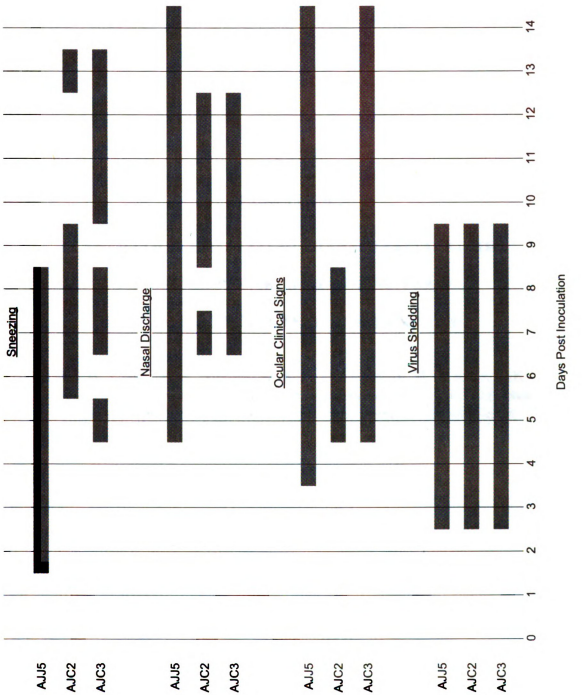


Figure 3.6 Durations of clinical signs and virus shedding in cats inoculated with the C-27 parent strain (Cat AJJ5) or the FHV1ΔBAC virus (Cats AJC2 and AJC3). The Gantt chart shows the time frame during which a specific clinical sign or virus shedding persisted. The cat number is shown next to its respective bar. A bar is shown only if a clinical sign was observed or virus shedding occurred.

CHAPTER 4

GENERATION AND *IN VITRO* CHARACTERIZATION OF FELINE HERPESVIRUS 1 (FHV-1) MUTANTS LACKING GLYCOPROTEINS C (gC) AND E (gE)

Tai, S.H., Cheng, H.H., Niikura, M., Kim, T., Engstrom, M.D., Maes, R.K. Generation and *in vitro* characterization of feline herpesvirus 1 (FHV-1) mutants lacking glycoproteins C (gC) and E (gE). In preparation for submission.

Generation and *in vitro* characterization of feline herpesvirus 1 (FHV-1) mutants lacking glycoproteins C (gC) and E (gE)

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ABSTRACT

Feline herpesvirus 1 (FHV-1) mutants lacking the entire open reading frames encoding glycoprotein C (gC) or E (gE) were constructed, based on a FHV-1 BAC clone. The gC^- FHV-1 mutant virus produced primary plaques that enlarged slowly and had relatively few secondary plaques compared to the wild type virus. Analysis on multi-step growth kinetics showed that the mutant virus grew to titers that were reduced by approximately 6,000 – 9,000 folds, compared with the wild-type virus. Analysis of the mutant virus on single-step growth kinetics showed that the titer of intracellular virus was 3.5 – 10 folds higher than the extracellular virus titer. Taken together, the results indicate that the mutant virus is more cell-associated compared to the wild-type virus. Thus, FHV-1 gC, like is the case in other varicelloviruses, plays a significant role in initial attachment/penetration, replication, and egress of FHV-1. In contrast, the gE^- FHV-1 mutant had single-step growth kinetics that were indistinguishable from those of the FHV1ΔBAC, and grew to a titer that was approximately 10-fold lower than that of the FHV1ΔBAC in the multi-step growth kinetics. These results suggested that virus egress, and most likely cell-to-cell spread as shown in other alphaherpesviruses, were affected by gE deletion, while virus entry and replication were not.

Keywords: Feline herpesvirus 1, FHV-1, glycoprotein C, glycoprotein E, mutant, infectious BAC clone, virulence

INTRODUCTION

Feline herpesvirus 1 (FHV-1) is an important viral pathogen of Felidae and has a world-wide distribution. Infection with FHV-1 not only accounts for approximately 50% of all diagnosed viral upper respiratory infections, but is also a significant cause of ocular diseases in cats (Nasisse, 1990). Pathobiology and clinical manifestations associated with FHV-1 have recently been reviewed (Gaskell, 2007; Maggs, 2005; Stiles, 2000). Primary infection, acquired via the oronasal route, results in fever, sneezing, ocular and nasal discharge, conjunctivitis, and keratitis. Like herpesvirus infections in other species, the acute phase of the disease is followed by lifelong latency. During the latent stage, the FHV-1 genome persists in neural tissues but infectious virus is not produced. Different biological stresses, or administration of corticosteroids, can induce the necessary biochemical stimuli in latently infected cells that lead to renewed production of infectious virus, which then travel to the periphery and is a potential source of viral transmission. Despite the clinical significance and high prevalence of FHV-1 infection, currently available vaccines can not totally protect cats from field virus infection and, as a consequence, from field virus latency (Gaskell, 1993; Harbour et al., 1991; Nasisse et al., 1997; Tham and Studdert, 1987). Furthermore, most vaccines are safe only when administered subcutaneously (Kruger et. al., 1996).

FHV-1 has a double-stranded DNA genome and a genomic organization similar to that of other varicelloviruses of the Alphaherpesvirus subfamily (Grail et al., 1991; Rota et al., 1986), which includes the prototype varicella zoster virus (VZV), bovine herpesvirus 1 (BHV-1), bovine herpesvirus 5 (BHV-5), equine herpesvirus 1 (EHV-1), equine herpesvirus 4 (EHV-4), and pseudorabies virus (PRV), among others. The complete 135,797-bp sequence of the FHV-1 genome has been determined and 74 encoding proteins have been identified, including 11 glycoproteins (Tai et al., submitted). FHV-1 has been shown to contain 23 virion-associated proteins (Fargeaud et al., 1984). The functions of seven glycoproteins, gB, gC, gD, gE, gG, gH and gI, had been reviewed by Maeda et al. (1997).

Glycoprotein C (gC) homologues have been extensively studied in several alphaherpesviruses. gC homologues are non-essential for herpesvirus replication *in vitro*, but they mediate several important biological functions. gC is involved in the initial step of viral attachment by interacting with heparan sulfate on the cell surface, as demonstrated for herpes simplex virus-1 (HSV-1) , PRV, BHV-1, and EHV-1 (Herold et al., 1991; Mettenleiter et al., 1990; Okazaki et al., 1991; Osterrieder, 1999). gC deficient mutants attach to cells with reduced efficiency (Osterrieder, 1999). gCs of HSV-1 and -2 can bind the complement component C3b (Geraghty et al., 1998; Lubinski et al., 1999). Binding of this complement factor may protect herpesvirus-infected cells from complement-mediated lysis (Fries et al., 1986). Viruses lacking complement-binding domains are less virulent than wild-type virus (Frink et al., 1983; Herold et al., 1991; Lubinski et al., 1999). The gC of FHV-1 has been shown to be the dominant heparin-binding glycoprotein that mediates the initial stage of viral adsorption,

as observed in other herpesviruses (Maeda et al., 1997). However, it remains to be determined whether FHV-1 gC protect virus-infected cells from complement-mediated lysis.

A deficiency of gC is commonly seen in strains of herpesviruses attenuated by serial passage in cell cultures. The PRV Bartha vaccine strain carries mutations within the gC gene that include an alteration in the amino terminal signal sequence, resulting in inefficient intracellular translocation and incorporation of this protein into the viral envelope (Robbins et al., 1989). The VZV vaccine strain Oka is defective in gC expression compared to wild type VZV strains (Kinchington et al., 1990). In addition, a genetically engineered TK-gC- vaccine strain has been licensed for use in control of pseudorabies (Kit, 1990). A sensitive and highly specific ELISA kit was developed for use in conjunction with this vaccine to distinguish non-infected and/or vaccinated pigs from those infected with PRV field strains (Kit et al., 1990).

Glycoprotein E (gE) is a virulence factor of FHV-1. In alphaherpesviruses, glycoprotein E (gE) and glycoprotein I (gI) form a heterodimer that functions in cell-to-cell spread of the virus and spread of infection throughout the host nervous system, which is ultimately the cause of neurovirulence. Generally, alphaherpesvirus mutants that lack these glycoproteins are replication-competent in cell culture but produce smaller plaques, due to reduced capacity for cell-to-cell spread. gE/gI deletion mutants of herpes simplex virus type 1 (HSV-1) and other alphaherpesviruses such as pseudorabies virus (PRV) and bovine herpesvirus 1 (BHV-1), show impaired ability to spread from cell to cell (Balan et al., 1994; Dingwell et al., 1994; Dingwell and Johnson, 1998; Otsuka and Xuan, 1996; Zuckermann et al., 1988). The gE/gI heterodimer

appears to play an even greater role in the spread of varicella-zoster virus (VZV) (Frink et al., 1983; Mallory et al., 1997; Mallory et al., 1998) and in Marek's disease virus (MDV), in which the gE/gI heterodimer has been found to actually be essential for growth in cultured cells (Schumacher et al., 2001). Previous studies have shown that gE/gI complex of FHV-1 is not essential for virus growth, although virulence is significantly reduced and the virus produces smaller plaques when the gE/gI genes are deleted (Sussman et al., 1995). The FHV-1 gE/gI mutant, when administered via the oronasal route, can protect cats from clinical signs and significantly reduce viral loads in subsequent challenge with a high dose of field virus. However, this mutant at higher dose levels still retained partial virulence as it can produce mild clinical signs in a dose-dependent manner (Kruger et al., 1996; Sussman et al., 1997).

Bacterial artificial chromosome (BAC) cloning and recombination-mediated genetic engineering (recombineering) are two state-of-the-art techniques to facilitate the mutagenesis of herpesviruses. BACs are single copy F-factor-based plasmid vectors which can stably hold 300 kb or more of foreign DNA (Shizuya et al., 1992). The BACs' larger capacity and greater stability over the other vectors have made BAC the vector of choice for the cloning of entire herpesvirus genomes. Many alphaherpesvirus genomes have been cloned as BACs since the first infectious BAC of a herpesvirus was reported in 1997 (Messerle et al., 1997). These include HSV (Saeki et al., 1998; Stavropoulos and Strathdee, 1998), PRV (Smith and Enquist, 1999), EHV-1 (Rudolph et al., 2002), and MDV (Niikura et al., 2006; Schumacher et al., 2000). Recently, we have constructed a BAC clone that contains the entire FHV-1 genome, and virus derived from this BAC clone has been shown to possess *in vitro* and *in vivo*

characteristics very similar to the prototype C-27 strain (Tai et al., submitted). This FHV-1 BAC clone is therefore a suitable starting platform for characterizing virulence factors and development of vaccine candidates.

