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RNA Interference of the Glycoprotein D and DNA Polymerase Genes of Feline Herpesvirus 1 by Synthetic siRNAs

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To the Graduate Council:

I am submitting herewith a dissertation written by Rebecca Penrose Wilkes entitled "RNA Interference of the Glycoprotein D and DNA Polymerase Genes of Feline Herpesvirus 1 by Synthetic siRNAs." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Stephen A. Kania, Major Professor

We have read this dissertation and recommend its acceptance:

Melissa A. Kennedy, Karla J. Matteson, Leon N.D. Potgieter

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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genes of feline herpesvirus 1 by synthetic siRNAs**

**A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville**

**Rebecca Penrose Wilkes
August 2007**

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Dedication

This dissertation is dedicated to the three women who raised me, my mom Marilyn Penrose; my aunt, Sherilyn Neel; and my granny, Marguerite Neel. Thank you for showing me what it is to be a strong woman in the face of adversity and heartache and maintain dignity and grace. Mom, thank you for your unending support and all the years you worked so hard so my dreams could come true.

Marguerite Sinclair Neel 1919-2000

Sherilyn Joyce Neel 1950-2006

Abstract

Feline herpesvirus 1 (FHV-1) is a linear double-stranded DNA virus that causes approximately 50% of the upper respiratory tract infections and produces the most severe respiratory disease in domestic cats. Primary ocular infection, as occurs in humans with the related virus, herpes simplex virus type 1 (HSV-1), consistently produces conjunctivitis and minimal corneal involvement; however, clinical manifestations of disease due to repeated recrudescence involve the cornea and can potentially lead to blindness. Vaccines only produce partial protection from clinical disease, and antiviral medications approved for treatment of HSV-1 in humans are only minimally effective for treatment of the chronic cases in cats. Therefore, RNA interference (RNAi), a RNA-guided gene regulatory mechanism that is found in a variety of eukaryotic organisms and provides anti-viral immunity in plants, was used as a therapeutic method for prevention of FHV-1 infection in feline cells. Previous RNAi experiments in related herpesviruses demonstrated that the DNA polymerase gene and a gene coding for a viral attachment protein are effective targets for inhibiting herpesvirus replication. Therefore, a region coding for highly conserved amino acid motifs of herpesvirus DNA polymerase genes, which is unique to each viral species and lacks DNA sequence drift for alphaherpesviruses, including herpes simplex virus type 2 and also feline herpesvirus as shown in this study, was targeted by RNAi. The attachment proteins for FHV-1 are unknown, but the highly conserved glycoprotein D (gD) gene was also chosen as a target for this study. HSV-1 gD is an essential receptor-binding polypeptide and is necessary for penetration of the virus into cells. FHV-1 gD, an envelope protein, is an inducer of

virus-neutralizing antibodies and may play an important role in the restriction of the host range of the virus to feline cells.

Two synthetic siRNAs targeting the DNA polymerase gene and the gD gene of FHV-1 were effective in knockdown of their intended targets by 69-83% for DNA polymerase mRNA and 77-87% for gD mRNA, determined by quantitative real-time RT-PCR. Based on flow cytometry results, glycoprotein D mRNA knockdown decreased gD cell surface expression by 27-43%, and DNA polymerase mRNA interference decreased cell surface FHV-1 glycoprotein expression by 29-71%. Knockdown of the mRNAs from these genes also resulted in decreased infective virus *in vitro*, with decreased viral replication by 83-96% by DNA polymerase RNAi and 77-84% by gD RNAi, determined by plaque assays. Interference of each mRNA also resulted in a decrease in the amount of the opposite mRNA, independent of interferon β production. These results indicate that FHV-1 glycoprotein D, like its homolog in HSV-1, is essential for *in vitro* replication and is likely involved with viral attachment and/or penetration, and both this gene and the FHV-1 DNA polymerase gene are suitable targets for RNAi anti-viral treatment. This study lays the groundwork for potential *in vivo* investigations in cats. Such studies may provide unique insights into the prevention/treatment of herpesvirus infections by RNAi.

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Part 1 General Introduction

Introduction

Feline herpesvirus 1 (FHV-1) is a linear double-stranded DNA virus (185) that causes approximately 50% of the upper respiratory tract infections (140) and produces the most severe respiratory disease in domestic cats (173). This virus is labile in nature but maintains itself by producing latent infections in its host (55). Killed and modified live vaccines are available for FHV-1, but because the virus is poorly immunogenic (8), the vaccines do not prevent infection and only produce partial protection from clinical disease (21). Acute infections are usually localized to the respiratory tract and clinical signs, though potentially severe, usually resolve in a few weeks (173). However, chronic disease in latently infected cats is problematic. First, diagnosing the disease is difficult in these cats (140). Approximately 70% of cats are seropositive for the virus (106), and about 80% of infected cats become latently infected (55). Though the majority of latently infected cats do not develop chronic clinical disease (6), there is still a large percentage of adult cats that have this problem (210). Therefore, a positive test result may mean FHV-1 is the cause of the disease, found coincidentally as a result of intermittent shedding, or present secondary to stress caused by a separate disease that is primarily responsible for the clinical signs (27, 137).

Second, the clinical manifestations of disease due to repeated recrudescence, mainly affecting the eye, are significant and can potentially lead to blindness (6). Antiviral medications approved for treatment of a related virus, herpes simplex virus type 1 in humans, are only minimally effective for treatment of these chronic cases in cats (212). Therefore, development of a new therapeutic for FHV-1 would be beneficial.

Recently, RNA interference has been used for prevention of various mammalian viral infections both *in vitro* and *in vivo* (98). RNA interference is a RNA-guided gene regulatory mechanism that is found in a variety of eukaryotic organisms, including yeast, plants, and mammals. One of its biological functions is anti-viral immunity in plants (39). This defense mechanism is triggered by double stranded RNA, and small interfering RNAs can be chemically produced and delivered to cells to silence specific genes of interest (47). The purpose of this study was to determine the feasibility of using RNA interference to prevent feline herpesvirus 1 infections *in vitro*.

Feline Herpesvirus 1 Literature Review

Feline herpesvirus 1 was first isolated in the United States in 1957 from a group of 5-10 week old kittens experiencing upper respiratory tract disease (38). The virus was subsequently detected in many other countries, including Switzerland in 1963 (35), Hungary in 1965 (82), Canada in 1965 (46), Britain in 1966 (88), Australia in 1970 (216), and Japan in 1974 (125).

Classification and viral properties: FHV-1 is a member of the family *Herpesviridae* (46) and is classified in the subfamily *Alphaherpesvirinae* (184). Based on phylogenetic analysis of its proteins, FHV-1 is grouped in the *Varicellovirus* genus (126, 246).

FHV-1 has a diameter between 141 and 173 m μ , contains an icosahedral capsid comprising 162 capsomers, and has an envelope (34, 35). The genome of FHV-1 is approximately 126-134 kbp, based on restriction endonuclease mapping (67, 185). It has a G+C content of approximately 46% (172). The genome consists of a single linear molecule of double-stranded DNA. It is composed of a unique long region approximately 99-104kbp, which is covalently linked to a unique short sequence of 8-9 kbp that is flanked by inverted repeats of 7-11 kbp each (67, 185). The short region can invert its orientation relative to the long region, so the genome exists in two isomeric forms. FHV-1 has a group D genome, which includes other alphaherpesviruses varicella-zoster virus, pseudorabies virus, equine herpesvirus-1, and bovine herpesvirus-1. The genome is most similar in size and arrangement to equine herpesvirus-1 (185).

Viral transcription occurs in the host cell nucleus. Three classes of mRNA, α (immediate early), β (early), and γ (late) are transcribed in sequence. The immediate

early and early genes encode enzymes and DNA-binding proteins, and the late genes encode mainly structural proteins. Viral DNA replication occurs in the nucleus and viral DNA is packaged into capsids within this compartment. The virions acquire their envelopes by budding from the nuclear membrane (151).

Host/tissue range and cytopathology: FHV-1, unlike other alphaherpesviruses such as herpes simplex virus type 1 and pseudorabies virus, is restricted *in vivo* to Felidae and *in vitro* to feline cells (34, 35, 82, 111, 172, 175). The virus replicates in cultures of feline origin including kidney, thymus, tongue, lung (175), cornea (188), tracheal organ cultures (113), and T-lymphocytes (78), and the virus has been isolated only in feline cells (35).

FHV-1 grows rapidly in cell culture. Infectious intracellular virus can be detected six hours post infection, and cell-to-cell spread of virus can be detected six to seven hours post infection. Extracellular spread of the virus occurs at nine to ten hours post infection, concurrent with detection of infectious extracellular virus (236).

The characteristic cytopathologic feature of FHV is the occurrence of type A intranuclear inclusion bodies, which are spherical or oval, acidophilic and separated from the nuclear membrane by a clear halo (34, 38). FHV also develops multinucleated giant cells or syncytia in cell cultures, which consist of foci of cells that have lost their distinct cellular membranes and have fused with adjacent cells. Infected cells are rounded or elongated in shape and are more retractile than uninfected cells (36).

Antigenic and genomic properties: There is antigenic homogeneity between isolates from many parts of the world compared to the prototype strain (177). All isolates belong to one serotype, confirmed by virus neutralization, complement fixation,

immunodiffusion, and hemagglutination inhibition assays (175). Minor differences between strains can be identified with immunoblot and restriction endonuclease analysis. Some isolates lack a 36 kDa protein, and the restriction enzyme *Mlu*I produces variable restriction patterns among strains, suggesting isolates can be divided into different groups based on genomic heterogeneity. The importance of these differences in genomes with regard to virulence is unknown (80, 129). The apparently low rate of variability between isolates suggests the genome has evolved to a fairly constant state, possibly through an interaction with the host cell DNA during latency or reflecting some inherent property of the virus (67).

Viral genes and proteins: FHV-1 contains at least twenty-three viral proteins (49). Seven FHV-1 glycoproteins have been identified and each share sequence homology with other alphaherpesvirus glycoproteins. Genes encoding glycoproteins B, C and H are located in the unique long segment of the genome (125). FHV-1 glycoprotein B homolog was identified in 1992 (126). It is 2829 base pairs and contains two potential proteolytic cleavage sites (204). In nonreducing conditions, glycoprotein B in virus infected cells has molecular masses of 143 kDa and 108 kDa, the latter likely represents the precursor form of the glycoprotein, which lacks glycosylation (79, 126). Under reduced conditions, glycoprotein B has molecular masses of 100 kDa, 64 kDa, and 58 kDa. Due to the potential cleavage sites in the sequence encoding this gene, the protein likely consists of a heterodimer or trimer (124). Glycoprotein B is a major envelope glycoprotein of herpes simplex virus-1 and is crucial for virus infectivity. This glycoprotein is involved with virus adsorption and penetration and cell-to-cell spread of the virus by fusion of susceptible cells (126). Glycoprotein B is the most conserved

glycoprotein among herpesviruses, and some epitopes of FHV-1 cross-react with herpes simplex virus-1 antisera (79) and with monoclonals directed against canine herpesvirus glycoprotein B (118).

The gene for glycoprotein C is 1602 base pairs in length and homologs are non-essential for replication *in vitro*. Glycoprotein C has molecular masses of 113 kDa and 75 kDa (125). The glycoprotein C of herpes simplex virus type 1 mediates the initial stage of virus attachment by binding heparan sulfate (133). FHV-1 glycoprotein C has been demonstrated to bind heparin, indicating the role of FHV-1 glycoprotein C may be similar to herpes simplex virus glycoprotein C (132). Some FHV-1 isolates have genetic rearrangements in the glycoprotein C gene, resulting in insertions of repeat sequences, but the relevance of the rearrangements is unknown (69).

The gene encoding glycoprotein H is the second most highly conserved gene among herpesviruses. In herpes simplex virus type 1 and pseudorabies virus, glycoprotein H is implicated in viral entry and direct cell-to-cell spread of virus and in membrane fusion. This gene is located immediately downstream of the thymidine kinase gene and is predicted to encode 821 amino acids (127).

Glycoproteins G, D, I, and E have been identified in the unique short segment of the genome. FHV-1 glycoprotein D has a molecular weight of 53-59 kDa (77, 205). The FHV-1 glycoprotein D gene appears highly conserved; nucleotide sequences from three different strains are identical (243). Glycoprotein D has variable functions in different alphaherpesviruses. Herpes simplex virus type 1 glycoprotein D is necessary for virus growth. Glycoprotein D negative mutants are unable to infect susceptible cells or spread by direct cell-to-cell transmission. However, glycoprotein D in pseudorabies virus is not

essential for virus growth, and varicella-zoster virus lacks a glycoprotein D homolog (125). The glycoprotein D homolog in FHV-1 has been identified as a hemagglutinin of feline red blood cells (63, 77, 128). Hemagglutination titers are low (63), but the hemagglutination activity of FHV-1 is enhanced by treatment of the virus with polyoxyethylene sorbitan monooleate (Tween 80) and ether, resulting in separation of the glycoprotein from the envelope of the virus (147).

FHV-1 glycoprotein D may play an important role in the restriction of the host range of the virus to feline cells (131). FHV-1 does not hemagglutinate dog, chicken, or guinea pig red blood cells (63). Insect cells expressing FHV-1 glycoprotein D bind only to feline cell lines, not porcine, bovine, or canine (131). Monoclonal antibodies directed against glycoprotein D inhibit hemagglutination (79). The hemagglutinins of FHV-1 and canine herpesvirus share common epitopes. A monoclonal directed against the FHV-1 hemagglutinin inhibits the hemagglutination activity of canine herpesvirus (118).

Glycoprotein E and glycoprotein I form a hetero-oligomeric complex (146) and are involved in cell-to-cell transmission. The complex is found on the surface of infected cells and these genes are non-essential for growth in cell culture (125). However, an FHV-1 mutant containing a disrupted glycoprotein I gene produces a smaller plaque size than the parent virus (145). Deletion of glycoprotein E and a portion of glycoprotein I results in reduced virulence when the mutant virus is administered oronasally, suggesting these are virulence genes (101).

Glycoprotein G is expressed as a secreted protein or is bound to the membrane of infected cells. It is also a structural protein, incorporated into the viral envelope.

Glycoprotein G functions as a chemokine binding protein, modulating the anti-viral chemokine defense system in the host (33).

Glycoproteins B, C and D are also expressed on the surface of infected cells. Cell membrane fluorescence can be demonstrated at 12 hours post-infection for glycoprotein C, 16 hours post-infection for glycoprotein D, and 20 hours post-infection for glycoprotein B. Glycoprotein D has at least four distinct epitopes, glycoprotein C has two antigenic domains (one neutralizing domain consisting of three overlapping epitopes and a non-neutralizing domain), and glycoprotein B has only one antigenic site, consisting of five similar or overlapping epitopes (117). Monoclonal antibodies produced against these glycoproteins exhibit virus neutralizing activity. The neutralizing activity against glycoproteins B and C requires complement, but the neutralizing activity against glycoprotein D shows varying complement requirements. This is possibly due to recognition of the different epitopes, but the majority of the anti-glycoprotein D monoclonals neutralize without need for complement (79).

Latency: Like other alphaherpesviruses, FHV-1 establishes latency in the trigeminal ganglion, as determined by explant /isolation techniques (53, 153) and polymerase chain reaction (PCR) (181, 218, 237). More than 80% of cats become latently infected after acute FHV-1 infection, and at least 45% of those shed the virus (55). The carrier state is characterized by periodic episodes of shedding. Re-excretion of the virus can be stimulated by stress, such as changes in housing or parturition and lactation, or induced by injections of steroids (55, 57). The mean lag time between the induction of stress and re-excretion of virus is seven days, with excretion lasting 1-13 days. Re-excretion may be associated with clinical signs such as mild conjunctivitis or

rhinitis or may not be associated with any clinical signs at all (55, 57). Spontaneous shedding also occurs without an association of clinical signs (55).

Latency associated transcripts are detected in trigeminal ganglia of latently infected cats (163, 224). These transcripts are the opposite strand of the immediate early gene of FHV-1 (163), and are considered to indicate a true state of latency when detected in tissue (211). Other suggested latently infected tissues, determined by detecting non-replicating virus by PCR, include the olfactory bulbs (56, 181, 218, 237), brain stem (218), optic nerves (237), and nasal turbinates (181). The virus may also establish latency in the cornea (181, 224, 237), but latency associated transcripts have not been detected in the corneas of latently infected cats (224). Isolation of infectious virus from tissues of latently infected cats is difficult, even from cats actively shedding virus. This suggests either very little virus is present in the tissues, with few cells infected (163), or the virus is in a form that is non-infectious (56, 181).

Transmission: FHV-1 has been perpetuated in nature as a result of its ability to produce latent infections, and latently infected cats represent the most important reservoir of the virus (55, 166). Herpesvirus is unable to survive outside the host for more than three days. Samples collected on swabs must be suspended in protein medium to allow survival up to 72 hours at room temperature. Virus remains viable at room temperature no more than 18 hours on swabs suspended in saline (176). Direct transmission between cats and persistence of the virus within cats is necessary for the perpetuation of the virus. Transmission occurs between shedding and susceptible cats only if there is intimate contact between them. Widespread airborne transmission does not seem important in viral spread and FHV-1 is unlikely to be spread between cats by aerosol (58, 173). The

virus can be spread by sneezed droplets and the probable effective distance for transmission is about three feet (177). Spread by fomites is also a mode of transmission that is more likely to occur in cattery situations (175).

Cats are territorial and do not naturally commingle. This behavior reduces the contact between cats, decreasing the opportunity to spread respiratory infections. However, in situations in which cats are brought together from different sources and housed in close quarters, severe respiratory disease outbreaks may occur (92). Respiratory disease is most likely to occur in breeding colonies and rescue catteries and disease is most likely to be detected in younger cats, approximately 4-11 months old (16). Cats that have recently visited a boarding facility are also most likely to have respiratory disease (16), and cats in contact with cats outside the household are most likely to have detectable FHV-1 (219). Poor hygiene within multi-cat households, including breeding facilities and rescue shelters, is also a risk for upper respiratory tract disease (75).

Upper respiratory infections in cats is second only to overcrowding as the leading cause of euthanasia in shelters (9). FHV-1 is one of the most important causes of shelter respiratory disease. In one study, 41% of shelter cats had active FHV-1 infection (9), and in another study, shedding increased from 4% in cats upon relinquish to 52% after just one week within the shelter. Though many cases are probably due to recrudescence, this also represents new cases, suggesting FHV-1 is spread rapidly and efficiently within shelters (170).

Prevalence: FHV-1 is widespread within the feline population, with at least 70.7% of cats serologically positive (106), but the seroprevalence may be as high as 97%, based on results from a more sensitive test (42, 140). Prior to the development of

vaccines, the rate of infection in healthy cats, as determined by isolation from oropharyngeal swabs, was 1% from household cats and 1.75% from cat show cats. However, the true rate is likely higher because carriers shed intermittently (235). Recent studies suggest the rate of carriers, determined by isolation of FHV-1 from conjunctival swabs (140) and detection of FHV-1 DNA by nested PCR from conjunctival snip biopsies (213) from clinically normal cats is 10.9% and 12%, respectively. FHV-1 has been detected in 13.7%-54% of cats with naturally acquired conjunctivitis or respiratory disease (27, 140, 213).

Clinical disease: The disease produced by FHV-1, designated feline viral rhinotracheitis (36), is the clinically most significant infection of the feline respiratory diseases (173). The incubation period is usually 2-4 days (175), but the infecting dose is a significant factor in determining the length of the incubation period (54). The acute disease is characterized by pyrexia, salivation, leukocytosis (primarily neutrophilia), depression, conjunctivitis, serous to mucopurulent ocular and nasal discharge, and accompanying coughing, sneezing and dyspnea (37, 38, 222). Dendritic corneal ulcers may also develop but are less common during the acute infection (158), and are considered pathognomonic if present (160, 183). The most significant lesions are produced in the upper respiratory tract, especially in the mucosa of nasal passages and turbinates, as a result of the cytopathic effects of the virus (37). Osteolytic lesions can occur in nasal turbinate bone (76). The virus has a limited ability to replicate at core body temperature, so the primary sites of replication are the cooler epithelial surfaces (211). Thus, the main sites of viral replication are the nasal turbinates, conjunctivae, tonsils, soft palate, and trachea (37, 38, 56). Experimental infection can be produced by

application of the virus to the mucosal membranes of the eyes, nose, or mouth, but disease does not occur with intramuscular injection of the virus (177). Large amounts of virus are produced and released upon rupture of infected cells. Shedding occurs in all discharges from the eyes, nose, and throat and infectious virus can be demonstrated in these secretions for a period of 1-3 weeks after primary infection (175, 177).

Acute infection can occur in kittens, resulting in generalized disease and death (51, 206). Less common manifestations of FHV-1 include skin ulcers (73, 90), interstitial pneumonia (120), and lingual ulcers (197). Bronchial pneumonia can develop secondary to bacterial infection and conjunctivitis can lead to adhesion of the eyelids (82). The clinical signs may be worse in immunosuppressed cats co-infected with feline leukemia virus or feline immunodeficiency virus (180, 217). Mortality is high in young or debilitated cats, but the majority of cats recover in 7-10 days; however, the recovery may take weeks for severe cases (175).

Abortions have also been associated with FHV-1 infection (76, 206). Herpesvirus associated abortion in animals is due to viremia, rarely resulting from the spread of the disease from the lower genital tract. Vaginitis can be experimentally produced by infecting the vaginal area. Kittens born to queens infected in this manner in the later stages of gestation have high mortality rates due to generalized infection. Some kittens in the study were born with respiratory disease, suggesting in utero infection (20). However, genital tract lesions have not been detected in natural infections (175). Queens experimentally infected with FHV-1 intravenously or intranasally in the sixth week of gestation either abort or have dead fetuses. Lesions are detected in the placenta, uterus, and in a fetus from queens inoculated intravenously, but not in those inoculated

intranasally (76). In a study evaluating the transmission of the virus, no *in utero* infections were demonstrated (58). Therefore, abortions occurring in natural infections are more likely due to severe maternal infection than to fetal infection with FHV-1. FHV-1 rarely causes viremia and has a low abortogenic potential, and abortions in naturally occurring infections are rare (201).

FHV-1 is the only documented viral cause of feline keratitis, and corneal ulceration is primarily seen in adult cats with recrudescence of latent virus. Stromal keratitis is a less common manifestation of FHV-1 infection and results from chronic recurrent episodes of FHV-1 keratitis. Only a small percentage of cats will have recurrent bouts of keratitis, but this manifestation of FHV-1 infection is vision threatening, due to the associated stromal fibrosis and opacification. The mechanism of stromal disease is unknown, but prolonged absence of corneal epithelium results in stromal keratitis (6). This may be a function of the duration of the cornea's exposure to virus, and after virus gains access to the stroma, keratitis may be mediated by an immune response to viral antigens with infiltration of infected tissues with neutrophils, followed by B and T lymphocytes (155). Stromal keratitis does not occur in acute infection unless cats are treated intraocularly with steroids. Intraocular steroid injections preceding FHV-1 infection result in increased severity and prolonged infection with delayed epithelialization and suppressed collagen synthesis, leading to stromal keratitis, characterized by geographic epithelial ulceration, interstitial edema and deep vascularization. Other complications include decreased tear production and corneal sequestration, likely a nonspecific sequelae to significant stromal damage (155, 158). Recurring FHV-1 ocular disease may be unilateral or bilateral (212).

FHV-1 DNA has been detected in the corneas of a higher percentage of cats with eosinophilic keratitis or corneal sequestration than in corneas from clinically normal cats. Eosinophilic keratitis is a superficial proliferative inflammatory response of the cornea, predominated by mast cells and eosinophils. Corneal sequestration results from degeneration of stromal collagen and accumulation of a dark brown or black pigment presumed to be of tear film origin (156). FHV-1 has also been associated with uveitis. DNA has been detected in the aqueous humor from cats with uveitis. It is believed FHV-1 gains intraocular access via axonal transport along sensory nerve axons from the trigeminal ganglion, which innervates intraocular structures including the anterior uvea (139).

FHV-1 may also play a role in chronic rhinosinusitis, but in one study, detection of FHV-1 DNA was not statistically different in cats with chronic rhinosinusitis versus control cats. In the study, no actively replicating virus was detected in cats with chronic rhinosinusitis, so the disease does not appear the result of direct cell lysis (86). However, increased nasal cytokine expression has been detected in cats with replicating FHV-1, suggesting FHV-1 does have some role in nasal inflammation (87).

Immunoprophylaxis and immune response: Many attenuated (intranasal (41, 107) and parenteral) vaccines and an inactivated (parenteral) vaccine are available for FHV-1 in the United States (107). The first documented successful vaccine was the F-2 strain. This was an attenuated vaccine, produced by multiple passages in cell culture, including some passages at low temperature, and was used intramuscularly. The vaccine, like all FHV-1 vaccines developed since, produced significant reduction in the disease in vaccinated cats compared with unvaccinated cats, but it did not prevent disease in all the

cats tested (21, 22). Vaccination also does not prevent chronic ocular manifestations of FHV-1 (212). Vaccination does not prevent shedding of virus following challenge, and according to different studies, it may or may not decrease the course and amount of virus shedding (31, 107, 166, 195, 222, 238). Vaccination does not prevent the establishment of a latent infection (165); although, vaccination with at least one strain showed a reduced latency load in the trigeminal ganglia of cats post challenge (218). This may be beneficial, because attenuation of the amount of virus that reaches the sensory nerves results in a smaller reservoir of latent virus from which subsequent reactivations could arise (109).

Intranasal vaccination may be superior to intramuscular vaccination, requiring one dose instead of two, and may provide better protection than parenteral vaccines because a local and or cell mediated immune response may be more important for protection than serum antibody (41, 166). However, cats shed intranasal vaccine virus (166), and attenuated intranasal vaccine virus may establish latency (238). Intranasal use of attenuated parenteral vaccine strains produces severe disease (41, 101, 218).

The use of subunit vaccines may be useful in preventing establishment of a latent infection with a modified live recombinant vaccine (117). Glycoproteins D and B are potential targets for subunit vaccines. Glycoprotein D expressing vaccinia recombinants produced complement-independent virus neutralizing antibodies in immunized rabbits (205). Also, FHV-1 glycoprotein D expressed in a recombinant baculovirus produced high virus neutralizing antibody titers in immunized mice (130). Plasmid DNA expressing glycoprotein B injected into mice also produced virus neutralizing antibodies (123).

FHV-1 stimulates a poor immune response. Cats are susceptible to re-infection six months after primary infection. FHV-1 may evade the immune system. Because it is highly cell associated, matures intracellularly, and can spread cell to cell without contact with the interstitium, contact with neutralizing antibody is minimized (8). The immune response develops with serum neutralizing antibodies appearing about day 16 post exposure, but antibodies may not be detectable in some cats until three weeks post exposure (35, 82, 175).

Vaccination does appear to produce long term partial protection from clinical disease. Vaccination subcutaneously with an inactivated vaccine produced reduced severity of clinical disease following viral challenge 7.5 years after vaccination in cats. The vaccine provided persistent antibody titers for three years, and there was a rapid anamnestic response in the vaccinated cats post-challenge (195). In another study, vaccination induced a serologic response that lasted at least 48 months (150), and in a separate study, vaccination provided protection from severe clinical disease 10 and 31 months post vaccination (106). Though detection of FHV-1 antibodies in vaccinated cats appears to correlate with protection from clinical disease, some cats lacking antibody titers may also be protected from disease. This suggests the importance of cell mediated immunity or a rapid anamnestic response to FHV-1 infection (106).