Recombineering is a powerful method for fast and efficient manipulation of the BAC. It allows DNA cloned in *E. coli* to be modified via lambda (λ) Red-mediated homologous recombination, obviating the need for restriction enzymes and DNA ligases. Site-specific and "scarless" deletions, insertions, and point mutations can be introduced efficiently using a recombineering procedure developed by Tischer et al. (2006), known as two-step Red-mediated recombination (See Figure 4.1 for an overview.)

In this report, we present the construction and *in vitro* characterization of the first FHV-1 gC-deletion mutant, as well as a gE-deletion mutant. Both were constructed based on an infectious FHV-1 BAC clone by the two-step Red-mediated recombination approach.

MATERIALS AND METHODS

Cells and Viruses

Crandell-Reese feline kidney (CRFK) cells (ATCC CCL-94, Manassas, VA) were cultured in Eagle's Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS) and 10 μg/ml of ciprofloxacin. The FHV-1 prototype strain C-27 (ATCC

VR-636, Manassas, VA) was propagated in the CRFK cells and used as the wild-type virus throughout this study.

Construction of Mutant FHV-1 BACs

"Scarless" deletions of the entire open reading frame (ORF) of gC or gE were introduced into the FHV-1 BAC clone (Tai et al., submitted) using the two-step Red-mediated recombination method described by Tischer et al. (2006) and illustrated in Figure 4.1. First, a mutating DNA fragment that contained 50-bp of upstream recombination arm, 50-bp of downstream recombination arm, kanamycin resistance gene expression cassette (*kan*), and a restriction site of homing endonuclease I-*Sce*I was generated by PCR using the Extended High Fidelity PCR System (Roche Applied Science, Indianapolis, IN). The 25 µl reaction consisted of 1x Expand High Fidelity buffer, 1.5 mM of MgCl2, 200µM of each dNTP, 600 nM of each dUL44F and dUL44R primer (Integrated DNA Technologies, Coralville, IA) (Table 1), 2 ng of pEPkan-S as template, and 1.4 U of Expand High Fidelity enzyme mix. PCR amplification was carried out in a Thermal Cycler 2720 (Applied Biosystems, Foster City, CA). The program started with an initial denaturation step at 94°C for 2 minutes, followed by 40 cycles of 94°C for 1 minute, 55°C for 1 minute, and 68°C for 1 minute. Following the last elongation step at 68°C for 10 minutes, the template plasmid was degraded by adding 40 U of *Dpn*I (New England BioLabs, Ipswich, MA) to the PCR reaction and incubating at 37°C for 1 hour. The 1,145-bp amplicon was resolved in a 1% agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The gel-purified DNA was then transformed into *E. coli* SW105 cells (Warming et al., 2005)

containing the FHV-1 BAC clone, which was induced for the expression of recombination genes *exo*, *bet* and *gam* at 42°C for 15 min. Candidate colonies grown on LB agar plates containing 50 μg/ml of chloramphenicol and 75 μg/ml of kanamycin were checked by PCR using primers RM1206-RM1207 and RM1208-RM1209 (Table 1), and restriction pattern analysis. Positive clones were further engineered to remove the *kan* insertion. The second recombination was carried out by inducing the expression of recombination genes at 42°C for 15 min and the expression of I-*Sce*I by 1% of arabinose. The successfully engineered gC- and gE-deleted FHV-1 BAC clones were identified based on the restriction pattern, results of colony PCR, and sequence analysis, and designated as FHV1BACΔUL44 and FHV1BACΔUS8, respectively.

Reconstitution of Infectious Viruses from BACs

To reconstitute mutant FHV-1 virions from the mutant BAC clones and excise the BAC cassette from the viral genome, CRFK cells were co-transfected with either FHV1BACΔUL44 or FHV1BACΔUS8 DNA, and pcDNA-Cre plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) (Tai et al., submitted). Briefly, 300,000 CRFK cells were seeded in each well of a 24-well plate and incubated overnight at 37°C in an atmosphere of 5% CO_2 until the cultures reached ~95% confluence. To form DNA-Lipofectamine 2000 complexes, 1 μg of either FHV1BACΔUL44 DNA or FHV1BACΔUS8 DNA, and 1 μg of pcDNA-Cre plasmid were diluted in 50 μl of EMEM without antibiotics or FBS, and combined with 3 μl of Lipofectamine 2000, also diluted in 50 μl of EMEM without antibiotics or FBS. The cell culture medium was replaced with 100 μl of each DNA-Lipofectamine 2000 complex and 500 μl of fresh

EMEM containing 10 μg/ml of ciprofloxacine and 10% FBS. After 5 – 7 days of incubation, the supernatants were harvested and progeny virus that produced non-fluorescent plaques was plaque-purified before being propagated in CRFK cells. Reconstituted gC-deleted virus that had the BAC cassette removed thru Cre-loxP recombination was designated as FHV1ΔgC. Reconstituted gE-deleted virus that had the BAC cassette removed was designated as FHV1ΔgE. The removal of BAC cassette was verified by PCR using primhers RM1101 and RM1102 (Table 4.1) and direct sequencing of the purified PCR product. Deletions of gC and gE was verified again by PCR and direct sequencing of the purified PCR product as described above.

Plaque Morphology and Plaque Size Analysis

Fifty $TCID_{50}$ of the viruses to be tested were inoculated on confluent CRFK monolayers in a 6-well plate. After a one-hour adsorption at room temperature, the inoculum was removed, and fresh growth medium was added. The cells were then incubated at 37°C in an atmosphere of 5% CO_2 and observed daily for 5 days. Plaques produced by each virus were photographed using a QImaging camera (QImaging, Surrey, BC, Canada).

To perform plaque size analysis, 150 $TCID_{50}$ of the viruses to be tested were inoculated on confluent CRFK monolayers grown in a 6-well plate. After a one-hour adsorption at room temperature, the inoculum was removed, and fresh growth medium containing 0.8% carboxymethylcellulose was added. The cells were then incubated at 37°C in an atmosphere of 5% CO_2. After 5 days of incubation, the infected monolayers were fixed and plaques were stained using fluorescein-conjugated anti-FHV-1

antibodies. One hundred plaques produced by each virus were photographed and the diameters were determined using a QImaging camera and the QCapture Pro software (QImaging, Surrey, BC, Canada). ANOVA was used to assess whether differences in plaque size were statistically significant.

Growth Kinetics

Single-step growth curves were constructed as follows: monolayers of CRFK cells were infected in triplicate with the C-27 strain or the deletion mutants at a multiplicity of infection (MOI) of 3. After an adsorption period of 1 hour, cells were washed with 0.1 M phosphate buffered saline, pH 7.2 (PBS), overlaid with growth medium, and incubated at 37°C in an atmosphere of 5% CO_2. The cell culture supernatant and infected cells were harvested separately at successive intervals between 4 and 48 hours post inoculation (p.i.) and the amount of infectious virus was titrated on CRFK cells, as described previously (Sussman et al, 1995).

Multi-step growth curves were constructed by infection of CRFK monolayers in triplicate with the C-27 strain or the deletion mutants at an MOI of 0.01. After an adsorption period of 1 hour, cells were washed with PBS, overlaid with growth medium, and incubated at 37°C in an atmosphere of 5% CO_2. Culture supernatants and infected cells were harvested separately at successive intervals between 6 and 120 hours p.i. The amount of infectious virus was titrated on CRFK cells as described previously.

RESULTS

Construction of Mutant FHV-1 BACs

Using two-step Red-mediated recombination techniques (Tischer et al., 2006), FHV-1 mutants lacking the entire ORF of gC or gE were generated, based on the FHV-1 BAC clone previously constructed in our laboratory (Tai et al., submitted). The mutagenesis of the BAC clone was confirmed by colony PCR (data not shown), restriction pattern analysis (Figure 4.2), and sequence analysis (data not shown).

Reconstitution of Infectious Virus from BACs

The removal of the BAC vector in the reconstituted FHV1ΔgC and FHV1ΔgC viruses was verified by the loss of fluorescence resulting from the EGFP gene in the BAC vector, PCR and direct sequencing of purified PCR products. The deletions of gC and gE were also verified by PCR (Figure 4.3) and direct sequencing of purified PCR products (data not shown).

Plaque Morphology and Plaque Size Analysis

Figure 4.4 shows the development of plaques produced by the FHV1ΔgE and the parent C-27 strain on CRFK monolayers without carboxymethycellulose overlay. On day 1 p.i., plaques produced by both FHV1ΔgC and C-27 parent strain were very small. After day 2 p.i., FHV1ΔgC plaques expanded gradually, while C-27 strain plaques spread rapidly. Overall, plaques produced by FHV1ΔgC developed slower and remained focal, while the FHV-1 C-27 strain produced extensive CPE on day 4 p.i.

Relatively few secondary plaques were produced by FHV1ΔgC compared to the C-27 strain.

Preliminary plaque size analysis did not show a significant difference between the FHV1ΔgC and the C-27 strains.

Growth Kinetics

Single-step (Figures 4.5 and 4.7) and multiple-step (Figures 4.6 and 4.8) growth curves were constructed for FHV-1 C-27 strain, FHV1ΔBAC, FHV1ΔgC, and FHV1ΔgE. FHV1ΔBAC grows to a titer that is approximately 2 – 12 fold lower than the C-27 strain.

When inoculated at an MOI of 3 on CRFK monolayers, both the wild type and the gC-deletion mutant started to replicate extensively in CRFK cells after 8 hours post inoculation (Figure 4.5). Compared to the wild type, the gC-deletion mutant replicated much less efficiently and released fewer virions into the medium. In case of the wild type, the amount of extracellular virus surpassed the amount of intracellular virus between 20 and 24 hours p.i. In contrast, the amount of extracellular virus of the gC-deletion mutant never surpassed the intracellular virus. For both viruses, CPE was nearly complete at 24 hours p.i., all the cells were showing CPE. The amount of intracellular viruses remained constant, and the amount of extracellular viruses continued to increase. In contrast, the gE-deletion mutant has a growth curve very similar to FHV1ΔBAC (Figure 4.7).