A cell mediated immune response to FHV-1 develops in the cat and can be demonstrated *in vitro*. The response is comprised of cytotoxic T cells and antibody dependent cell mediated cytotoxicity (221, 236). FHV-1 infected cells can also be destroyed by antibody and complement mediated lysis (236). Protection to FHV-1

appears to be due to a specific immune response, and local responses are likely to be involved (31).

Passive immunity in kittens is acquired through colostrum and provides protection for 5-8 weeks but cannot be correlated with the antibody levels of the queen (58, 177). Shedding associated with the later stages of lactation, during the time of waning passive immunity, can result in subsequent infection and clinical disease in kittens (176, 177). Vaccination of kittens earlier than the recommended age of 9 weeks may be useful in high risk situations such as in breeding colonies. In one study, a significant portion of six week old kittens with maternal antibodies responded to parenteral vaccination with a modified live vaccine (43). In a separate study, vaccination of kittens with an intranasal vaccine at 5 weeks and a killed vaccine at 5 and 7 weeks also provided protection against disease in kittens with and without maternal antibodies, though seroconversion was delayed in kittens with maternal antibodies (89). In some cases, kittens with maternal antibodies may become subclinically infected from the queen and become latent carriers of the virus (58).

Diagnosis: Identification of FHV-1 infection has been by detection of FHV-1 antibodies by serum neutralization (178), enzyme linked immunosorbent assay (ELISA) (42, 191), or hemagglutination inhibition tests (63). ELISA has been shown to be more sensitive than serum neutralization (42, 191) and hemagglutination inhibition tests (191). Diagnosis of FHV-1 by serology is problematic. The magnitude of serum neutralization or ELISA titers is independent of the presence or absence of clinical signs of FHV-1 associated disease, and seroprevalence does not differ between clinically normal cats or cats with acute respiratory disease or with chronic ocular disease. Detection of serum

antibodies does not distinguish between infection by wild-type strain or attenuated vaccine strain. Cats with chronic ocular disease have been shown to have lower serum ELISA titers than clinically normal cats. Chronic or recrudescent infection, latency, prior infection, or vaccination may affect titers and limit the usefulness of paired serum samples for detection of active infection in chronically or latently infected cats (140).

Diagnosis of FHV-1 infection is commonly done by detection of the virus. Cats shed virus in ocular, nasal, and oropharyngeal secretions for at least 1-3 weeks during the initial infection, for several days during re-infection, and irregularly during periods of recrudescence (175). Virus is detected by immunofluorescent antibody tests (28), virus isolation (38), or PCR (27, 72, 181, 214, 220, 237). Virus may also be detected in tissues by immunohistochemistry and in situ hybridization (217).

Virus isolation has been considered the gold standard for FHV-1 detection (160). Virus isolation and immunofluorescent antibody testing may be of limited value in diagnosing FHV-1 disease in cats with chronic conjunctivitis because cats shed less virus for a shorter period of time during periods of recrudescence or chronic disease (27), and virus isolation and immunofluorescent antibody tests have low sensitivity. Positive results are more likely in cats with disease of shorter duration (159). However, both tests can detect virus in clinically normal cats (27, 140). In one study, virus isolation was found to be more sensitive than fluorescent antibody testing for detecting FHV-1 in cases of chronic conjunctivitis (159), but in a different study, the reverse was found in cats with chronic ocular disease (140).

Problems exist with virus isolation and immunofluorescent antibody assays. Interpretation of immunofluorescent antibody assay results is subjective, and nonspecific

immunofluorescence can be falsely interpreted as positive. Also, staining of the eye with a fluorescein dye to test for ulcers prior to collecting specimens for fluorescent antibody testing results in false-positive immunofluorescent antibody results (160). False negative results may occur with low virus antigen or interference by host derived antibody (27). Virus isolation requires proper specimen handling to ensure presence of viable virus for this assay to be successful (140).

There have been numerous PCR assays developed to detect FHV-1 DNA (27, 72, 181, 214, 220, 237). PCR assays have been determined to be more sensitive in detecting FHV-1 than virus isolation in latently infected cats (181), in cats with acute disease that have been previously vaccinated (220), and in cats with naturally acquired FHV-1 infection with current signs of conjunctivitis or nasal discharge (27). PCR may be more sensitive because as previously mentioned, virus isolation requires viable virus, and enveloped viruses are relatively labile and may be destroyed in transport and with freezing and thawing of samples. In contrast, DNA is stable and transit time and temperature appear not to affect the ability to detect FHV-1 DNA in samples (30). Virus infectivity may also be destroyed by enzymes in saliva or tears. The virions may be complexed with antibodies, decreasing infectivity, which may explain the increased sensitivity of PCR in vaccinated cats or in cats with chronic disease. Immature virions may also give positive PCR results but negative isolation results (181).

Nested PCR has been determined to be more sensitive than single round PCR for detecting FHV-1 in cats with signs of upper respiratory tract disease (72). Nested PCR is also more sensitive than virus isolation and fluorescent antibody detection (27, 214).

To increase the likelihood of detecting viral DNA in samples from cats with ocular disease, more than one specimen should be collected. There appears to be no sampling technique (conjunctival or corneal swabs and scrapings, or biopsies of the conjunctiva or cornea) that is more likely than another to harvest viral DNA, except in cats with corneal sequestrum, in which no virus could be detected in swabs (232).

Though more sensitive than isolation, different PCR protocols show marked variation in sensitivity, which affects the ability to detect FHV-1 DNA. Six published PCR assays were compared to diagnose FHV-1, and detection rates varied from 29-86% in cats with keratitis and/or conjunctivitis. Each assay also detected five vaccines tested, suggesting a positive PCR result may be due to detecting a vaccine strain (137).

A more sensitive test may not necessarily be better due to the potential to increase detection of virus in subclinical shedders. PCR results must be interpreted carefully, because a positive result may mean FHV-1 is the cause of the disease, found coincidentally as a result of intermittent shedding, or present secondary to stress caused by a separate disease that is primarily responsible for the clinical signs (27, 137).

In one of the PCR studies, FHV-1 DNA was detected in samples from 31% of clinically normal shelter cats. The author suggests PCR may be of limited value for diagnosing FHV-1 disease due to detection of healthy carriers (27). In an additional study, FHV-1 DNA was also detected in 6/13 keratectomy specimens from clinically normal shelter cats (213), and in another study, FHV-1 DNA was detected in 45/92 corneas from cats without a recent history of respiratory or ocular disease. Twenty-nine of the positive samples were obtained from shelter cats. The high rate of detection of FHV-1 DNA in normal corneal samples may represent detection of latent infection,

because the cornea may be a latently infected tissue, or may represent detection of subclinical shedders (224). Each of these studies used shelter cats as the clinically healthy control groups, which may have exaggerated the problem of detecting subclinical shedders, because as previously mentioned, cats in that environment are at an increased risk of being exposed to the virus or having reactivated virus (27).

Real time PCR assays have also been developed for detection of FHV-1 DNA and are more sensitive than virus isolation (74, 231). Real time PCR and isolation results correlate well in the acute phase of the disease, but real time PCR is more likely to detect FHV-1 DNA in later phases of disease in which there is a marked decline of virus titer (231). Real time PCR has an advantage over conventional PCR because of the ability to determine viral load and track the course of disease. Real time PCR is also less prone to contamination than conventional PCR due to the lack of post-amplification processing (231). This is especially true for nested PCR, in which contamination from the original product can arise (214).

Antiviral drugs: There are currently no anti-herpetic drugs approved for veterinary use in the United States, but many antiviral drugs marketed for treatment of herpes simplex virus type 1 infections in humans have been explored as therapeutic agents for treatment of FHV-1 infections in cats (136). Several acyclic nucleoside analogs have been tested, including acyclovir, ganciclovir, and penciclovir (136, 157). These drugs work by incorporating into the viral DNA chain, terminating its growth and inactivating viral DNA synthesis. Acyclic nucleoside analogs require three phosphorylation steps to be converted to their active form, and the first step is performed by the viral thymidine kinase, giving these drugs specificity for virally infected cells and

making them relatively safe (136). Acyclovir is relatively ineffective against FHV-1 *in vitro* (136, 157, 228), but it appears to be effective in cats for treatment of FHV-1 related conjunctivitis and corneal ulcers if administered topically several times a day (244). Ganciclovir and penciclovir show greater efficacy than acyclovir *in vitro*, but *in vivo* studies have not been performed (136, 228).

Other types of antiviral drugs have also been tested. Ribavirin, an inhibitor of nucleic acid synthesis, has only a slight antiviral effect on FHV-1 and reduces the rate of cell proliferation in treated cultures (174). Foscarnet, a drug that inhibits DNA polymerase by interacting with the pyrophosphate binding site of the enzyme, has intermediate to low antiviral efficacy *in vitro* (135, 228), but another viral DNA polymerase inhibitor, PMEDAP, has potent FHV-1 inhibition *in vitro* (228). Cidofovir, a cytosine analog, has been shown to be effective against FHV-1 in both CRFK cells (136, 228) and feline primary corneal cells (189). Cidofovir does not require phosphorylation by the viral kinase, and it inhibits the viral DNA polymerase 1000 times more potently than it inhibits the host cell polymerase (136). However, cidofovir causes increased cell death and decreased cell proliferation in corneal cells at effective antiviral doses (189).

The antiviral drugs that have shown the greatest effectiveness *in vitro* include idoxuridine and trifluridine (135, 157). These compounds also have non selective antiviral activity and inhibit CRFK cell proliferation at effective antiviral doses (157). The antivirals are epitheliotoxic and chronic use may lead to corneal or conjunctival disease (157), or may reduce cellular proliferation enough to negatively affect healing of corneal lesions (189). An additional problem with the current antiviral drugs is that these drugs are virostatic and not virocidal. Therefore, to be effective, these drugs must be

applied 4-6 times a day. Idoxuridine, vidarabine, and trifluridine have been used clinically as topical treatments for ocular lesions, but one study suggests that the overall response to these treatments is poor. This is possibly due in part to the need for frequent application of the drugs to be effective and poor owner compliance (212).

There is currently no effective systemic antiviral drug available for use in cats (210). Acyclovir is the only systemic drug that has been adequately tested and has been used clinically for treatment of FHV-1, but it has limited efficacy due to poor bioavailability and may also produce toxic side effects in cats (135, 167). Because of the poor bioavailability of acyclovir, a pro-drug of acyclovir, valacyclovir, has been tested in cats. After oral administration, valacyclovir is almost completely converted to acyclovir and provides much higher plasma concentrations of acyclovir than obtained with oral acyclovir administration. Despite achieving effective doses in cats in the study, this drug did not suppress FHV-1 replication in the acutely infected cats and was found to be toxic, causing hepatic and renal necrosis and severe bone marrow suppression (154).

Idoxuridine has also been tested systemically for treatment of FHV-1 in cats, and it produced vomiting, depression, and loss of appetite when administered intraperitoneally (208). Therefore, any currently available systemic antiviral drug must be used with caution in cats (154).

Adjunctive therapies: There are also adjunctive therapies for chronic FHV-1 infections. Oral lysine supplementation is used clinically to treat FHV-1 infected cats. Oral L-lysine supplementation reduces the severity of conjunctivitis in cats undergoing primary infection (215), and reduces viral shedding in latently infected cats following changes in husbandry and housing but not following corticosteroid administration (141).

In vitro, reduction of FHV-1 replication by lysine supplementation only results with reduction of arginine concentrations. Arginine exerts a substantial growth-promoting effect on FHV-1 and is an essential amino acid for viral protein synthesis, and it appears lysine antagonizes this effect. Lysine and arginine competitively inhibit transport of each other by using a common transport system, and lysine induces arginase, an enzyme that causes the degradation of arginine. Arginine deficiency inhibits synthesis of infectious viral particles and down regulates synthesis of viral proteins (138).

Human recombinant interferon alpha has also been used as a treatment for FHV-1. Oral administration of low doses has been associated with reduced clinical signs but not reduced FHV-1 shedding (134). The antiviral effect *in vitro* is modest (190) and dose dependent (198). Interferon treatment may only be effective when administered in high doses early in the course of infection. Human recombinant alpha-2b interferon is less effective against FHV-1 than feline recombinant omega interferon *in vitro*. Interferon is probably most effective when administered as an adjunct treatment (198). When used in combination with human recombinant interferon alpha, acyclovir could be used at a nearly eight fold reduction to achieve maximal inhibition of FHV-1 *in vitro*, and synergistic interactions were seen between the two treatments (239).

Additional therapies that have been tested for FHV-1 infection include bovine lactoferrin and cat-mouse chimeric antibodies. Bovine lactoferrin is a mammalian iron-binding glycoprotein that is produced by mucosal epithelial cells and found in secretions including milk, colostrums, saliva, and tears. It has antiviral, antibacterial, and antimycotic properties. It appears to inhibit FHV-1 attachment or penetration into cells and has an inhibitory effect on FHV-1 replication in CRFK cells (12).

Mouse-cat chimeric antibodies targeting FHV-1 glycoprotein D have been used in two separate studies (226, 227). In one study, the antibodies were administered intravenously to cats on day 2 post-infection with FHV-1. The treated cats had decreased clinical signs compared to the control cats (226). In the second study, the antibodies were used prophylactically, administered subcutaneously 15 days prior to infection with FHV-1, and resulted in decrease clinical signs in the treated group (227).

RNA Interference Literature Review

Currently, there is no effective therapy that specifically targets FHV-1.