When inoculated at an MOI of 0.01 on CRFK monolayers, the wild type virus started to replicate extensively in CRFK cells between 6 and 16 hours p.i. (Figure 4.6).

The amount of the intracellular virus reached a plateau at 48 hours p.i. The amount of extracellular virus surpassed intracellular virus between 48 and 72 hours p.i., and reached a plateau at 72 hours p.i. After 96 hours p.i., cell lysis was extensive and the virus titer started to decrease. The amounts of extracellular and intracellular virus of the gC-deletion mutant started to increase between 16 and 24 hours p.i., and were very similar between 24 and 72 hours p.i. The gC-deletion mutant also reached a plateau at 72 hours p.i., but the titer was 6,000 – 9,000 fold lower than the wild type titer. The gE-deletion mutant grew to a titer that was approximately 10-fold lower than the FHV1ΔBAC (Figure 4.8).

DISCUSSION

Previous work in our laboratory involved cloning of the entire FHV-1 genome as an infectious BAC. Virus generated from this BAC clone was shown to behave very similarly to the parent C-27 strain, both *in vitro* and *in vivo* (Tai et al., submitted). Therefore, it was considered to be a suitable starting platform for mutagenesis and virulence studies. Based on this BAC clone, we constructed FHV-1 mutants in which the entire ORF of gC or gE was deleted.

The FHV-1 gC-deletion mutant had *in vitro* characteristics that suggested attenuation. The results of this study suggested that gC could play a role at three different stages of viral infection: attachment/penetration, replication, and/or egress.

FHV-1's attachment/penetration abilities were impaired by gC deletion. In plaque assays without carboxymethylcellulose overlay, the gC-deletion mutant produces relatively few secondary plaques, while the wild type produces many secondary plaques and spreads to the entire monolayer very quickly. Adsorption conditions can influence virus titer. When the inocula were not removed from the monolayers after an hour of adsorption, essentially allowing longer time for adsorption, the titer of combined intracellular and extracellular virus stock of the FHV-1 gC-deletion mutant could reach 10^5 $TCID_{50}$/ml, about 10 times higher than as shown in Figure 4.6. A stock of 10^6 $TCID_{50}$/ml could be grown by co-cultivation of uninfected and FHV1ΔgC-infected CRFK cells (data not shown). It is well-documented that herpesvirus gC orthologues are the major protein that confers the initial attachment of the virion to the host cell, by electrostatically binding to heparin- and chondroitin-like glycosaminoglycans on the cell surface (Herold et al., 1991; Mettenleiter et al., 1990; Okazaki et al., 1991; Osterrieder, 1999). This binding step can be blocked with soluble heparin (Okazaki et al., 1991). In addition to attachment, PRV gC was shown to play a role in penetration, independently from its attachment function (Mettenleiter, 1989; Rue and Ryan, 2002). A gC-deficient mutant of EHV-1, a very close relative of FHV-1, attaches to cells with reduced efficiency, and penetration assays showed that this mutant's penetration ability was impaired in primary equine cell culture, and severely impaired in a rabbit cell line (Osterrieder, 1999).

The FHV-1 gC-deletion mutant grew to a lower titer and showed a lower growth rate, as shown in the single-step (Figure 4.5) and multi-step (Figure 4.6) growth curves. This observation is not consistent with all studies in other herpesviruses. An EHV-1 gC-

negative mutant has been shown to have a 5- to 10-fold reduction in titer when grown in rabbit and equine cell lines, and a 48- to 210-fold reduction when grown in primary equine cells (Osterrieder, 1999). gC-negative mutants of HSV-1 and PRV grow to titers that are only reduced by approximately 10-fold in cultured cells (Osterrieder, 1999). In contrast, deletion of gC in BHV-1 did not change its growth kinetics significantly in a bovine kidney cell line (Liman et al., 2000), and gC-negative VZV Oka strain exhibited accelerated and more efficient growth in cultured melanoma cells compared to the wild-type Oka strain (Cohen and Seidel, 1994). The reason for FHV1ΔgC's lower titer and slower growth may be due to reduced ability of the mutant virus to attach, thus resulting in lower virus load compared to the wild type. It is possible that a higher titer can be reached by inoculating the cells with more virions, as done in the studies of EHV-1 and BHV-1, which used 5 MOI to construct single-step growth curves. However, in order to carry out an inoculation at 5 MOI, pelleting of FHV1ΔgC virions would have been necessary. In addition, different cell type could affect the virus's growth, as shown in the EHV-1 study. This issue can be addressed by using different types of cells, such as feline corneal epithelial cells (Sandmeyer et al., 2005) and *Felis catus* whole fetus cells (Jacobse-Geels and Horzinek, 1983; Pedersen et al., 1981).

It is also possible that FHV-1 gC plays a role in virus replication. In BHV-1, gC has been shown to be important for maintaining the efficacy of viral replication in the natural host (Liang et al., 1992).

Yet another reason for the reduced growth could be the reduced ability of the mutant virus to egress. The gC-deletion mutant is more cell-associated than the wild type, suggesting a possible impairment in viral egress. As demonstrated in single-step

growth curve (Figure 4.5), which characterizes virus replication in individual cells, most of the gC-deleted mutant virus remained in the cell. Specifically, the titer of intracellular virus was 3.5 – 10 times higher than the extracellular virus. This is in contrast to the wild type, where the titer of extracellular virus can be ~29-fold higher than the intracellular virus. This finding is less obvious in the multi-step growth curves. Herpesvirus gC is not considered to have a major role in viral egress or release. Nonetheless, it was noted that the reduction in titer of the gC- mutant of EHV-1 in primary equine cells was partially due to impairment in viral egress (Osterrieder, 1999).

The limited ability of the FHV-1 gC- mutant to spread in cultured epithelial CRFK cells, as demonstrated in Figure 4.4, suggest that when administered via the natural oronasal route *in vivo*, it could spread slower on the mucosal epithelial cells at the primary infection site, allowing more time for the immune system to respond, and hence induce a stronger immune response. In addition, it is known that herpesvirus gC plays an important role in immune evasion. gC inhibits the activation of the complement cascade by binding to complement component C3b, and by blocking the binding of properdin and C5 to C3b (Frink et al., 1983; Lubinski et al., 1999, Roizman et al., 2007). It has been shown in HSV that gC-negative mutants are more easily inactivated by complement than the wild-type (Hidaka et al., 1991). Therefore, by deleting FHV-1 gC, it is likely that the virus will induce a stronger immune response and fewer clinical signs. Although *in vivo* experiments are necessary to substantiate this hypothesis, it is reasonable to assume that the FHV-1 gC- mutant will show reduced virulence in the natural host.

The fact that single-step growth property of the gE-deletion mutant was practically indistinguishable from that of the FHV1ΔBAC (Figure 4.6) and the multi-step growth curve showed reduced titer suggests that virus egress but not entry and replication was affected by gE deletion. This observation is consistent with previous studies that showed gE plays an important role in cell-to-cell spread (Balan et al., 1994; Dingwell et al., 1994; Dingwell and Johnson, 1998; Otsuka and Xuan, 1996; Zuckermann et al., 1988).

In summary, the gC- and gE- mutants have potential as vaccine candidates that are safer and more immunogenic than existing vaccines. An additional advantage of these mutants over current commercially available vaccines is that they can be serologically differentiated from field virus. A distinct advantage of the recombineering-based approach to create deletion is that additional deletions could be made in either one of these mutants, should it be evident from future *in vivo* experiments that they still have residual virulence.

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TABLES

Table 4.1 Primers used in this study

Primer	Sequence (5' – 3')	Used for
dUL44F	*taagtagtcccgcgagacgatttacatcccgggatcaccaacaa tctgcg*AGGATGACGACGATAAGTAGGG	Generation of gC-mutating fragment
dUL44R	ataaccgctgaaacacggttatgataagtaatttatacgaactataag *agcgcagattgttggtgatcccgggatgtaaatcgtctcgcggga ctactta*CAACCAATTAACCAATTCTGATTAG	
dUS3F	*agacgtagggcaggtttctgttatttaggaagtgtatacgtttggct aat*AGGATGACGACGATAAGTAGGG	Generation of gE-mutating fragment
dUS3R	*ctctgggtgaacctttagagtggttataactttacgaagatggttgc* gggattagccaaacgtatacacttcctaaataacagaaacctgccct acgtctCAACCAATTAACCAATTCTGATTAG	
RM1188	AGTCGAAATCACAGGCAGTG	Differentiation of gC-mutant and wild type
RM1189	TGGAGTCGGTTTATATCCATAC	
RM94	GGTCATGTGTAATGTTGACG	Differentiation of gE-mutant and wild type
RM1193	ATACAATATACGCGTTTGACG	
RM1206	GCGGTTGTGTCTAATAACTG	Confirmation of *kan* insertion in gC (5'-end)
RM1207	GCTTCCCATACAATCGATAGAT	
RM1208	GATTTTGATGACGAGCGTAAT	Confirmation of *kan* insertion in gC (3'-end)
RM1209	CGGTTTATATCCATACGAATGA	
RM1210	CGCATAGTGGGTGGTGACCTAA	Confirmation of *kan* insertion in gE (5'-end)
RM1211	ATCATTGGCAACGCTACCTTT	
RM1212	TTGCCATTCTCACCGGATTCA	Confirmation of *kan* insertion in gE (3'-end)
RM1213	CCCGGATCGACTAGTGTGAAC	
RM1101	TGCCCCTCCCCCGTTCATGCTGTG	Confirmation of the excision of BAC cassette
RM1102	TGGAGCCTGTTTCCGATTCTGTGT	

Sequences in upper-case, nucleotides annealing to template; lower-case, hanging nucleotides; *italic*, upstream homologous recombination arm; underlined, downstream homologous recombination arm.