Vaccination against FHV-1 provides only partial protection from clinical disease (195), and antivirals are minimally effective (212). Therefore, the development of a therapy to specifically target this virus to decrease clinical signs associated with acute infections, to decrease establishment of latent infections, or to prevent recrudescence in latently infected cats to prevent viral shedding should be explored. Such a therapy would be useful for cats in shelters, catteries, or multiple cat households, environments which are known to be associated with an increased risk for FHV-1 infection (75).

Recently, RNA interference (RNAi) has been shown to be a possible therapeutic method for viral infections. RNA interference therapy is sequence specific post-transcriptional gene silencing based on an ancient self-defense mechanism conserved in many eukaryotic species (64).

Discovery: Silencing based on sequence homology was first discovered in transgenic plants and fungi. In 1990, in a study in petunias, attempts to over-express endogenous genes by introduction of additional copies (transgenes) resulted in suppression of endogenous gene expression as well as the transgenes (152). In a study in 1993, infection by an RNA virus was shown to be restricted in plants by artificial expression of one of the viral genes, an observation apparently mediated by cytoplasmic activity that targets specific RNA for inactivation (119). In 1996, quelling (transgene induced gene silencing) in *Neurospora crassa*, was determined to be mediated by an RNA molecule, and silencing was discovered to be associated with a decrease in

messenger RNA (mRNA) from the silenced gene (32). The breakthrough discovery occurred in the nematode, *Caenorhabditis elegans*, in 1998, in which this process of post-transcriptional silencing was linked with double-stranded RNA (dsRNA), and the process was named RNA interference (50).

Cellular machinery and mechanism: Introduction of dsRNA into cells can specifically lead to the degradation of mRNAs containing homologous sequences, resulting in gene silencing (50, 194). RNA interference is initiated by a member of the RNase III family of ATP-dependent ribonucleases called Dicer, which binds with high affinity to double-stranded RNA and chops it into 21-23 nt. small interfering RNA (siRNA) duplexes, containing 19 base-pairs and 2 nucleotide 3' overhangs (14, 48). The siRNA duplexes, containing 3' phosphates and 5' hydroxyls, are incorporated into the RNA induced silencing complex (RISC) (71, 179). For each duplex, one strand becomes the guide strand, and the other becomes the passenger strand. The strand of the duplex that has the 5' end less tightly bound to its complement becomes the guide strand (95, 194). The endonuclease component of RISC, Argonaute, cleaves the passenger strand, and it is removed from the complex, activating RISC, which contains the single-stranded guide (143, 179). RISC then targets homologous mRNA transcripts by base pairing with the guide strand and cleaves the messenger RNA (performed by the Argonaute protein). The mRNA is cleaved between bases 10 and 11 relative to the 5' end of the siRNA guide strand (48). The mRNA is further degraded by exonucleases (70, 98).

Amplification of the silencing signal and systemic spread of silencing occurs in plants and *C. elegans*, but this phenomenon has not yet been demonstrated in mammals (11). In addition to the primary RNAi response, in which double stranded RNA interacts

with Dicer, resulting in 21-nt dsRNAs that interact with RISC to target complementary mRNA sequences, in plants and *C. elegans*, amplification of the trigger RNA occurs. The amplification contributes to the potency and persistence of RNAi in these organisms. Secondary siRNAs are produced in *C. elegans* and in the plant *Arabidopsis* by RNA dependent RNA polymerase (RdRP), which copies the mature mRNA template (11). These secondary siRNAs in *C. elegans* are 21-22 nt in length and have triphosphate 5' termini, unlike primary siRNAs, which have a monophosphate at the 5' end. It is assumed in *C. elegans* that primary siRNAs act as guides to recruit the RdRP to the non-degraded target transcript, resulting in the unprimed synthesis of short antisense RNAs, mainly upstream from the targeted sequence but also downstream, which are removed from the target transcript and incorporated into complexes capable of finding additional targets (168, 199). Currently, there have not been any RdRP genes discovered in mammalian genomes (11).

RNAi in mammalian cells and the interferon response: Long dsRNAs activate RNAi in *C. elegans* (50) and in fruit flies, *Drosophila melanogaster* (71), but initial attempts to activate RNAi in mammalian cells with long dsRNAs failed. dsRNA larger than 30 base pairs in the cytoplasm of mammalian cells can trigger the interferon response (47). However, after the discovery that RNAs 21-22 nucleotides in length mediate RNA interference (48), the use of synthetic 21 nucleotide (nt) siRNAs was demonstrated to induce RNAi in mammalian cells without inducing interferon (47).

Interferons are cytokines that function as the host's first line of defense against viral infections and are part of the mammalian innate immune response. Type 1 interferons (alpha and beta) are produced in response to dsRNA (common viral

replication intermediates). dsRNA binds and activates dsRNA-activated protein kinase PKR and 2', 5'-oligoadenylate synthetase. Activated PKR halts translation by phosphorylation of translation initiation factor eIF2 α (eukaryotic initiation factor subunit α), resulting in a general inhibition of cellular protein synthesis. Activated 2'-5'-oligoadenylate synthetase causes mRNA degradation by activating RNase L in a non sequence specific manner (47). Interferon alpha is primarily produced by leukocytes, and interferon beta is produced by fibroblasts and epithelial cells. The role of interferon induction is to induce antiviral, antiproliferative, and immunomodulatory activities in cells (207).

RNAi as an antiviral defense mechanism: RNAi is an innate antiviral defense mechanism in plants (39) and *Drosophila* (233). The dsRNA develop as a result of replication intermediates or from secondary structure of viral RNA or from DNA viruses by annealing of overlapping complementary transcripts produced by symmetrical transcription (5). An antiviral role for the RNAi machinery appears to also exist in *C. elegans*. Though no natural virus of *C. elegans* is known, experimental infection with vesicular stomatitis virus results in resistance to the viral infection in the nematode (192, 242). It is unclear if RNAi is a natural defense mechanism against viruses in mammalian cells. Because long dsRNA viral replication intermediates appear to be the natural precursors for siRNAs, and long dsRNA induces interferon and general shutdown of transcription in mammalian cells, it is unknown how this process may be naturally activated by viruses in mammalian cells (39, 64). Mammals have also developed a highly sophisticated acquired immune system based on protein recognition that protects against infection in a highly specific manner (64).

miRNA pathway: Naturally occurring siRNAs have not yet been detected in mammalian cells (64). However, related small RNAs, called microRNAs (miRNA) have been discovered in mammalian cells (108, 112). miRNAs result from endogenous non-coding genes distinct from the mRNA they control or come from introns of pre-mRNAs (10). miRNAs were first discovered in *C. elegans* (108, 112), and have also been identified in fish, worms, flies, and plants and are generally conserved. miRNAs are expressed during developmental stages and in a tissue specific manner and have important regulatory roles for development and differentiation in plants and animals (10). Long primary transcripts (pri-miRNA) are cleaved by an RNase III endonuclease, Drosha, in the nucleus, resulting in 60-70 nucleotide stem loop precursors (pre-miRNA). The pre-miRNAs are transported from the nucleus by the export factor, Exportin-5. In the cytoplasm, the pre-miRNAs are processed by Dicer into approximately 22 nucleotides. miRNAs are then incorporated into RISC and regulate mRNA expression by either target destruction or translation inhibition. Partially complementary sequences, predominately in the 3' untranslated region of the target, result in blockage of translation. Complementary results in destruction of the target by RISC, which mainly occurs in plants (10). Apparently siRNAs and miRNAs program RISC equivalently and can have the same effects on mRNA expression, despite different origins (251).

RNAi in human cells likely results from incorporation of siRNAs into the miRNA pathway (70). While *Drosophila* has two Dicer proteins, humans have only one Dicer protein. In *Drosophila*, miRNA and siRNA pathways use separate Dicer proteins. The Dicer protein in human cells resembles the DICER-1 protein in *Drosophila*, essential for miRNA processing. Also, the Argonaute proteins of mammalian cells are related to

Drosophila Ago1, which preferentially binds miRNAs. Mammalian cells lack a homolog of *Drosophila* Ago2, which preferentially binds siRNAs and protects *Drosophila* from infection with RNA viruses (52, 70). This suggests the human genome has retained the miRNA processing machinery, but not the specific machinery for processing long double-stranded RNAs (70).

Viral suppressors of RNAi: If RNAi is a natural defense mechanism against viruses in mammals, then viral infection should result in the production of siRNAs of viral origin, but as previously stated, no naturally occurring siRNAs have been identified in mammalian cells. Also, inhibition of RNAi should result in increased viral replication, and as an adaptive response, the viruses should have developed proteins that inhibit the RNAi response. Each of these criteria has been demonstrated in plants, in which RNAi is an important viral defense mechanism. In an attempt to prove RNAi is a naturally occurring process in mammalian cells, recent studies have attempted to identify RNAi suppressors in mammalian viruses. In nature, the best examples of viral suppressors of RNA interference are found in plant viruses (39).

RNAi suppressor dsRNA binding appears to be a key element in the silencing suppression strategy that has evolved independently many times. Many unrelated plant RNA virus RNAi suppressors bind dsRNA. Many of these suppressors are size selective, preferentially binding 21 nucleotide dsRNA. This is the fragment size generated by Dicer, so expression of a protein that binds these RNAs, preventing their incorporation into RISC to form active silencing effector complexes, is an ideal viral defense strategy. This may also be true for invertebrate virus RNAi suppressors, such as the flock house

virus B2 suppressor, also a double stranded RNA binding protein, which could potentially inhibit viral suppression by RNAi in *C. elegans* (144).

dsRNA binding proteins have been the target of investigation for potential human virus silencers of RNAi. Two such proteins, the non-structural (NS1) protein of influenza A and the E3L protein of vaccinia have been shown to suppress RNAi in experiments in *Drosophila* cells (115). NS1 was shown to bind siRNAs in a plant experiment, suggesting it suppresses RNAi by sequestering siRNAs, a mechanism which occurs in many plants (25). But it has been discovered that influenza NS1 does not suppress RNAi in mammalian cells, though it does inhibit the interferon response (100). Both NS1 and E3L are important for the pathogenesis of these viruses in the natural host because they function as inhibitors of the innate antiviral response regulated by interferon. So the suppression of RNAi by these proteins is likely due to their over-expression in cells not naturally infected with these viruses and to their ability to bind dsRNA (39). Interestingly, a size specific dsRNA binding virus silencing suppressor has not yet been identified in animals (144).

A suppressor of RNAi has also been proposed for HIV-1. The gene Tat encodes an activator of the HIV-1 LTR, and according to some researchers, Tat inhibits Dicer. Therefore, siRNAs cannot be produced and viral RNAs are not targeted (13). However, Tat can also act as a nonspecific dsRNA binding protein and the observed effect is probably due to the massive over-expression of the protein. At physiological concentrations, the protein shows no inhibition of RNAi (39).

Human adenovirus type 5 encodes two non-translated RNA polymerase III transcripts, which act as decoy RNA to prevent the induction of the interferon system in

infected cells. The virus associated (VA) RNAs also appear to inhibit RNAi by acting as competitive substrates for Dicer and are processed into 21nt dsRNAs that are incorporated into RISC. These siRNAs may act more like miRNAs to suppress viral or cellular gene expression during infection (5). VA1 was also shown to compete with cellular pre-miRNAs for transport from the nucleus by the factor Exportin-5 (121). VA1 RNA is highly expressed during viral infection, so the inhibitory effect of this RNA could be inadvertent. Its activity as an RNAi inhibitor may be coincidental based on its structure, which resembles pre-microRNAs. It is unknown if VA RNA specifically evolved to function as an RNAi inhibitor (39).

Cellular miRNA may have a role in antiviral defense in mammalian cells. Human miR-32 inhibits primate foamy virus type 1 in human cells as the result of “fortuitous” near perfect sequence homology between a portion of the virus sequence and the cellular miRNA. The homology is adequate enough to produce virus translation inhibition. Also, the virus produces a protein, Tas, that inhibits the miR-32 translational inhibition, in a non-sequence specific manner (110). However, it remains unclear whether Tas does indeed act as an RNAi suppressor in primate foamy virus infected cells (39).

Experimentally induced antiviral RNAi in mammalian cells: The RNAi machinery likely exists in vertebrates only for post-transcriptional regulation by genome encoded miRNA. Despite this fact, the machinery can be reprogrammed for antiviral immunity by the introduction of exogenous RNA to generate an artificial RNAi response (39). Numerous experiments demonstrate that exogenous siRNAs can prevent *in vitro* and *in vivo* infections with many different viruses, including positive and negative stranded RNA viruses, retroviruses, and DNA viruses (64).

RNA silencing could be used to complement the protein-based immune response to viruses. RNAi occurs in the cytoplasm of cells, where recognition of virus by B and T lymphocytes cannot take place. The RNAi response is rapid, taking only hours to exert its effect; whereas, the immune response, even the anamnestic response, takes days (29). In cases in which virus causes severe acute disease due to significant cytopathic effect resulting from high multiplicity of infection, low immunogenicity, high replication capacity, direct toxic effects, or a combination of each, RNAi could support or enhance the antiviral effects for a short period of time until innate or adaptive immune responses allow the host to overcome the pathogen (94).

Incoming positive-stranded viral RNA may be targeted by RNAi, as is the case for West Nile virus (62) and hepatitis C virus (247). These viruses initially act as mRNA after infecting cells, and the viral target of the RNAi machinery is likely the mRNA (strand that directs protein synthesis) and not viral genomic RNAs (strands acting as templates for replication) (7, 18, 61). This is demonstrated by the superiority of RNAi targeting the positive-strand of a positive single-stranded RNA virus, versus targeting the negative strand replication intermediate (193). Genomic strands kept inside specialized compartments in the cell cytoplasm, such as occurs with rotavirus (7) and West Nile virus (62), are likely inaccessible to the RNAi machinery.

Various phases of the life cycle of viruses have been targeted by RNA interference. Targeting viral transcription factors, such as E6 in the human papillomavirus, results in an inhibition of viral gene expression and inhibited growth in culture (94). Targeting a portion of the influenza RNA transcriptase effectively inhibits production of all viral mRNAs (61). Targeting the 5' untranslated region of hepatitis C

virus, which has an internal ribosomal entry site for translation of the entire viral polyprotein, inhibits protein synthesis. Blocking synthesis of non-structural proteins required for replication of subgenomic viral RNA results in suppression of viral infection (250).

Inhibiting viral replication is likely the best method of suppressing viral infection (94). In many studies, the RNA dependent RNA polymerase of RNA viruses, including SARS (234), West Nile virus (164), and coxsackievirus B3/picornavirus (193) has been targeted to inhibit viral infection. Virus encoded DNA polymerase is also an effective target for inhibiting viral replication of DNA viruses such as herpesviruses (241). Also, silencing of the nucleocapsid gene, which produces a protein necessary for genome RNA replication, has been shown to inhibit influenza replication *in vitro* and in embryonated chicken eggs (61).

Attachment proteins have also been targeted effectively, including the spike protein of SARS (2), and the envelope glycoprotein B of herpes simplex virus type 2 (169). Silencing the attachment protein VP4 of rotavirus has been shown to be effective in reducing infectious viral progeny, whereas all other structural proteins were unaltered (45).

Also, RNAi can be used to target genes whose products are involved in assembly and budding of the virus particle from the plasma membrane. In a study with the Sendai virus, a member of the *Paramyxoviridae* family, targeting of the matrix protein resulted in reduction of virus production, but had no effect on viral replication or transcription (149). Knockdown of viral genes used in late stages of the intercellular viral life cycle,

such as for viral assembly, is expected to be less effective than RNAi in early steps of the viral life cycle (94).

Down regulation of cellular factors associated with viral infections has proven effective for inhibiting replication of HIV-1. However, many molecules are essential for normal cell processes and are inappropriate targets (94).

Methods of siRNA delivery into cells: Effective therapeutic use of siRNAs involves the development of delivery systems that stabilize the siRNAs but not interfere with their gene silencing activity, provide efficient delivery to the target organs, and maintain a siRNA half life sufficient enough to be therapeutic (1). Currently, cationic lipids and polymers are commonly used to transfect cells *in vitro*. However, problems associated with these methods include lack of transfection efficiency and toxicity. A commonly used transfection reagent, Lipofectamine 2000, by itself and complexed with siRNAs is toxic to cells at the doses required for optimal transfection, and use of high concentrations of siRNA results in cellular detachment (203). However, lipid transfecting reagents have been used successfully in *in vivo* studies (169) (19).

Polyethylenimine (PEI), a synthetic polymer containing a high cationic charge, has been used in many studies. PEI is able to complex negatively charged siRNAs, forming colloidal particles which are endocytosed by the cell. The buffering capacity of PEI protects the siRNAs from destruction within the endosomes and the effects of the PEI result in osmotic swelling and disruption of the endosomes, with release of the complexes (1). PEI has been effectively used to deliver influenza virus gene specific siRNAs into lung cells by intravenous administration for therapeutic/prophylactic treatment of influenza in mice (60). But in other studies, PEI used intranasally resulted in

sickness and death in mice (19), and when used intratracheally or intravenously, resulted in severe lung inflammation in mice (114).

Additional methods of delivery to increase transfection efficiency have been studied, including using cell-penetrating peptides, which interact with negatively charged particles on the cell surface and appear to be taken into the cell by endocytosis. The problem with transfection efficiency may not be inefficient uptake of molecules, but the lack of release of bioavailable siRNA molecules from endocytosed vesicles (230).

Early *in vivo* studies used hydrodynamic intravenous injection to force siRNAs into cells. However, this is impractical for use in humans, because it may cause transient right sided heart failure and liver damage by requiring injection of a bolus of liquid equivalent to 3 liters (59). Transfection is good for local delivery of siRNAs, especially for delivery to mucosal surfaces (19, 169). In fact, local delivery of naked siRNAs in cell culture medium (19) or in a dextrose solution (114) into the nasal cavity has been successful for prevention/treatment of respiratory syncytial virus (RSV) in mice and SARS in macaques. Currently, siRNAs delivered by aerosol administration are being used in a phase I clinical trial for RSV (98). However, systemic delivery is more of a challenge. Ideally, the goal is to deliver siRNAs into specific cells to decrease the amount of drug necessary for therapeutic effect and avoid nonspecific silencing in bystander cells. A method for targeted delivery is antibody mediated delivery. The Fab fragment directed against the HIV-1 envelope protein gp160, fused to siRNAs, has been used to deliver siRNAs specifically to cells expressing the HIV-1 envelope protein. This method was successful for targeted delivery both *in vitro*, and *in vivo*, when injected intravenously in mice (202).

Down regulation of target genes with siRNAs is transient because intracellular concentrations are diluted over consecutive cell divisions (98). The effect lasts only 3-5 days in cell culture. This duration of silencing is acceptable for acute infections such as SARS and influenza, but not for chronic infections such as HIV and hepatitis C (94). Silencing can be enhanced by consecutive applications of siRNAs to virus infected cells; however, this can lead to selection of mutants that have changes in the target region (247), especially in chronic infections with RNA viruses that produce quasispecies populations (104).

Stable expression of RNA effector molecules is possible with plasmids or viral vectors. The most common approach uses short hairpin RNAs (shRNAs), which are generated from DNA vectors that express the short hairpin structures, resembling pre-miRNA, from RNA polymerase III promoters (4, 24). Polymerase II promoters have also been used to produce shRNAs that mimic pri-miRNAs (98). The short hairpin structures are processed into siRNAs by Dicer (24). In *Drosophila*, Dicer has been shown to be instrumental in handing over the 21 nucleotide duplexes to RISC, and Dicer substrates, such as shRNAs, are more potent inducers of RNAi than 21mer siRNAs (4). The shRNAs are incorporated into the miRNA pathway, and over expression of shRNAs *in vivo* can be fatal. In one study, shRNAs expressed from a viral vector in mouse liver cells resulted in liver failure and death in the mice. There was associated down regulation of liver miRNAs, presumably due to the competition of the cellular miRNAs with the shRNAs for cellular components of the miRNA pathway, including the nuclear factor exportin-5 (68).

Dicer substrates incorporated into the miRNA pathway beyond the nuclear processing stages may potentially be safer for use *in vivo*. Recently, longer siRNAs, 27-mers, were also shown to be more potent inducers of RNAi. These longer siRNAs are processed by Dicer and therefore, the resultant siRNAs are more efficiently incorporated into RISC (96).

Viral vectors, such as lentivirus or adenovirus vectors, have been used extensively *in vitro* to deliver shRNA plasmids into cells for long term expression of RNA effector molecules. These vectors are currently the best vehicles for effective transfer of genetic material into cells (223). Problems with viral vectors used in the past *in vivo* for gene therapy have included a massive immune response to an adenoviral vector that had disseminated into the circulation of a patient, and oncogenesis (leukemia) in patients by a retrovirus vector that inserted in or near an oncogene. However, improvements have been made in these viral vectors (223). In fact, in an upcoming clinical trial, a lentiviral vector will be used to deliver a shRNA targeting HIV-1 into hematopoietic stem cells *ex vivo* as part of a combinatorial RNA therapy for HIV-1 infection (98).

Toxicity associated with experimental RNAi: Design of siRNAs may be more important for effective targeting than the method of delivery. The thermodynamic properties of siRNA molecules are critical to the functionality of the molecules. These properties facilitate association with RISC and subsequent degradation of target mRNA (95).

siRNAs must be designed to prevent off-target effects, which are toxic to the cell. Off-target effects associated with synthetic siRNAs have been shown to be concentration dependent, with increased concentrations of siRNA leading to a stress response within

the cell. The effects may include stimulation and suppression of expression of genes unrelated to the intended target (171, 196). These effects appear to be sequence-related; siRNAs may cross-react with mRNAs of limited sequence homogeneity. As few as 11 contiguous nucleotides of sequence homogeneity, specifically located in the 5' end of the antisense strand, are capable of cross-hybridizing to transcripts of non-targeted genes, resulting in their silencing. Both the sense and antisense strands are capable of causing off-target effects (83). Thus, siRNAs should be designed to direct the antisense strand (mRNA complement) into RISC (85). In a more recent study, off-target effects were shown to result from homology between sequences of 7-8 nucleotides in the 5' end of the antisense strand and the 3' untranslated region of the unintended mRNA targets. Thus, the off-target silencing occurs in a manner similar to silencing by miRNAs (84). Scrambled siRNAs used as controls may translationally suppress untargeted genes by forming bulging loops and initiate the miRNA mechanism. In functioning like miRNAs with partial homology, mRNA levels of off-target genes may be unaffected, but protein expression is decreased (84). Considering as few as seven nucleotides of homogeneity are all that is necessary to potentially produce off-target effects, specificity in siRNA design is difficult to achieve, and it is unlikely that all off-target effects can be prevented (84). Therefore, observing the same phenotype with multiple individual siRNAs targeting the same gene increases the confidence that the knockdown of the intended gene can be attributed to the observed phenotype (84, 85).

Toxicity may also be due to activation of the innate immune response by siRNAs. Long dsRNA is detected in vertebrate cells by the dsRNA-activated protein kinase PKR and by Toll-like receptor 3. Therefore, as previously mentioned, long dsRNAs cannot be

used as triggers for the RNAi response in mammalian cells, but short siRNAs can be used successfully (47). However, production of siRNAs with a bacteriophage T7 RNA polymerase induces a type 1 interferon response. The response has been shown to result from a 5' triphosphate on the siRNAs, which is used by the polymerase to initiate transcription (97). shRNA plasmid vectors have also been shown to trigger an interferon response. The authors suggest the response may be due to the accumulation of unprocessed polymerase III transcripts that trigger interferon expression, so the lowest effective dose of vector must be used to alleviate the problem (23).

Therefore, chemically synthesized siRNAs represent the gold standard for RNAi applications (4). However, one report suggests 21 nucleotide dsRNAs (including chemically produced siRNAs) transfected into cells can activate PKR in a concentration dependent manner, resulting in interferon beta production and nonspecific suppression in cells (200). A different study suggests siRNAs mediate a type 1 interferon response by binding Toll-like receptor 3, which recognizes dsRNA and induces interferon (93). However, use of naked siRNAs *in vivo* is not associated with much of an interferon response, but when coupled with cationic lipids, a marked interferon (type I and II) response can be generated (122). In two additional studies, siRNAs delivered by lipid transfection reagents induced production of interferon alpha in plasmacytoid dendritic cells *in vitro* and production of interferon alpha and cytokines, IL-6 and TNF α , with activation of T cells and dendritic cells in the spleens of mice (81, 91). It appears siRNA activation of the innate immune response is dependent on the use of delivery vehicles and the recognition of the siRNAs occurs within the endosomal pathway by toll-like receptor 7, which is located in the endosomal membrane and recognizes single-stranded RNA

(81). Therefore, PKR and Toll-like receptor 3, binders of long dsRNA, likely do not represent the major mechanisms by which siRNAs are detected by the innate immune system (81). The activation of Toll-like receptor 7 is dose dependent and sequence dependent. Poly (U) or GU rich sequences can be immunostimulatory, and siRNAs containing a UGUGU motif or a GUCCUCAA motif have been determined to activate the innate immune response (81, 91). Therefore, testing for interferon induction is important before attributing knockdown of gene expression to RNAi in mammalian cells (23).

siRNA design: Many siRNA design criteria have been developed to increase the effectiveness of individual siRNAs and to augment the production of toxicity due to off-target effects, activation of the innate interferon response, or some nonspecific effect. The most important criterion appears to be asymmetry in the stability of the duplex ends. The 5' end of the antisense strand should be less thermodynamically stable than the 3' end (3, 182). This facilitates the use of the antisense strand as the guide strand by RISC (95, 194). The A/U differential of the 3 terminal base pairs at either end of the duplex is important for this asymmetry (3). The 5' end of the antisense strand should be A/U rich, especially the first five nucleotides (182, 225). According to evaluations of siRNAs, functional siRNAs (defined as producing 70% or greater knockdown of the intended mRNA target (3)) have an A/U at position 1 of the antisense strand, but no G/C, and have a G/C at position 19 of the antisense strand, but no U (3, 182, 225). Other single nucleotide bases associated with effective silencing include an A at positions 17 and 14, a U at position 10, and no G at position 7 of the antisense strand (3, 182). Additional recommendations include, a G/C content of approximately 30-53% (3, 182), with absence

of a long G/C stretch of more than 9 nt in length (225), a low melting temperature, and lack of inverted repeats which would lead to hairpin structures in the antisense strand (182). The 3' overhangs have little to no effect on the activity (3). Despite using rational design rules, it is still difficult to predict if a particular siRNA will work. It is expected about 1 out of every 3 siRNAs will give highly effective silencing (66), and some siRNAs that should not work according to the design rules are sometimes the most effective (19).

Viral RNAi escape mechanisms: The mRNA secondary structure also determines siRNA silencing efficiency (103, 240). This was demonstrated in an HIV study in which a point mutation outside the siRNA target sequence resulted in changes in the secondary structure of the mRNA. The mutation caused the target sequence to be incorporated into a hairpin structure, thereby inhibiting its ability to base-pair with the siRNA, resulting in escape from RNAi (240).