FIGURES AND FIGURE LEGENDS

Figure 4.1 An overview of the two-step Red-recombination approach (Tischer et al., 2006) used to construct the gC-deleted FHV-1 BAC. Diagrams at the left column depict events at cellular level, while the right column depicts events at DNA level. Recombination arms with the same sequence are shown by a line in the same color (red and blue). *Kan*: kanamycin resistance gene expression cassette; S: I-*Sce*I recognition site; arrows: priming sites of RM1188 and RM1189. 1: *E. coli* SW105 cells carrying FHV-1 BAC were induced for expression of recombination proteins, which were encoded in mini-lambda DNA. 2: Cells from step 1 were transformed with a mutating DNA fragment produced by PCR. 2A: Homologous recombination results in the replacement of the gC gene by *kan*. 3: Cells from step 2 were transformed with pBAD-I-*Sce*I, a plasmid that carries endonuclease I-*Sce*I. 4: Expression of I-*Sce*I was induced by arabinose. 5: Cells were induced for expression of recombination proteins. 5A: BAC DNA was excised by I-*Sce*I, and *kan* was removed by the second homologous recombination, resulted in a scarless gC deletion. 6: The gC-deleted FHV-1 BAC was isolated from *E. coli*.

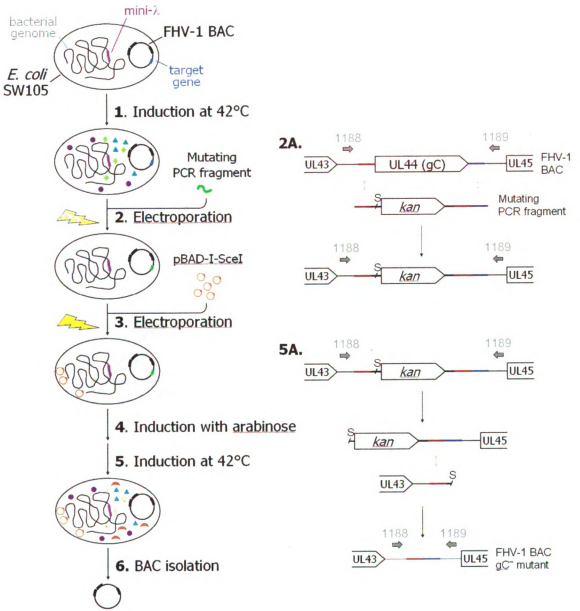
bacterial genome
mini-λ
FHV-1 BAC
target gene
E. coli SW105
1. Induction at 42°C
Mutating PCR fragment
2. Electroporation
pBAD-I-SceI
3. Electroporation
4. Induction with arabinose
5. Induction at 42°C
6. BAC isolation
2A.
1188
1189
UL43
UL44 (gC)
UL45
FHV-1 BAC
S
kan
Mutating PCR fragment
5A.
FHV-1 BAC gC⁻ mutant

Figure 4.1

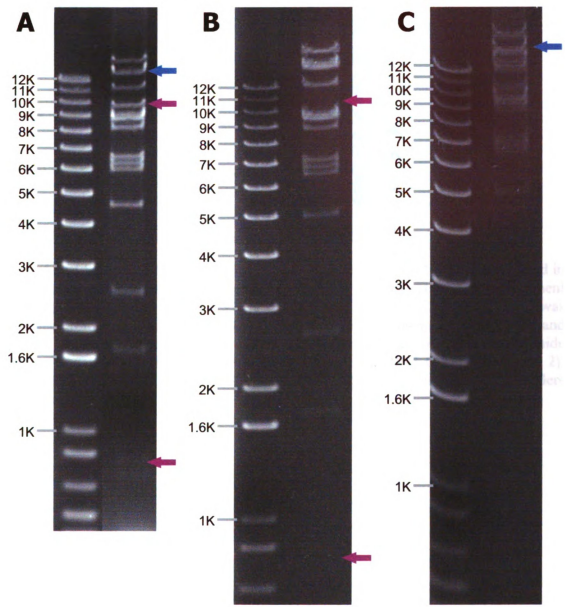

Figure 4.2 *Sal*I fragments of purified FHV-1 BAC (A), FHV1BACΔUL44 (B), and FHV1BACΔUS8 (C) were resolved in a 0.7% agarose gel and visualized by ethidium bromide staining. DNA fragments of 10,120 bp and 769 bp (pink arrows) merged into a 10,120 bp fragment because of gC deletion. DNA fragments of 14,020 bp (blue arrows) became 12,417 bp because of gE deletion. Sizes of the ladder are indicated in bp.

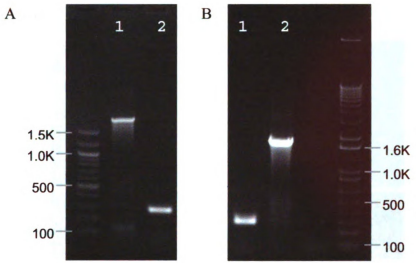

Figure 4.3 (A) PCRs carried out using primers RM1188 and RM1189 were resolved in a 1% agarose gel and visualized by ethidium bromide staining. The 1,879-bp fragment was amplified from the FHV-1 C-27 strain (Lane 1), and the 274-bp fragment was amplified from FHV1ΔgC (Lane 2). (B) PCRs carried out using primers RM94 and RM1193 were resolved in a 1% agarose gel and visualized by ethidium bromide staining. The 1,867-bp fragment was amplified from the FHV-1 C-27 strain (Lane 2), and the 268-bp fragment was amplified from FHV1ΔgC (Lane 1). Sizes of the ladders are indicated in bp.

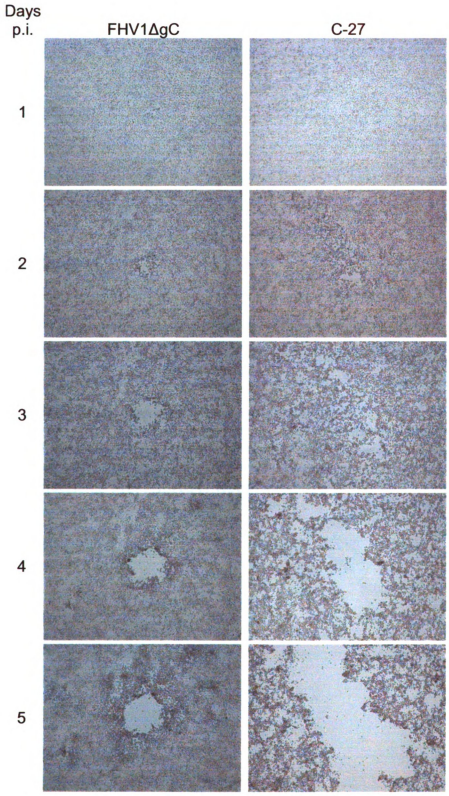

Figure 4.4 Development of one FHV1ΔgC plaque (right panel) and one C-27 strain plaque (left panel) over a period of 5 days. Magnification, 10X.

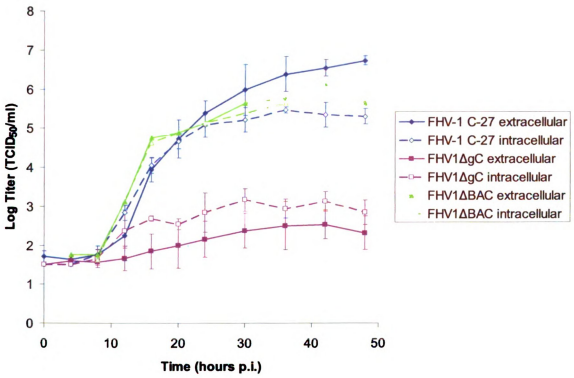

Figure 4.5 Single-step growth kinetics of the FHV-1 C-27 strain, FHV1ΔBAC, and the FHV1ΔgC. The CRFK cells were infected with the respective virus strain at an MOI of 3. At the indicated times intracellular and extracellular virus were harvested and titrated on CRFK cells. All growth kinetics was performed in triplicate except for intracellular and extracellular FHV1ΔBAC (1 repeat). Error bars represent ±1 standard deviation.

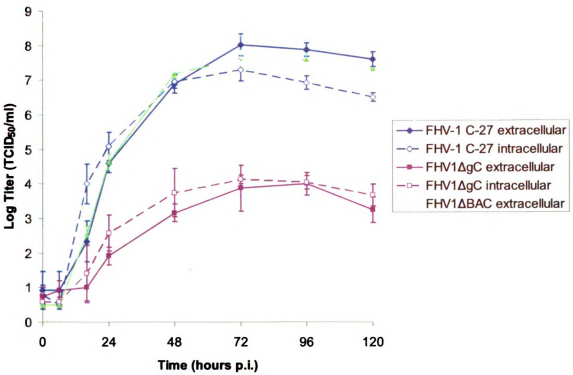

Figure 4.6 Multi-step growth kinetics of the FHV-1 C-27 strain, FHV1ΔBAC, and the FHV1ΔgC. The CRFK cells were infected with the respective virus at an MOI of 0.01. At the indicated times intracellular virus (open symbols) and extracellular virus (filled symbols) were harvested and titrated on CRFK cells. All growth kinetics was performed in triplicate. Error bars represent ±1 standard deviation.

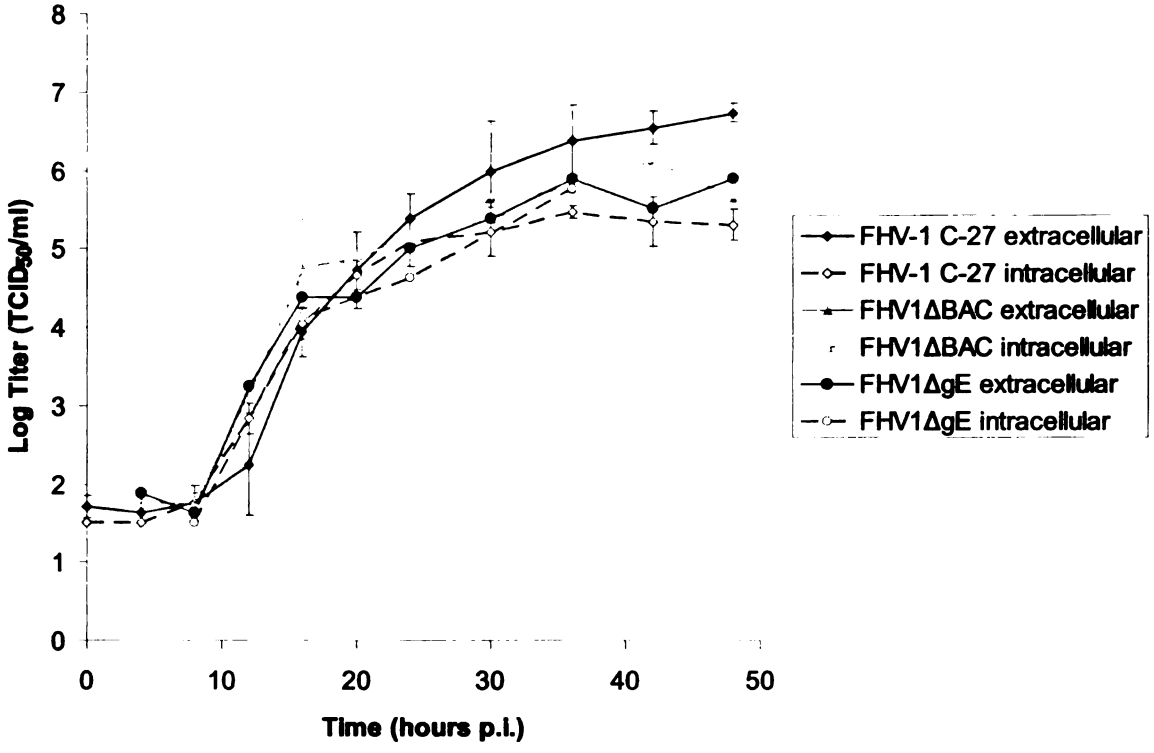

Figure 4.7 Single-step growth kinetics of the FHV-1 C-27 strain, FHV1ΔBAC, and the FHV1ΔgE. The CRFK cells were infected with the respective virus strain at an MOI of 3. At the indicated times intracellular and extracellular virus were harvested and titrated on CRFK cells. Growth kinetics for FHV-1 C-27 strain was performed in triplicate. Growth kinetics for FHV1ΔBAC and FHV1ΔgE is shown in 1 repeat. Error bars represent ±1 standard deviation.

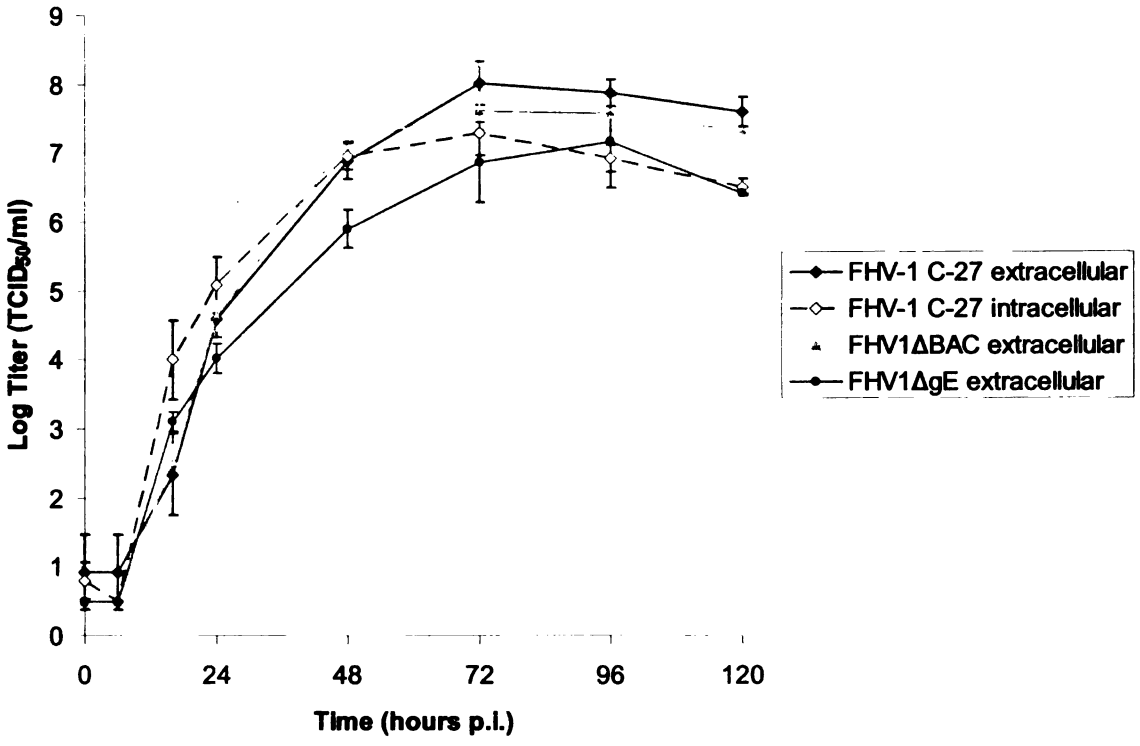

Figure 4.8 Multi-step growth kinetics of the FHV-1 C-27 strain, FHV1ΔBAC, and the FHV1ΔgE. The CRFK cells were infected with the respective virus at an MOI of 0.01. At the indicated times intracellular virus (open symbols) and extracellular virus (filled symbols) were harvested and titrated on CRFK cells. All growth kinetics was performed in triplicate. Error bars represent ±1 standard deviation.

CHAPTER 5

GENERATION OF $gC^{-}gE^{-}$, US3 PROTEIN KINASE $(PK)^{-}$, AND $PK^{-}gE^{-}$ MUTANTS OF FELINE HERPESVIRUS 1 (FHV-1)

5.1 Introduction

In addition to the glycoprotein C (gC) and E (gE) deleted FHV-1 mutants described in Chapter 4, additional FHV-1 genes were selected, individually or in combination, as targets for mutagenesis.

As reviewed in Chapters 1 and 4, glycoprotein E (gE) is a virulence factor of FHV-1. The gE/gI heterodimer functions in cell-to-cell spread of the virus and spread of infection throughout the host nervous system, ultimately contributing to neurovirulence. A partial gE/gI deletion mutant previously generated in our laboratory using traditional methods produced smaller plaques and exhibited significantly reduced virulence (Sussman et al., 1995). This FHV-1 gE/gI mutant, when administered via the oronasal route, can protect cats from clinical signs and significantly reduce viral loads in subsequent challenge with a high dose of field virus (Kruger et al., 1996; Sussman et al., 1997). However, this mutant at higher dose levels still retained partial virulence as it can produce mild clinical signs. We hypothesize that further attenuation can be achieved by deletion of additional virulence factors, resulting in a vaccine candidate that is safer and more efficacious.

The *US3* gene of FHV-1 encodes a serine/threonine protein kinase (PK), and its amino acid sequence is conserved in the subfamily *Alphaherpesvirinae* (Frame et al., 1987; McGeoch and Davison, 1986; Purves et al., 1987). Possible functions of PK include blocking of apoptosis induced by both viral and cellular proteins (Leopardi et al., 1997; Munger et al., 2001; Munger and Roizman, 2001; Ogg et al., 2004), regulation of the nuclear egress of progeny nucleocapsids (Reynolds et al., 2001; Reynolds et al., 2002), and control of the morphology of infected cells (Kato et al., 2008; Murata et al.,

2002). Kimman et al. demonstrated that a PK^- mutant of pseudorabies virus (PRV) has strongly reduced virulence, and animals inoculated with PK^-gE^- PRV mutant and subsequently challenged with wild-type virus has reduced virus shedding (Kimman et al., 1994).

We expect that single deletion of PK will attenuate FHV-1, and an additional gC or PK deletion in the gE-deleted FHV-1 mutant will further attenuate the virus, and thus improve its safety as a vaccine.

5.2 Materials and Methods

Cells, Viruses, Plasmids, and DNA Preparation

The methodologies are presented in Chapters 3 and 4.

Construction of Mutant Viruses

FHV-1 PK^- mutant was constructed using the FHV-1 BAC clone described in Chapter 3. FHV-1 gC^-gE^-, and PK^-gE^- mutants were constructed using the FHV-1 gE^- BAC clone described in Chapter 4. Site-specific and "scarless" deletions of FHV-1 open reading frames (ORFs) *UL44* (encoding gC) and *US8* (encoding gE) were carried out using the two-step Red-mediated recombineering methods developed by Tischer et al. (2006), as described in Chapter 4. Briefly, mutating DNA fragments were generated by PCR using the primers dUL44F/dUL44R (for gC deletion), dUS8F/dUS8R (for gE deletion), or dUS3F/dUS3R (for PK deletion) (Table 5.1). In the first recombination,

these PCR mutating fragments replaced target gene with kanamycin resistance gene (*kan*), an I-*Sce*I recognition site, and an additional 50-bp upstream recombination arm. The entire ORF of gC and gE was deleted. Because part of the *US3* gene was present twice in the FHV-1 BAC (Chapter 3), only the first 793 bp at the N-terminal of the entire 1,056-bp ORF were deleted. The *kan*, I-*Sce*I site, and additional recombination arm were subsequently removed in the second recombination, resulting in a scarless deletion. Mutant FHV-1 virions were reconstituted from the mutated BAC as described in Chapters 3 and 4. PCR assays were designed for differentiation of deletion mutants and wild type virus throughout the entire process (Table 5.1).

Table 5.1 Primers used in this study

Primer	Sequence (5' – 3')	Use
dUL44F	*taagtagtcccgcgagacgatttacatcccgggatcaccaacaat ctgcg*AGGATGACGACGATAAGTAGGG	Generation of gC$^-$ mutating fragment
dUL44R	*ataaccgctgaaacacggttatgataagtaatttatacgaactata agagcgcagattgttggtgatcccgggatgtaaatcgtctcgcggga* ctacttaCAACCAATTAACCAATTCTGATTAG	
dUS3F	*agacgtagggcaggtttctgttatttaggaagtgtatacgtttggcta at*AGGATGACGACGATAAGTAGGG	Generation of gE$^-$ mutating fragment
dUS3R	*ctctgggtgaacctttagagtggttataactttacgaagatggttgcg ggattagccaaacgtatacacttcctaaataacagaaacctgccctac* gtctCAACCAATTAACCAATTCTGATTAG	
RM1188	AGTCGAAATCACAGGCAGTG	Differentiation of gC$^-$ mutant and wild type
RM1189	TGGAGTCGGTTTATATCCATAC	
RM1343	AGGCACTCAGTGGGCCAAAGT	Differentiation of PK$^-$ mutant and wild type
RM1346	AGGCTGTCTTACACATGAGGCA	

Sequence in upper-case, nucleotides annealing to template; lower-case, hanging nucleotides; *italic*, upstream homologous recombination arm; underlined, downstream homologous recombination arm.