As previously mentioned, viruses can also escape destruction by experimentally induced RNAi by developing mutations in the target region. These escape mutants have been described in RNAi experiments with viruses including Polio (65), HIV (40), and hepatitis C (247), appearing in cell culture after a few weeks. One single nucleotide substitution in the central target region has been associated with interference escape (65, 104). Deletion (partial or complete) of the target region has been shown in HIV experiments, as well as accumulation of several point mutations (40). It has been suggested that in some cases, a single point mutation increases the level of resistance of the virus, allowing further selective pressure to accumulate more point mutations, with successive increases in the level of resistance (247). The difference observed in the level of resistance conferred by point mutations is associated with the location of the mutation

in the target sequence. Complete homology within the central region of the targeted sequence is essential for silencing and substitutions at a few positions within the 3' and 5' ends of the target sequence are only partially tolerated (187). Nucleotides 2-8 in the guide strand (antisense strand) are important for target recognition (seed region). The first nucleotide does not contribute to target recognition; instead, it is sequestered in the binding pocket of the endonuclease domain of the Argonaute protein. Nucleotides 9 through the 3' terminal end have reduced importance for target recognition (70).

Targeting conserved sequences in essential viral genes is unlikely to result in deletion of the target sequence as an escape mechanism. This would not likely be maintained because it would not be consistent with life of the virus. Silent mutations are more likely to occur, leading to changes in nucleotides but not amino acids (40). Mutational associated escape is more likely to occur with RNA viruses, which lack proofreading capability in the RNA polymerase (247). The viruses least likely to escape RNAi are DNA viruses due to lower mutation frequencies (64). Development of escape mutants has been shown to be prevented or decreased by using a combination of siRNAs targeting different parts of the viral genome (247). Targeting multiple genes has been shown to be more effective than targeting a single gene (169). However, with use of multiple siRNAs, the RNAi machinery can become saturated, with no enhanced effect (94) or result in degradation of untargeted genes (105).

Feline herpesvirus 1 is a suitable virus for targeting by RNAi. The virus, in the vast majority of cases, produces a localized respiratory infection (37). Therefore, no systemic administration of siRNAs is necessary, eliminating the challenge of targeted delivery. *In vivo* studies have already demonstrated the effective uptake of siRNAs from

mucosal surfaces (169), including effective intranasal delivery of siRNAs for treatment of respiratory viruses (19, 114). FHV-1 is a DNA virus and due to lower mutation frequencies of DNA viruses, escape mutation is not expected to be a significant problem (64). Targeting conserved regions in essential genes is also less likely to lead to mutational escape, and in targeting these genes, replication of the virus can be inhibited (169). Targeting conserved regions also mitigates the problem of sequence diversity between viral isolates (169). Fortunately, FHV-1 has an apparently low rate of genetic variability between isolates (67). Previous studies have shown that targeting essential genes in herpesviruses, including a herpesvirus DNA polymerase gene (241) and glycoprotein B, an attachment protein gene (169), can effectively inhibit herpesvirus replication. The glycoproteins that are essential for FHV-1 attachment and cell penetration have not yet been determined, but the essential herpes simplex virus glycoproteins are gD, gB, and gH-gL (162).

FHV-1 glycoprotein D and viral DNA polymerase genes were chosen as targets for silencing for this study. FHV-1 glycoprotein D, an envelope protein, is the viral hemagglutinin and an important contributor to viral infectivity. This glycoprotein is an inducer of virus-neutralizing antibodies (125, 128). This gene also appears to be highly conserved (243). FHV-1 glycoprotein D may play an important role in the restriction of the host range of the virus to feline cells (131). In the related virus, herpes simplex virus type 1, glycoprotein D is necessary for penetration of the virus into cells (116). It is an essential receptor-binding polypeptide, binding to herpesvirus entry mediator A and nectin-1 receptors on human cells (102). Therefore, like the DNA polymerase gene,

FHV-1 glycoprotein D is expected to be essential for viral replication in feline cells. The hypotheses for this study are as follows:

1. FHV DNA polymerase and gD genes are essential for FHV growth *in vitro*.
2. Silencing of either gene through mRNA destruction by RNA interference will result in decreased viral infection in Crandell-Reese feline kidney cells.
3. Decreased viral infection will result in decreased viral titer, decreased viral mRNAs, and decreased viral proteins.

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**Part 2 Inhibition of feline herpesvirus 1 replication in vitro by
small interfering RNAs targeting the viral DNA polymerase**

Abstract

Feline herpesvirus 1 (FHV-1) is an alphaherpesvirus and produces the most severe upper respiratory disease of cats. Symptoms include an ocular infection that resembles the infection produced by the related virus, herpes simplex virus type 1, in humans. Like all alphaherpesviruses, FHV-1 produces latent infections, and this virus is widespread in the feline population. Vaccines are available but do not prevent infection and antivirals are only minimally effective for treatment of chronic ocular infections in cats. We evaluated RNA interference as a method to prevent FHV-1 infection *in vitro*. Two siRNAs designed to target the viral DNA polymerase were effective in suppressing viral replication. Therefore, RNA interference has potential as an antiviral therapeutic for FHV-1 and this study lays the groundwork for potential *in vivo* investigations in cats.

Introduction

Feline herpesvirus 1 (FHV-1) is a member of the subfamily *Alphaherpesvirinae* and consists of a single linear molecule of double-stranded DNA (31). FHV-1 is widespread in the feline population, with as many as 71% of cats seropositive for this virus (20), and it causes 50% of the cases of upper respiratory disease in cats (23). Of these infections, FHV-1 causes the most severe clinical disease (30). Acute infections are usually localized to the respiratory tract, and clinical signs include pyrexia, ocular and nasal discharge, rhinitis, tracheitis, and depression (7, 22). Primary ocular infection, as occurs in humans with the related virus, herpes simplex virus type 1, consistently

produces conjunctivitis and minimal corneal involvement. Acute signs, though potentially severe, usually resolve in a few weeks (30).

FHV-1 is very fragile in nature and does not survive long outside the host, so transmission requires close contact, especially mucosal contact, and sneezing and short distance droplet spread are important in transmission in large confined populations, such as in breeding colonies and rescue catteries (25). Upper respiratory infections in cats is second only to overcrowding as the leading cause of euthanasia in shelters, and FHV-1 is one of the most important causes of shelter respiratory disease (3).

FHV-1 has been perpetuated in nature as a result of its ability to produce latent infections, and latently infected cats represent the most important reservoir of the virus (11, 24). Approximately 80% of cats infected with the virus become latently infected (11). During periods of stress such as changes in housing, parturition and lactation, or with corticosteroid administration, recrudescence occurs with associated viral shedding, with or without clinical disease. Also, 29% of latently infected cats are spontaneous shedders.(11) Though the majority of latently infected cats do not develop chronic clinical disease (1), there is still a large percentage of adult cats that have this problem (34). The clinical manifestations of disease due to repeated recrudescence, including corneal ulcerations (4), eosinophilic keratitis, or corneal sequestration (26), are significant and can potentially lead to blindness (1).

Antiviral medications approved for treatment of herpes simplex virus type 1 in humans are only minimally effective for treatment of these chronic cases in cats (35). This is possibly due in part to the need for frequent application of the virostatic drugs to be effective and poor owner compliance (35). Vaccines are available for FHV-1, but

because the virus is poorly immunogenic (2), the vaccines do not prevent infection or shedding and only produce partial protection from clinical disease (6). Therefore, development of a new therapeutic for FHV-1 would be beneficial.

Recently, a mechanism called RNA interference (RNAi) has been manipulated for prevention of various mammalian viral infections both *in vitro* and *in vivo* (18). RNA interference is a double stranded RNA-guided gene silencing pathway that is found in a variety of eukaryotic organisms, including yeast, plants, and mammals (for a review, see reference (14)). The double stranded RNA, small interfering RNAs (siRNA), that triggers the pathway can be supplied exogenously to silence specific genes (10, 14).

The best method of suppressing viral infection is inhibiting viral replication (17). Previous studies have shown that targeting essential genes in herpesviruses, including a herpesvirus DNA polymerase gene (37), can effectively inhibit herpesvirus replication. We have shown that siRNA designed to specifically target DNA polymerase gene expression of FHV-1 can significantly reduce viral replication *in vitro*, and this gene is an excellent target for antiviral therapy.

Materials and Methods

Cells and viruses: Crandell-Reese feline kidney (CRFK) cells (ATCC, Manassas, VA) were propagated and maintained in Dulbecco's minimal essential medium (DMEM) (Cambrex, Charles City, IA) supplemented with heat-inactivated 5% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 100 U/ml penicillin, 100µg/ml streptomycin, and Fungizone (Cambrex) at 37°C in a 5% CO₂ incubator. The prototype FHV-1 strain C-27 (ATCC) was used for the study. A calicivirus wild-type strain used in the study and

10 FHV-1 isolates from 2003-2006 were a generous gift from the Clinical Virology Laboratory, University of Tennessee, Knoxville, TN.

siRNAs and transfection: Five siRNAs designed to target the DNA polymerase mRNA of FHV-1 were produced by Ambion- Applied Biosystems (Austin, TX) (Table 2.1). The FHV-1 sequences targeted by the siRNAs are identical in each of the FHV-1 DNA polymerase sequences contained in GenBank and in an additional 10 isolates obtained from cats from 2003-2006. Transfections of 100 nM siRNA per well in six well plates were performed with 5 μ L of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) per sample using Opti-MEM (Gibco-Invitrogen), according to manufacturer's protocol. Approximately 5×10^4 CRFK cells diluted in DMEM supplemented with 10% FBS were added to each well containing the transfection mixtures. Plates were incubated for 24 hours prior to infection with FHV-1. Each siRNA was tested in duplicate, and functional siRNAs were retested for a total of three experiments per siRNA. A non-targeting negative control siRNA was purchased from Ambion. siRNAs targeting the feline GAPDH gene, also obtained from Ambion, were used as a positive control to verify effective siRNA transfection (Table 2.1). Other controls used for each experiment included uninfected/mock-transfected CRFK cells, FHV-1 infected/non-transfected cells, and a type 1 interferon control. The type 1 interferon control consisted of non-transfected CRFK cells infected with feline calicivirus, an RNA virus that activates interferon β in CRFK cells.

Virus infection and plaque assays: The FHV-1 strain C-27 (ATCC) was grown in CRFK cells until 50% cytopathic effect was visible. Plaque forming units were

TABLE 2.1 siRNA designed to target mRNA.

| siRNA | Antisense sequence (5'→3') | Location targeted |
|---------------------------|--|--------------------------|
| Feline GAPDH | Based on GenBank accession AB038241 | |
| GAP 1 | UGCUUCACCACCUUCUUGAtg ^a | 785-767 |
| GAP 2 | UGAGCUUCCCAUUCAGCUCtg | 679-661 |
| GAP 3 | AGAAGCAGGGAUGAUGUUCtg | 624-606 |
| FHV DNA Polymerase | Based on GenBank accession AF079125 (15) | |
| DNA 1 | UGAGUAGCAUAUCUCUUCCTa | 70-52 |
| DNA 2 | GGUCAUAGCUUCUGGAAAAtc | 144-126 |
| DNA 3 | UUUCGACUCCUUCUCUAGUtg | 121-103 |
| DNA 4 | CUAGACUCCACGUAAUGUUta | 95-77 |
| DNA 5 | AGACAUGGGAGAAGACCAUta | 23-5 |

a. Upper case letters represent guide sequence, lower case letters represent 3' overhangs

determined by plaque assay as previously described (8). However, instead of using an agarose overlay, serum from a cat with an IFA titer of >1:2560 (a gift from the University of Tennessee Clinical Virology Lab) was used at a dilution of 1:50. Aliquots of virus were prepared and frozen at -80°C, and each aliquot was used only once for each experiment.

Transfected CRFK cells were infected with FHV-1 at a MOI of 0.1. One hour after incubation, the cells were washed with DMEM and fresh DMEM supplemented with 10% FBS was added to each well. Infected cells were incubated for a total of 48 hours. 500 µL of cell culture medium was removed from each well at 48 hours and stored at -80°C until plaque assays could be performed.

Flow Cytometry: Forty-eight hours after infection, the cells in each test and control well were trypsinized, washed with phosphate-buffered saline (PBS) (Gibco-Invitrogen), and resuspended in 1 mL of PBS per sample. 100µL of each suspension was removed and placed on ice for later RNA extraction. An additional 100 µL of each suspension was removed and processed for Western blot analysis, and the rest of each sample was processed for flow cytometry.

The flow cytometry samples were washed in flow buffer (60 mL 0.5% sodium azide solution, 87 mL PBS, and 3 mL FBS) and the cells were pelleted. The cells from each sample were stained with 200 µL fluorescein isothiocyanate-labeled cat anti-FHV polyclonal antibody (Accurate Chemical and Scientific Corp., Westbury, NY) for one hour on ice. The cells were washed in PBS and were resuspended in 1mL PBS per sample. Cell surface fluorescence was assessed with an Epics XL flow cytometer (Beckman Coulter, Fullerton, CA).

Western blot analysis: Cells were combined with SDS sample buffer and boiled for five minutes. Samples were stored at -20°C until later assayed. Proteins were electrophoresed on 10% polyacrylamide gels and transferred to nitrocellulose membranes (Bio- Rad, Hercules, CA). The membranes were probed with CRFK adsorbed cat anti-FHV-1 polyclonal antibody (Accurate Chemical and Scientific Corp.) or an anti-GAPDH monoclonal antibody (Chemicon International- Millipore, Billerica, MA), and an anti- β actin monoclonal antibody (Ambion) was used as a loading control. Samples were incubated for 1 hour on a shaker at room temperature. The membranes were washed five times and probed with peroxidase labeled goat anti-mouse IgG (GAPDH and beta actin monoclonals) or peroxidase labeled goat anti-feline IgG (FHV polyclonal) secondary antibodies (KPL, Gaithersburg, Maryland) for one hour on a shaker at room temperature. Five additional washes were performed, and proteins were detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). The density of the protein bands was determined with a visible scanning densitometer (Quick Scan 2000, Helena Laboratories, Beaumont, TX).

RNA extraction and real-time RT-PCR: Total RNA was extracted from 100 μ L aliquots of each test and control sample with the RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's protocol. Two on-column DNase digestions (Qiagen) were performed for each RNA sample, and the samples were diluted 1:1000 to reduce DNA contamination. Purified RNA samples were stored at -80°C until tested by real-time RT-PCR. Primers and probes for real-time RT-PCR were designed with Primer3 (32) to detect FHV-1 DNA polymerase and interferon β mRNA (Table 2.2). 28S rRNA was used as a control to standardize RNA concentration (Table 2.2). Primers, probe, and

TABLE 2.2 Primers and probes used to detect mRNA.

| Primer/Probe | Sequence (5'→3') | Location |
|---------------------------|--|-----------------|
| FHV DNA polymerase | Based on GenBank accession AJ224971 (38) | |
| Forward | CGGAGGGAAAATGCTTATGA | 3328-3347 |
| Reverse | ATCCATTCTCTGGGATGCAC | 3486-3467 |
| Probe | TCAATACATACGCTCGCCGATTAGTGGATA | 3387-3416 |
| Interferon beta | Based on GenBank accession AB021707 | |
| Forward | ATTGCCTCAAGGACAGGATG | 237-256 |
| Reverse | CAGGATCGTTTCCAGGTGTT | 454-435 |
| Probe | TTTTCAGTAGAAGCACCTCTAGCACGGGAT | 354-383 |
| 28S rRNA | Based on GenBank accession AF353617 (16) | |
| Forward (16) | CGCTAATAGGGAATGTGAGCTAGG | 663-686 |
| Reverse (16) | TGTCTGAACCTCCAGTTTCTCTGG | 783-760 |
| Probe ^a | AGACCGTCGTGAGACAGGTTAGTTTTACCC | 690-719 |
| GAPDH (28) | Based on GenBank accession AB038241 | |
| Forward | GCTGCCCAGAACATCATCC | 598-616 |
| Reverse | GTCAGATCCACGACGGACAC | 731-712 |
| Probe | TCACTGGCATGGCCTTCCGT | 677-696 |

- a. Probe designed from sequence obtained from real-time RT-PCR product from CRFK cells

RT-PCR cycling parameters for feline GAPDH were previously published (28) (Table 2.2). RT-PCR was performed for each transcript using the SuperScript III Platinum One-Step qRT-PCR kit (Invitrogen). 5 μ L of diluted RNA was used in 25 μ L total volume reactions, which contained 200nM of each probe and 300nM of each primer, and 40U of RNaseOut recombinant ribonuclease inhibitor (Invitrogen). Amplification of FHV-1 DNA polymerase was carried out in a SmartCycler® II (Cepheid, Sunnyvale, CA) with the following parameters: cDNA production at 45°C for 30 min, hot start Taq polymerase activation at 95°C for 2 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 30 seconds. The parameters for interferon β and 28S rRNA were as follows: cDNA production at 42°C (interferon) or 50°C (28S) for 30 minutes, 95°C for two minutes, followed by 45 cycles of 95°C for 15 seconds, 60°C (interferon) or 62°C (28S) for sixty seconds, and 72°C for 30 seconds. Extensive DNA contamination was ruled out by RTase negative controls, which were run with Platinum taq (Invitrogen) instead of SuperScript III. Each set of samples was run with three mRNA standard dilutions, generating curves for mRNA quantitation.

mRNA standards and standard curve production: mRNA standards were produced for FHV-1 DNA polymerase and feline GAPDH by ligating each PCR product into a pCR2.1 plasmid vector, and the recombinants were transduced into *Escherichia coli* (TA Cloning Kit, Invitrogen). The plasmids were isolated (SNAP MiniPrep Kit, Invitrogen), and the identity and orientation of the cloned products were determined by sequencing (Molecular Biology Resources Service, University of Tennessee, Knoxville, TN). The plasmids were linearized (Hind III, Fisher Scientific), and used for *in vitro*

transcription (AmpliCap T7 High Yield Message Maker Kit-Epicentre Biotechnologies, Madison, WI). Following treatment with DNase (Qiagen), the mRNA transcripts were purified (RNeasy Mini Kit, Qiagen). These standards were used to produce standard curves for absolute quantitation of FHV-1 DNA polymerase and feline GAPDH mRNA transcripts isolated from the samples. The RNA concentration and purity of the standards were determined by spectrophotometrical analysis at OD 260/280 (BioPhotometer 6131 spectrophotometer, Eppendorf, Westbury, NY). The number of RNA copies in the sample was estimated based on the molecular weight of the RNA standards and the RNA concentration. Ten fold serial dilutions of the RNA stock were prepared, and aliquots were made and frozen immediately at -80°C . Each aliquot was used only once for real-time RT-PCR.

A standard curve was generated by testing 10-fold serial dilutions of the standard RNA by real-time RT-PCR and the standard curve was generated by the SmartCycler® II software (Cepheid). The intra-assay and inter-assay coefficients of variation of the reactions were determined using dilutions of the standard RNA as previously described (33). To ensure the standard RNA and the target RNA were amplified with similar efficiencies according to the calculation: $\text{Efficiency} = [10^{(-1/\text{slope})}] - 1$ (40), four 10-fold serial dilutions of RNA extracted from a CRFK control well and an FHV infected control well were also prepared and tested in quadruplicate by real-time RT-PCR.

Statistical analysis: The results for the DNA polymerase specific siRNA test and control groups were compared by using univariate ANOVA (SPSS software) because all assumptions were met, based on the Levene's test for equality of variances and the Shapiro-Wilk test for normality. The real-time RT-PCR results for GAPDH specific

siRNA control groups were compared using the Mann-Whitney Test because the results lacked a normal distribution. Univariate ANOVA and a Post Hoc Dunnett T3 test were used to compare controls with GAPDH specific siRNA treatment groups because there were unequal variances between treatment groups.

Results

RNAi of feline GAPDH: In order to determine if RNAi could be performed in CRFK cells with siRNAs transfected with Lipofectamine 2000, an endogenous gene, feline GAPDH, was chosen as a target. CRFK cells were transfected with each GAPDH specific siRNA separately and compared to negative control siRNA transfected cells and mock-transfected cells. Forty-eight hours following transfection, knockdown of GAPDH mRNA was assessed by quantitative real-time RT-PCR. RNA was standardized by 28S rRNA, which had a reaction efficiency of 102% (data not shown). GAPDH mRNA copy numbers were calculated from the standard curve, which was linear over five orders of magnitude (slope = -3.299) and resulted in a theoretical limit of detection of 24 copies (Figure 2.1). The intra-assay variation within the portion of the curve used for mRNA copy calculations was 10-35% based on copy numbers (0.58-1.95% based on Ct values), and the inter-assay variation was 18-43% based on copy numbers (1.15-2.26% based on Ct values). The GAPDH specific siRNA, GAP1, GAP2, and GAP3, resulted in decreased GAPDH mRNA by 86%, 83%, and 93%, respectively, compared to the control, which consisted of an average of the results from the negative control siRNA transfected and mock-transfected CRFK cells (Figure 2.2). The knockdown of GAPDH

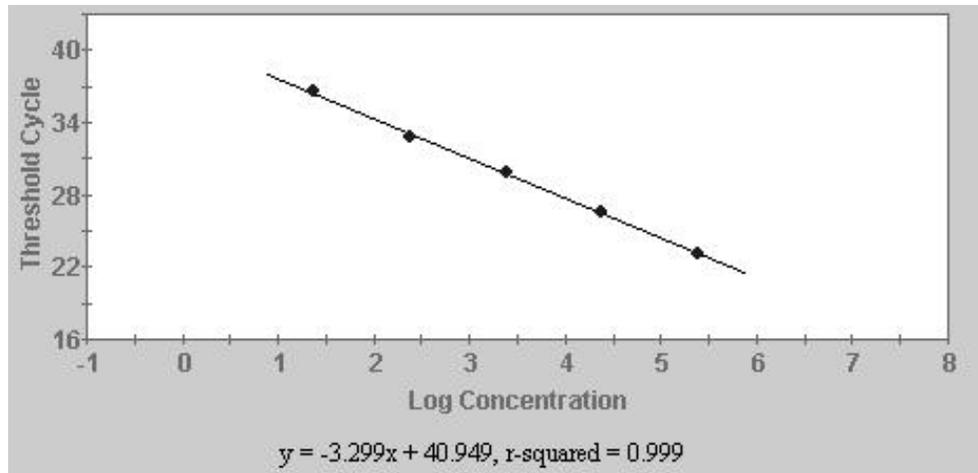


FIGURE 2.1 GAPDH standard curve. The GAPDH standard curve was generated by testing five 10-fold serial dilutions of GAPDH standard RNA, and the theoretical limit of detection was 24 copies. The efficiency of the reaction was 101%, compared to the efficiency of 10-fold serial dilutions of GAPDH mRNA of 102% (not shown).

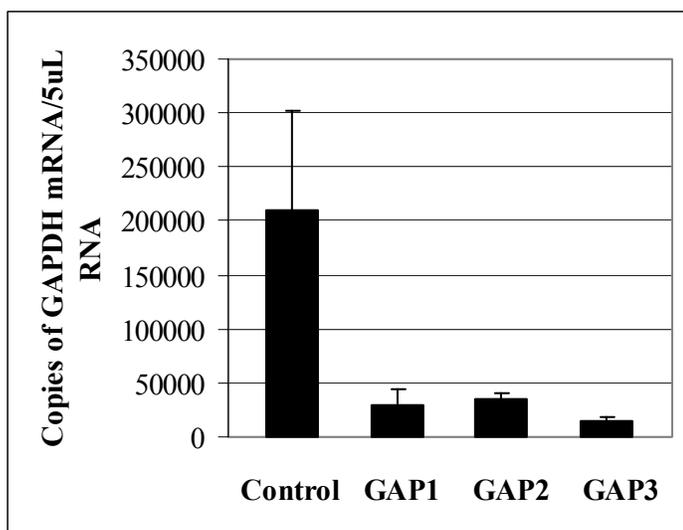


FIGURE 2.2 Knockdown of feline GAPDH mRNA by siRNAs. CRFK cells were transfected with feline GAPDH specific siRNAs (GAP1, GAP2, GAP3) or negative control siRNA. Following 48 hours incubation, total RNA was extracted from cells and tested by real-time RT-PCR with primers specific for GAPDH mRNA or 28S rRNA. The samples were normalized with 28S rRNA and copies of GAPDH mRNA were estimated from a standard curve generated by 10-fold serial dilutions of a GAPDH RNA standard. Three independent experiments were performed and the results show the means of the experiments; error bars represent 1 standard deviation from the means. GAP1 is statistically different than the control ($P= 0.026$), GAP2 is statistically different than the control ($P= 0.029$), and GAP3 is statistically different than the control ($P= 0.019$) based on statistical analysis. Control = average of results from mock-transfected and negative control siRNA transfected CRFK cells (not statistically different, $P= 1$).

mRNA was independent of type 1 interferon production, determined by insignificant interferon β mRNA production in siRNA treated cells (similar to the amount detected in mock-transfected cells) compared to the control, CRFK cells infected with feline calicivirus, an interferon β activator (data not shown).

GAPDH mRNA knockdown also resulted in reduced GAPDH protein expression. GAPDH protein was reduced by 43%, 24%, and 64%, by GAP1, GAP2, and GAP3, respectively, compared to non-treated and negative siRNA treated controls (Figure 2.3).

Knockdown of DNA polymerase mRNA by RNAi: Following the successful knockdown of GAPDH in CRFK cells by RNAi, we next evaluated RNAi directed against the DNA polymerase gene of FHV-1 in CRFK cells. CRFK cells were transfected separately with each of the five siRNAs designed to target the FHV-1 DNA polymerase mRNA or with negative control siRNA in six well plates 24 hours prior to infection with FHV-1, and these samples were compared to a non-transfected, FHV-1 infected control. Forty-eight hours following infection, total RNA was extracted and tested for FHV-1 DNA polymerase mRNA by quantitative real-time RT-PCR. The RNA in each sample was normalized with 28S rRNA. Copies of mRNA were calculated from the standard curve, which was linear (slope = -3.406), spanning six orders of magnitude, and resulted in a theoretical detection limit of 15 copies (Figure 2.4). The results were reproducible, with intra-assays of variation of 6-15% based on copy numbers (0.33-0.91% based on Ct values) and inter-assays of variation of 14-21% based on copy numbers (0.73-1.44% based on Ct values) within the portion of the curve used to calculate DNA polymerase mRNA copies. Two of the five siRNAs tested produced

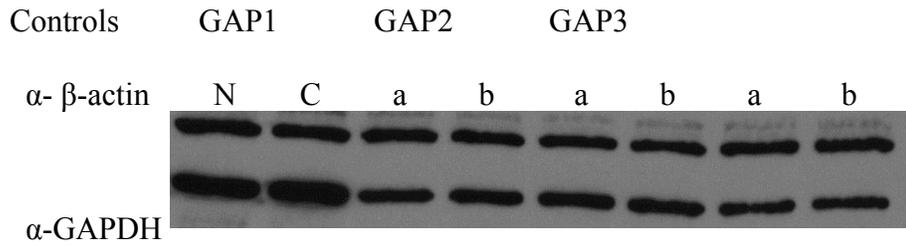


FIGURE 2.3 Reduction of GAPDH protein expression by siRNAs. CRFK cells were transfected with 100 nM each of feline GAPDH specific siRNAs (GAP1-3) and total cellular material was harvested after 48 hours incubation. Western blot analysis was performed with anti-GAPDH and anti-beta actin monoclonal antibodies. Each siRNA was run in duplicate (a,b) with a CRFK control transfected with negative control siRNA (N) and a CRFK mock-transfected control (C). Beta-actin was used as a loading control to normalize protein concentrations, and the density of the protein bands was determined with a visible scanning densitometer. The results of a representative experiment are shown.