5.3 Results and Discussions

Current progress of the construction and *in vitro* characterization of FHV-1 mutants are summarized in Table 5.2. Construction and *in vitro* characterization of FHV-1 gC^- and gE^- mutants has been completed and reported in Chapter 4. FHV-1 gC^- and gE^- mutant viruses that carry the BAC vector and EGFP marker was also generated, but not yet characterized. Construction of PK^- and gC^-gE^- mutant viruses, with and without the BAC vector, has been completed. Preliminary findings shows that these mutant FHV-1 viruses grew poorer in CRFK cells compared to the gC^- mutant, and needed to be propagated by co-cultivating infected and uninfected cells. The *US3* gene in a gE^- FHV-1 BAC clone has been replaced by *kan*, resulting in a $gE^-PK^-kan^+$ BAC clone. To obtain scarless deletion mutant viruses, either with or without the BAC vector and EGFP marker (i.e., $gE^-PK^-BAC^+$ or $gE^-PK^-BAC^-$), *kan* will need to be removed by the second Red-mediated recombination.

Table 5.2 Current progress of the construction and *in vitro* characterization of FHV-1 mutants.

Virus	Construction			*In vitro* characterization		
	First recombination	Second recombination	Propagation of mutant virus	Multi-step growth curve	Single-step growth curve	Plaque size analysis
gC^-BAC^+	●	●	●	○	○	○
gC^-BAC^-	●	●	●	●	●	◖
gE^-BAC^+	●	●	●	○	○	○
gE^-BAC^-	●	●	●	●	◖	○
PK^-BAC^+	●	●	●	○	○	○
PK^-BAC^-	●	●	●	◖	○	○
$gC^-gE^-BAC^+$	●	●	●	○	○	○
$gC^-gE^-BAC^-$	●	●	●	○	○	○
$gE^-PK^-BAC^+$	●	◖	O	○	○	○
$gE^-PK^-BAC^-$	●	◖	O	○	○	○

●: completed, ◖: in progress, ○: to be done.

5.4 Reference

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CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Conclusions

6.1.1 The FHV-1 BAC Clone

The entire FHV-1 genome was cloned as a BAC and transformed into *E. coli* for maintenance and future manipulation. The virus derived from this BAC clone grew to a high titer in cell culture, produced plaques that are similar in size to those produced by the C-27 parent strain, and is virulent *in vivo*. Therefore, this FHV-1 BAC clone is a very suitable starting platform for studies on viral virulence, as well as development of future vaccine candidates.

6.1.2 The Complete Sequence of the FHV-1 Genome

Complete genomic sequences were derived from both the FHV-1 BAC clone and purified virion DNA. The FHV-1 genome is 135,797 bp in size with an overall G+C content of 45%. A total of 78 open reading frames were predicted, encoding 74 distinct proteins. The gene arrangement is collinear with that of most sequenced varicelloviruses.

6.1.3 FHV-1 Mutants Lacking Glycoprotein C (gC) or E (gE)

FHV-1 mutants lacking the entire open reading frames encoding gC or gE were constructed, based on the FHV-1 BAC clone. Results of *in vitro* characterization indicate that gC plays a role in initial attachment/penetration, replication, and egress of FHV-1. The gC^- mutant is more cell-associated and has a reduced in growth rate. In contrast, the gE^- FHV-1 grew to a high titer in cell culture. Results of *in vitro* characterization suggest that virus egress, and most likely cell-to-cell spread were affected by gE deletion, while virus entry and replication were not. Both mutants are

likely to be attenuated, and further studies are necessary to evaluate their *in vivo* characteristics and potential as vaccine candidates.

6.1.4 Other Deletion Mutants

A $gC^{-}gE^{-}$, an US3 protein kinase $(PK)^{-}$, and a $gE^{-}PK^{-}kan^{+}$ mutant of FHV-1 were generated. Both the $gC^{-}gE^{-}$ and the PK^{-} mutants demonstrated reduced growth rates in CRFK cells.

6.2 Future Directions

6.2.1 Transcriptional & translational analyses

The construction of deletion mutants was verified by PCR, restriction pattern analyses, and sequencing. No parts of other predicted ORFs, promoters, or polyadenylation signals were deleted in our mutants. Nonetheless, it would be of interest to check the transcription and translation of both the deleted and adjacent genes. Transcriptional analyses can be carried out by infecting CRFK cells with respective mutant virus, harvesting infected cells when they reach 50% CPE, extracting total RNA from those cells, and performing RT-PCR using primer sets listed in Table 1. Monoclonal antibodies specific for the proteins encoded by UL44, US3, US8, and their adjacent genes will be needed to perform translational analyses. After infecting CRFK cells with respective mutant virus, and allowing for CPE to develop, total protein can be extracted, separated on a polyacrylamide gel, and transferred to a membrane for immunoblots.

Table 6.1 Primers for transcriptional analysis

Target gene	Primer	Sequence (5' – 3')
UL45	RM1409	acgacagctatttcatcgcgtagg
	RM1410	ctccaacagcagatgcggtgg
UL44	RM1350	acgcgacactcgggatccta
	RM1351	cactgcacataccgcctcaaagg
UL43	RM1411	gcctcggtattgcatgctcga
	RM1412	cggtagaccatcaaaggttccagg
US2	RM1419	agattcatacatacgacagataaac
	RM1420	ttggcccactgagtgcctaatgtg
US3	RM1421	aacgaagctcctcgtggtatgctg
	RM1422	aacgggtgtctcactccctgga
US4	RM1423	cggggatgttttcccgaattgg
	RM1424	catggttcggtcgtaggctcga
US7	RM1413	cctctatgcaccaatacgatcagc
	RM1414	gtccgcatgatgtcgatgaccac
US8	RM1352	caccaacgcttctgggttgtacg
	RM1353	tctccgtggtaggcgactgc
US8A	RM1415	agctgctcgtaaactccgtgag
	RM1416	cataggacggatagtcccatgtg
US9	RM1417	tgccacagatgataattgcttagacac
	RM1418	tgtcggatagagtttcatccgcaa

6.2.2 Testing of the mutants in other feline cells

The mutants may behave differently in different types of cells. Other cell lines that can be used include *Felis catus* whole fetus (FCWF-4) cells, feline corneal epithelial (FCE) cells, feline alveolar macrophages, alveolar pneumocytes, and CD4+ T-lymphoblastoid cells (MYA-1 and FL74 cells). Tracheal organ culture and mucosal explants can also be used for future characterization of the mutants.

6.2.3 *In vivo* testing of the FHV-1 mutants

Attenuation and potential of the FHV-1 mutants as vaccine candidates need to be assessed *in vivo*. To evaluate residual virulence, a safety study can be carried out with groups of SPF cats, each inoculated intranasally with the FHV-1 mutant under study, or

mock-inoculated. Clinical signs would be scored, and swab and serum samples analyzed as described in Chapter 3. To evaluate the protection against field virus infection, each group of cats would be challenged with a high dose of wild-type virus. Clinical signs are scored, and swab and serum samples analyzed as described in Chapter 3.

6.2.4 A gC-complementing Cell Line

Ideally, a vaccine candidate should grow to a high titer to reduce production costs. This is not always feasible, as *in vitro* high growth titer is often associated with virulence. It is very likely that the FHV-1 gC^- and gC^-gE^- mutants are safe and can induce strong immune response *in vivo*. The reduction of the gC^- and especially the gC^-gE^- mutant can be complemented by supplying gC *in trans*. A gC-complementing cell line can be constructed by cloning the ORF of gC into an eukaryotic expression vector, and transfecting CRFK cells with the plasmid clone. A gC-complementing cell line can be obtained by screening transfected cells for the expression of marker genes (e.g., fluorescent proteins), and clonal expansion of positive cells. Expression of gC on the cell surface can be verified using polyclonal antibodies and direct or indirect FA staining procedures. Progeny virions produced by infecting this cell line with FHV-1 gC^- mutant should carry gC on their envelope but not the ORF encoding gC.

Using the same method, a gE-expressing cell line can also be constructed. It is not as necessary to complement gE in gE^- FHV-1 mutants, since the reduction in growth of the gE^- mutant is minimal. However, it can be useful as described in Section 6.2.5.

6.2.5 DIVA ELISA

Cell lines expressing FHV-1 gC or gE are useful for more than complementing respective glycoproteins. An enzyme-linked immunosorbent assay (ELISA) capable of differentiating between infected and vaccinated animals (DIVA) can be established using a gC- or gE-expressing cell line. Monolayers of gC- or gE-expressing cells are grown in wells of a 96-well plate, fixed in acetone and stored at -20°C until needed. Sera collected from cats are added to the individual wells, allowing binding of anti-gC or anti-gE antibodies. Secondary anti-cat IgG antibodies conjugated with an enzyme are added, followed by a substrate/chromogen mixture to develop color. Alternatively, fluorescein isothiocyanate (FITC) -lableled anti-species antibodies can be used. Cats infected by the field strains have antibodies against gC or gE, and can be detected by this assay. Cats infected by the deletion mutant do not have antibodies against gC and/or gE, and will be negative in this assay.

Another potential DIVA ELISA format consist of coating wells with affinity purified gC or gE produced by a eukaryotic expression vector.

The availability of DIVA is important for disease control. Since none of the current vaccines can be serologically differentiated from field strains, such DIVA ELISA assays add value to the FHV-1 deletion mutants as vaccine candidates.

6.2.6 Further engineering of the gE^- and gC^- mutants

To further attenuate the gE^- and gC^- mutants, mutations can be introduced into additional FHV-1 genes, using the same methodology. A reasonable target gene is *UL23*, encoding the thymidine kinase (TK). TK^- mutants of many alphaherpesviruses, including FHV-1, have been shown to be attenuated. Components of other important viruses infecting cats, e.g. feline calicivirus, feline immunodeficiency virus, or feline panleukopenia virus, can be inserted into the FHV-1 BAC as well.

APPENDICES

Appendix A USDA scoring method for clinical signs of FHV-1 infections.

Clinical signs	Days	Score
Fever		
103.0 to 103.9°F		1 each day
104.0 to 104.9°F		2 each day
≥105°F		3 each day
Conjunctivitis		
Serous discharge only	1 to 3	1
	≥4	2
Mucopurulent discharge	1 to 2	2
	3 to 5	4
	≥6	6
Rhinitis		
Serous discharge only	1 to 3	1
	≥4	2
Mucopurulent discharge	1 to 2	2
	3 to 5	4
	≥6	6
Sneezing		1 each day
Dyspnea		
Audible rales		2 each day
Coughing		2 each day
Open mouth breathing		3 each day
Depression		
Anorexia		1 each day
Dehydration	1 to 2	3
	≥3	4
Hypothermia <99°F		2 each day
Oral ulcers (linguinal or oral mucosa)		
1 ulcer <4 mm	1 to 5	2
	6 to 9	3
	≥10	4
Multiple ulcers <4 mm	1 to 4	3
	5 to 8	5
	≥9	7
Ulcer or ulcers >4 mm	1 to 4	5
	5 to 8	7
	≥9	9
Salivating		1 each day
Extenal ulcers (lip or nares)		
Nonbleeding ulcer		4
Bleeding ulcer		6
Death		15

Sussman, M.D., Maes, R.K., Kruger, J.M., Spatz, S.J., Venta, P.J., 1995. A feline herpesvirus-1 recombinant with a deletion in the genes for glycoproteins gI and gE is effective as a vaccine for feline rhinotracheitis. Virology 214, 12-20.

USDA Supplemental Assay Method 311 (U.S. Department of Agriculture, Animal and Plant Health Inspection Service, National Animal Veterinary Services Laboratory, 1985.)

Appendix B Clinical scores recorded in the pilot *in vivo* study (Chapter 3)

Cat Identification: AJD3 Group: Negative Control
Color: Orange Sex: F Age: 11-13 weeks
Scoring: Janice

DAY	Date	Sneeze	Cough	Rales	Open Mouth Breath	Bleph-spasm	Corneal Ulcertaion (Opacity)	Ocular Discharge
0	09/04/07	N	N	N	N	N	N	N
1	09/05/07	N	N	N	N	N	N	N
2	09/06/07	N	N	N	N	N	N	N
3	09/07/07	N	N	N	N	N	N	N
4	09/08/07	N	N	N	N	N	N	N
5	09/09/07	N	N	N	N	N	N	N
6	09/10/07	N	N	N	N	N	N	N
7	09/11/07	N	N	N	N	N	N	N
8	09/12/07	N	N	N	N	N	N	N
9	09/13/07	N	N	N	N	N	N	N
10	09/14/07	N	N	N	N	N	N	N
11	09/15/07	N	N	N	N	N	N	N
12	09/16/07	N	N	N	N	N	N	N
13	09/17/07	N	N	N	N	N	N	N
14	09/18/07	N	N	N	N	N	N	N
Score	–	0	0	0	0	–	–	0

DAY	Nasal Discharge	Salivating	Oral Ulceration	External Ulceration	De-hydration	Appetite	Temp (℃)	Temp (℉)
0	N	N	N	N	N	Y	36.6	97.9
1	N	N	N	N	N	Y	–	–
2	N	N	N	N	N	Y	–	–
3	N	N	N	N	N	Y	–	–
4	N	N	N	N	N	Y	–	–
5	N	N	N	N	N	Y	–	–
6	N	N	N	N	N	Y	–	–
7	N	N	N	N	N	Y	–	–
8	N	N	N	N	N	Y	–	–
9	N	N	N	N	N	Y	–	–
10	N	N	N	N	N	Y	–	–
11	N	N	N	N	N	Y	–	–
12	N	N	N	N	N	Y	–	–
13	N	N	N	N	N	Y	–	–
14	N	N	N	N	N	Y	–	–
Score	0	0	0	0	0	0	–	0

Total Score = 0

Y: yes; N: no; S: serous; MP: mucopurulent; –: not applicable.

Appendix B, continued

Cat Identification: AJD3 Group: Negative Control
Color: Orange Sex: F Age: 11-13 weeks
Scoring: Sheldon

DAY	Date	Sneeze	Cough	Rales	Open Mouth Breath	Bleph-spasm	Corneal Ulcertaion (Opacity)	Ocular Discharge
0	09/04/07	N	N	N	N	N	N	N
1	09/05/07	N	N	N	N	N	N	N
2	09/06/07	N	N	N	N	N	N	N
3	09/07/07	N	N	N	N	N	N	N
4	09/08/07	N	N	N	N	N	N	N
5	09/09/07	N	N	N	N	N	N	N
6	09/10/07	N	N	N	N	N	N	N
7	09/11/07	N	N	N	N	N	N	N
8	09/12/07	N	N	N	N	N	N	N
9	09/13/07	N	N	N	N	N	N	N
10	09/14/07	N	N	N	N	N	N	N
11	09/15/07	N	N	N	N	N	N	N
12	09/16/07	N	N	N	N	N	N	N
13	09/17/07	N	N	N	N	N	N	N
14	09/18/07	N	N	N	N	N	N	N
Score	–	0	0	0	0	–	–	0

DAY	Nasal Discharge	Salivating	Oral Ulceration	External Ulceration	De-hydration	Appetite	Temp (℃)	Temp (℉)
0	N	N	N	N	N	Y	36.6	97.9
1	N	N	N	N	N	Y	37.3	99.1
2	N	N	N	N	N	Y	–	–
3	N	N	N	N	N	Y	–	–
4	N	N	N	N	N	Y	–	–
5	N	N	N	N	N	Y	–	–
6	N	N	N	N	N	Y	–	–
7	N	N	N	N	N	Y	–	–
8	N	N	N	N	N	Y	–	–
9	N	N	N	N	N	Y	–	–
10	N	N	N	N	N	Y	–	–
11	N	N	N	N	N	Y	–	–
12	N	N	N	N	N	Y	–	–
13	N	N	N	N	N	Y	–	–
14	N	N	N	N	N	Y	–	–
Score	0	0	0	0	0	0	–	0

Total Score = 0

Y: yes; N: no; S: serous; MP: mucopurulent; –: not applicable.

Appendix B, continued

Cat Identification: AJC2 Group: BAC
Color: Grey Tabby Sex: F Age: 11-13 weeks
Scoring: Janice

DAY	Date	Sneeze	Cough	Rales	Open Mouth Breath	Bleph-spasm	Corneal Ulcertaion (Opacity)	Ocular Discharge
0	09/04/07	N	N	N	N	N	N	N
1	09/05/07	N	N	N	N	N	N	N
2	09/06/07	N	N	N	N	N	N	N
3	09/07/07	N	N	N	N	N	N	N
4	09/08/07	N	N	N	N	N	N	N
5	09/09/07	N	N	N	N	N	N	S
6	09/10/07	Y	N	N	N	N	N	S
7	09/11/07	Y	N	Y	N	N	N	S
8	09/12/07	Y	Y	Y	N	N	N	S
9	09/13/07	Y	N	Y	N	N	N	N
10	09/14/07	N	N	Y SLIGHT	N	N	N	N
11	09/15/07	N	N	Y SLIGHT	N	N	N	N
12	09/16/07	N	N	N	N	N	N	N
13	09/17/07	Y	N	Y SLIGHT	N	N	N	N
14	09/18/07	N	N	N	N	N	N	N
Score	–	5	2	12	0	–	–	2

DAY	Nasal Discharge	Salivating	Oral Ulceration	External Ulceration	De-hydration	Appetite	Temp (℃)	Temp (℉)
0	N	N	N	N	N	Y	37.2	99.0
1	N	N	N	N	N	Y	37.1	98.8
2	N	N	N	N	N	Y	33.1	91.6
3	N	N	N	N	N	Y	37.0	98.6
4	N	N	N	N	N	Y	37.8	100.0
5	N	N	N	N	N	Y	38.0	100.4
6	N	N	N	N	N	Y	37.0	98.6
7	S	N	N	N	Y SLIGHT	Y	37.5	99.5
8	N	N	N	N	N	Y SLIGHT	37.0	98.6
9	S	N	N	N	Y SLIGHT	N	37.6	99.7
10	N	N	N	N	N	Y	37.1	98.8
11	N	N	N	N	N	Y	37.0	98.6
12	S	N	N	N	N	Y	37.6	99.7
13	N	N	N	N	N	Y	37.0	98.6
14	N	N	N	N	N	Y	37.7	99.9
Score	1	0	0	0	3	2	–	0

Total Score = 27

Y: yes; N: no; S: serous; MP: mucopurulent; –: not applicable.

Appendix B, continued

Cat Identification: AJC2 Group: BAC
Color: Grey Tabby Sex: F Age: 11-13 weeks
Scoring: Sheldon

DAY	Date	Sneeze	Cough	Rales	Open Mouth Breath	Bleph-spasm	Corneal Ulcertaion (Opacity)	Ocular Discharge
0	09/04/07	N	N	N	N	N	N	N
1	09/05/07	N	N	N	N	N	N	N
2	09/06/07	N	N	N	N	N	N	N
3	09/07/07	N	N	N	N	N	N	N
4	09/08/07	N	N	N	N	N	N	N
5	09/09/07	N	N	N	N	N	N	S
6	09/10/07	Y	N	N	N	N	N	S
7	09/11/07	Y	Y	N	N	N	N	S
8	09/12/07	Y	Y	N	N	Y	N	S
9	09/13/07	Y	N	N	N	N	N	N
10	09/14/07	N	N	N	N	N	N	N
11	09/15/07	N	N	N	N	N	N	N
12	09/16/07	N	N	N	N	N	N	N
13	09/17/07	Y	N	N	N	N	N	N
14	09/18/07	N	N	N	N	N	N	N
Score	–	5	4	0	0	–	–	2

DAY	Nasal Discharge	Salivating	Oral Ulceration	External Ulceration	De-hydration	Appetite	Temp (℃)	Temp (℉)
0	N	N	N	N	N	Y	37.2	99.0
1	N	N	N	N	N	Y	37.3	99.1
2	N	N	N	N	N	Y	38.4	101.1
3	N	N	N	N	N	Y	37.0	98.6
4	N	N	N	N	N	Y	37.8	100.0
5	N	N	N	N	N	Y	38.0	100.4
6	N	N	N	N	N	Y	37.2	99.0
7	S	N	N	N	Y	Y	37.5	99.5
8	N	N	N	N	N	N	37.0	98.6
9	N	N	N	N	N	Y	37.6	99.7
10	S	N	N	N	N	Y	37.1	98.8
11	S	N	N	N	N	Y	37.5	99.5
12	S	N	N	N	N	Y	37.0	98.6
13	N	N	N	N	N	Y	37.0	98.6
14	N	N	N	N	N	Y	37.7	99.9
Score	2	0	0	0	3	1	–	0

Total Score = 17

Y: yes; N: no; S: serous; MP: mucopurulent; –: not applicable.

Appendix B, continued

Cat Identification: AJC3 Group: BAC
Color: Brown Grey Tabby Sex: F Age: 11-13 weeks
Scoring: Janice

DAY	Date	Sneeze	Cough	Rales	Open Mouth Breath	Bleph-spasm	Corneal Ulcertaion (Opacity)	Ocular Discharge
0	09/04/07	N	N	N	N	N	N	N
1	09/05/07	N	N	N	N	N	N	N
2	09/06/07	N	N	N	N	N	N	N
3	09/07/07	N	N	N	N	N	N	N
4	09/08/07	N	N	N	N	N	N	N
5	09/09/07	Y	N	N	N	N	N	N
6	09/10/07	N	N	N	N	Y	N	N
7	09/11/07	Y	N	Y	N	Y	N	S
8	09/12/07	Y	N	Y	N	Y	N	S
9	09/13/07	N	Y	Y	N	Y	N	S
10	09/14/07	Y	Y	Y	N	N	N	S
11	09/15/07	Y	N	Y SLIGHT	N	N	N	S
12	09/16/07	Y	N	Y SLIGHT	N	N	N	S
13	09/17/07	Y	N	Y SLIGHT	N	N	N	MP?
14	09/18/07	N	N	N	N	N	N	S
Score	–	7	4	14	0	–	–	2

DAY	Nasal Discharge	Salivating	Oral Ulceration	External Ulceration	De-hydration	Appetite	Temp (℃)	Temp (℉)
0	N	N	N	N	N	Y	37.2	99.0
1	N	N	N	N	N	Y	37.3	99.1
2	N	N	N	N	N	Y	37.4	99.3
3	N	N	N	N	N	Y	36.0	96.8
4	N	N	Y	N	N	Y	38.6	101.5
5	N	N	Y	N	N	Y	38.0	100.4
6	N	N	Y	N	N	Y	38.3	100.9
7	S	N	N	N	Y SLIGHT	Y	37.0	98.6
8	S	N	Y	N	Y	N	37.4	99.3
9	S	N	N	N	Y SLIGHT	N	37.7	99.9
10	MP	N	N	N	Y	N	36.3	97.3
11	S	N	N	N	Y SLIGHT	Y	37.2	99.0
12	S	N	N	N	N	Y	36.8	98.2
13	N	N	N	N	N	Y	37.5	99.5
14	S	N	N	N	N	Y	37.0	98.6
Score	2	0	2	0	4	3	–	0

Total Score = 38

Y: yes; N: no; S: serous; MP: mucopurulent; –: not applicable.

Appendix B, continued

Cat Identification: AJC3 Group: BAC
Color: Brown Grey Tabby Sex: F Age: 11-13 weeks
Scoring: Sheldon

DAY	Date	Sneeze	Cough	Rales	Open Mouth Breath	Bleph-spasm	Corneal Ulcertaion (Opacity)	Ocular Discharge
0	09/04/07	N	N	N	N	N	N	N
1	09/05/07	N	N	N	N	N	N	N
2	09/06/07	N	N	N	N	N	N	N
3	09/07/07	N	N	N	N	N	N	N
4	09/08/07	N	N	N	N	N	N	N
5	09/09/07	Y	N	N	N	N	N	S
6	09/10/07	N	N	N	N	Y	N	S
7	09/11/07	Y	N	N	N	N	N	S
8	09/12/07	Y	N	N	N	Y	N	S
9	09/13/07	N	N	N	N	N	N	S
10	09/14/07	Y	N	N	N	N	N	N
11	09/15/07	Y	N	N	N	N	N	N
12	09/16/07	Y	N	N	N	N	N	N
13	09/17/07	Y	N	N	N	N	N	N
14	09/18/07	N	N	N	N	N	N	N
Score	–	7	0	0	0	–	–	2

DAY	Nasal Discharge	Salivating	Oral Ulceration	External Ulceration	De-hydration	Appetite	Temp (℃)	Temp (℉)
0	N	N	N	N	N	Y	37.2	99.0
1	N	N	N	N	N	Y	37.4	99.3
2	N	N	N	N	N	Y	38.1	100.6
3	N	N	N	N	N	Y	36.8	98.2
4	N	N	1	N	N	Y	38.6	101.5
5	N	N	1	N	N	Y	38.0	100.4
6	N	N	1	N	N	Y	38.3	100.9
7	S	N	1	N	N	Y	37.0	98.6
8	N (MP?)	N	1	N	N	Y	36.8	98.2
9	N	N	N	N	N	Y	37.7	99.9
10	MP	N	N	N	N	Y	36.3	97.3
11	N (MP?)	N	N	N	N	Y	37.7	99.9
12	S	N	N	N	N	Y	36.8	98.2
13	N	N	N	N	N	Y	37.5	99.5
14	N	N	N	N	N	Y	37.0	98.6
Score	2	0	2	0	0	0	–	0

Total Score = 13

Y: yes; N: no; S: serous; MP: mucopurulent; –: not applicable.

Appendix B, continued

Cat Identification: AJJ5 Group: Positive Control
Color: Black Sex: F Age: 11-13 weeks
Scoring: Janice

DAY	Date	Sneeze	Cough	Rales	Open Mouth Breath	Bleph-spasm	Corneal Ulcertaion (Opacity)	Ocular Discharge
0	09/04/07	N	N	N	N	N	N	N
1	09/05/07	N	N	N	N	N	N	N
2	09/06/07	N	N	N	N	N	N	N
3	09/07/07	Y	N	N	N	N	N	N
4	09/08/07	Y	N	N	N	N	N	S
5	09/09/07	Y	N	N	N	N	N	S
6	09/10/07	Y	N	N	N	N	N	S
7	09/11/07	Y	N	Y	Y	N	N	S
8	09/12/07	Y	N	Y	N	N	N	S
9	09/13/07	N	N	Y	N	N	N	S
10	09/14/07	N	N	Y	N	N	N	S
11	09/15/07	N	N	Y SLIGHT	N	N	N	S
12	09/16/07	N	N	N	N	N	N	S
13	09/17/07	Y	N	Y	N	N	N	MP
14	09/18/07	N	N	N	N	N	N	MP
Score	–	7	0	12	3	–	–	2

DAY	Nasal Discharge	Salivating	Oral Ulceration	External Ulceration	De-hydration	Appetite	Temp (℃)	Temp (℉)
0	N	N	N	N	N	Y	37.1	98.8
1	N	N	N	N	N	Y	37.0	98.6
2	N	N	N	N	N	Y	37.2	99.0
3	N	N	N	N	N	Y	38.0	100.4
4	N	N	N	N	Y SLIGHT	Y	40.1	104.2
5	S	N	N	N	N	Y	37.8	100.0
6	S	N	N	N	N	Y	35.0	95.0
7	N	N	N	N	N	Y	38.1	100.6
8	S	N	N	N	N	Y	37.0	98.6
9	S	N	N	N	N	Y	37.0	98.6
10	S	N	N	N	N	Y	37.4	99.3
11	S	N	N	N	N	Y	37.6	99.7
12	S	N	N	N	N	Y	36.3	97.3
13	MP BLOODY	N	N	N	N	Y	37.0	98.6
14	MP BLOODY	N	N	N	N	Y	38.0	100.4
Score	2	0	0	0	3	0	–	1

Total Score = 30

Y: yes; N: no; S: serous; MP: mucopurulent; –: not applicable.

Appendix B, continued

Cat Identification: AJJ5 Group: Positive Control
Color: Black Sex: F Age: 11-13 weeks
Scoring: Sheldon

DAY	Date	Sneeze	Cough	Rales	Open Mouth Breath	Bleph-spasm	Corneal Ulcertaion (Opacity)	Ocular Discharge
0	09/04/07	N	N	N	N	N	N	N
1	09/05/07	N	N	N	N	N	N	N
2	09/06/07	Y	N	N	N	N	N	N
3	09/07/07	Y	N	N	N	N	N	N
4	09/08/07	Y	N	N	N	N	N	S
5	09/09/07	Y	N	N	N	N	N	S
6	09/10/07	Y	N	N	N	N	N	S
7	09/11/07	Y	N	N	N	N	N	S
8	09/12/07	Y	N	N	N	N	N	S
9	09/13/07	N	N	N	N	N	N	S
10	09/14/07	N	N	N	N	N	N	S
11	09/15/07	N	N	N	N	N	N	S
12	09/16/07	N	N	N	N	N	N	S
13	09/17/07	N	N	N	N	N	N	S
14	09/18/07	N	N	N	N	N	N	MP
Score	–	7	0	0	0	–	–	2

DAY	Nasal Discharge	Salivating	Oral Ulceration	External Ulceration	De-hydration	Appetite	Temp (℃)	Temp (℉)
0	N	N	N	N	N	Y	37.1	98.8
1	N	N	N	N	N	Y	37.7	99.9
2	N	N	N	N	N	Y	38.2	100.8
3	N	N	N	N	N	Y	38.6	101.5
4	N	N	N	N	N	Y	40.1	104.2
5	S	N	N	N	N	Y	37.8	100.0
6	S	N	N	N	N	Y	35.0	95.0
7	S	N	N	N	N	Y	38.1	100.6
8	N (MP?)	N	N	N	N	Y	37.0	98.6
9	N	N	N	N	N	Y	37.4	99.3
10	MP	N	N	N	N	Y	37.4	99.3
11	MP	N	N	N	N	Y	37.6	99.7
12	MP	N	N	N	N	Y	36.5	97.7
13	MP	N	N	N	N	Y	37.0	98.6
14	MP	N	N	N	N	Y	38.0	100.4
Score	4	0	0	0	0	0	–	1

Total Score = 14

Y: yes; N: no; S: serous; MP: mucopurulent; –: not applicable.