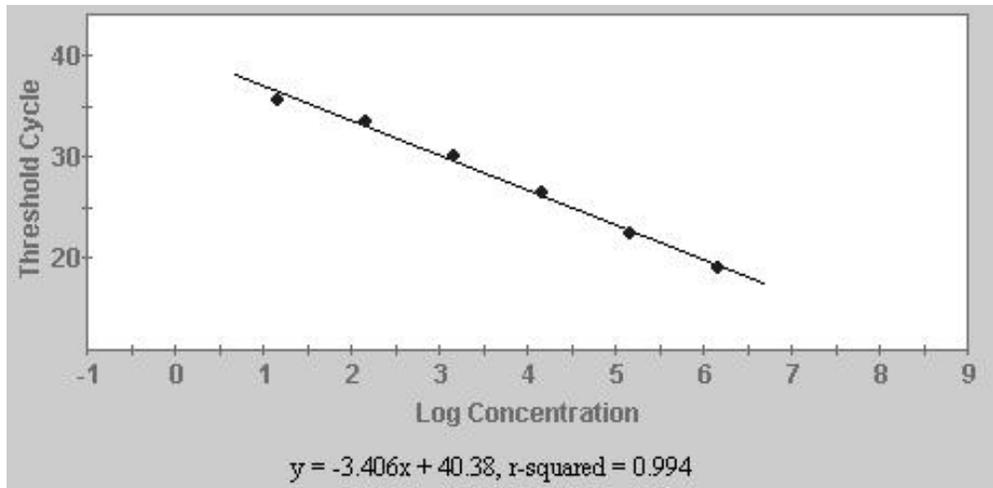


FIGURE 2.4 DNA polymerase standard curve. The FHV-1 DNA polymerase standard curve was generated by testing six 10-fold serial dilutions of DNA polymerase standard RNA, and the theoretical limit of detection is 15 copies. The efficiency of the reaction was 97-100%, compared to the efficiency of 10-fold serial dilutions of DNA polymerase mRNA of 105% (not shown). Therefore, this standard curve was suitable for estimation of copy numbers of DNA polymerase mRNA.

significant reduction in DNA polymerase mRNA compared to the control, resulting in 83% reduction by DNA1 and 69% reduction by DNA3 (Figure 2.5). RNAi knockdown of the DNA polymerase mRNA was independent of type 1 interferon production (Figure 2.6).

Reduction in FHV-1 protein expression by RNAi: Knockdown of FHV-1 DNA polymerase by DNA1 and DNA3 also resulted in decreased expression of FHV-1 proteins in treated cells compared to negative siRNA control treated cells and untreated, FHV-1 infected cells. Total protein was isolated from the infected cells and tested by Western blot (Figure 2.7); cells were also tested by flow cytometry to determine the amount of FHV-1 proteins on the surface of FHV-1 infected, DNA polymerase specific siRNA treated cells versus FHV-1 infected, negative siRNA control treated cells (Figure 2.8). DNA1 reduced cell surface FHV-1 glycoproteins on infected cells by 71% and DNA3 reduced cells surface FHV-1 glycoproteins by 29%, compared to the negative control siRNA treated cells.

FHV-1 DNA polymerase specific siRNAs inhibit FHV-1 replication in vitro: As expected and suggested by the decreased viral protein expression in DNA1 and DNA3 treated cells, RNAi directed against FHV-1 DNA polymerase mRNA inhibited replication of the virus in CRFKs. Infectious FHV-1 was detected in the cell culture supernatants of siRNA treated cells and controls by plaque assay. DNA1 siRNA inhibited FHV-1 replication by 96%, and DNA3 by 83%, compared to the control (Figure 2.9). Inhibition of replication was also evident by the indirect knockdown of the untargeted viral mRNA coding for glycoprotein D (Figure 2.10).

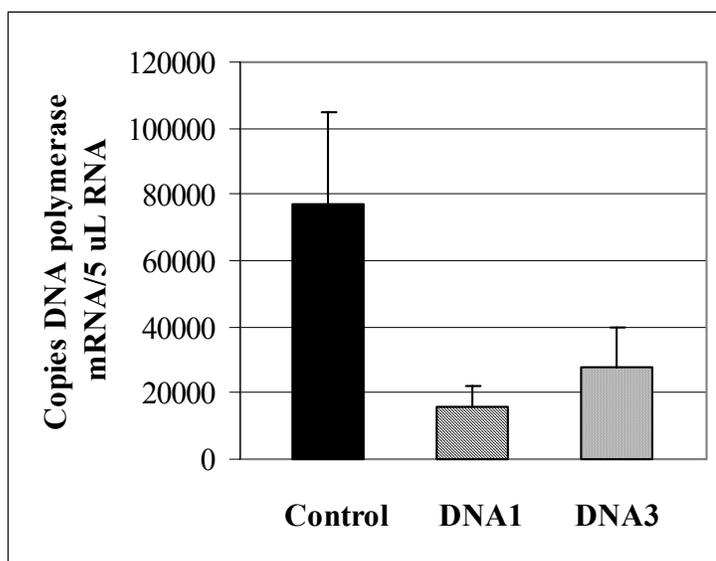


FIGURE 2.5 Knockdown of FHV-1 DNA polymerase mRNA by siRNAs. CRFK cells were transfected with FHV-1 DNA polymerase specific siRNAs (DNA1 and DNA3) or negative control siRNA 24 hours prior to infection with FHV-1 (MOI= 0.1). Following 48 hours incubation, total RNA was extracted from cells and tested by real-time RT-PCR with primers specific for DNA polymerase or 28S rRNA. The samples were normalized with 28S rRNA and copies of DNA polymerase mRNA were estimated from a DNA polymerase standard curve generated by 10-fold serial dilutions of a DNA polymerase RNA standard. Three independent experiments were performed and the results show the means of the experiments; error bars represent 1 standard deviation from the means. DNA1 is statistically different than the control (P= 0.006) and DNA3 is statistically different than the control (P= 0.015) based on univariate ANOVA analysis. Control = average of the results from non-treated and negative control siRNA treated FHV-1 infected cells (not statistically different, P = 0.33)

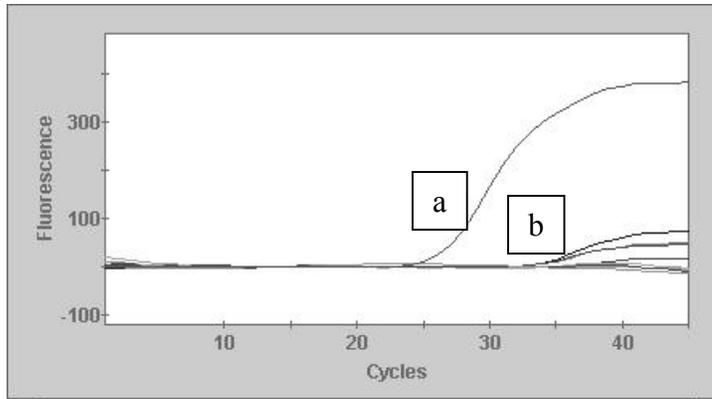


FIGURE 2.6 Interferon β real-time RT-PCR. Knockdown of FHV-1 DNA polymerase mRNA is siRNA specific and not the result of type 1 interferon induction. Interferon β mRNA copy numbers in samples treated with DNA1/DNA3 (b) are similar to the copy numbers detected in the untreated samples (b) and are much lower than the copy numbers detected in cells infected with calicivirus (a), an interferon β inducer.

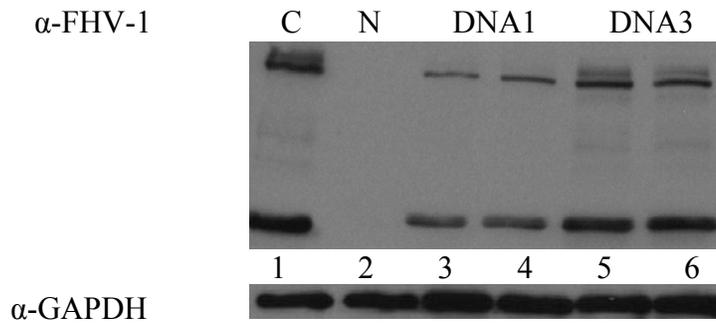


FIGURE 2.7 FHV-1 DNA polymerase specific siRNAs knockdown FHV-1 protein expression. Negative control siRNA (C) or FHV-1 DNA polymerase specific siRNAs (DNA1/DNA3) were transfected into CRFK cells 24 hours prior to infection with FHV-1 (MOI = 0.1). 48 hours following FHV-1 infection, total cellular material was electrophoresed on 10% SDS polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, and membranes were probed with anti-FHV-1 polyclonal antibodies or an anti-GAPDH monoclonal antibody, as indicated.

1- Negative control siRNA treated, FHV-1 infected cells (N); 2-non-treated, non-infected cells (C); 3 & 4- duplicates of DNA1 treated, FHV-1 infected cells; 5 & 6- duplicates of DNA3 treated, FHV-1 infected cells

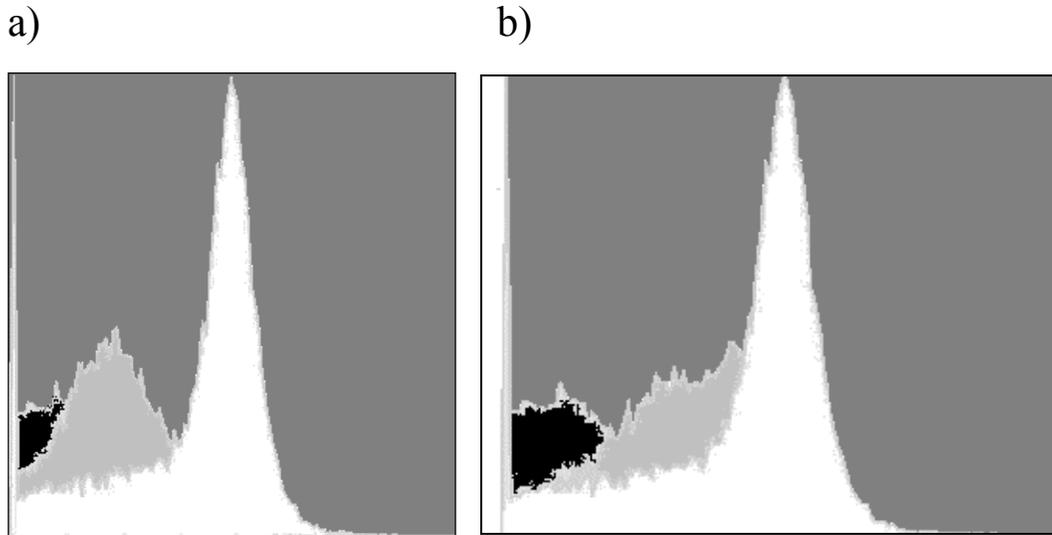


FIGURE 2.8 FHV-1 DNA polymerase specific siRNAs decrease expression of FHV-1 proteins on the surface of infected CRFK cells. Cells were transfected with negative control siRNA, or FHV-1 DNA polymerase specific siRNA, DNA1 (a) or DNA3 (b), 24 hours prior to FHV-1 infection (MOI = 0.1). 48 hours following infection, cells were incubated with anti-FHV-1 polyclonal antibodies. Fluorescence intensity increases from left to right, and 10^4 cells/sample were analyzed. The data are representative of three independent experiments. Color key: black- uninfected CRFKs, gray- FHV-1 infected, DNA polymerase specific siRNA treated CRFKs, white- FHV-1 infected, negative control siRNA treated CRFKs

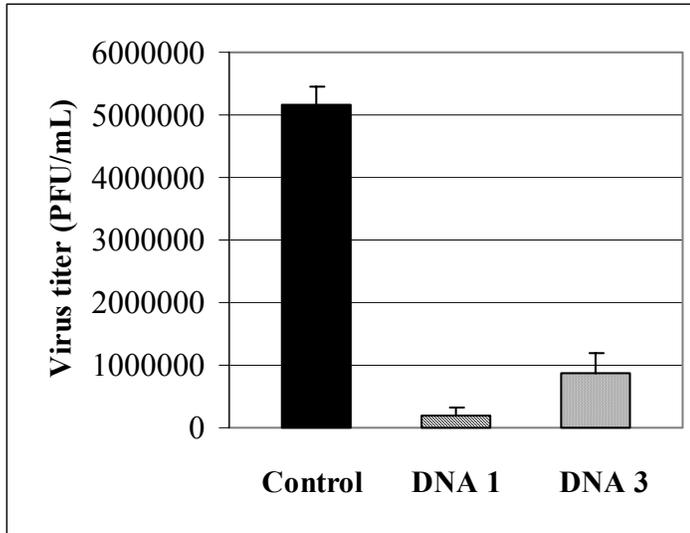


FIGURE 2.9 FHV-1 DNA polymerase specific siRNAs inhibit FHV-1 replication in CRFK cells. Cells were transfected with negative control siRNA, or DNA polymerase specific siRNA (DNA1/DNA3), 24 hours prior to FHV-1 infection (MOI= 0.1). 48 hours following infection, cell culture supernatant was collected and tested in duplicate by plaque assay. The experiment was repeated three times, and the results are shown as averages from the three experiments, with error bars representing 1 standard deviation from the means. The titers from the DNA1 and DNA3 treated samples are statistically different from the titers for the untreated and negative siRNA treated control samples (Control), with P values of <0.001 (univariate ANOVA).

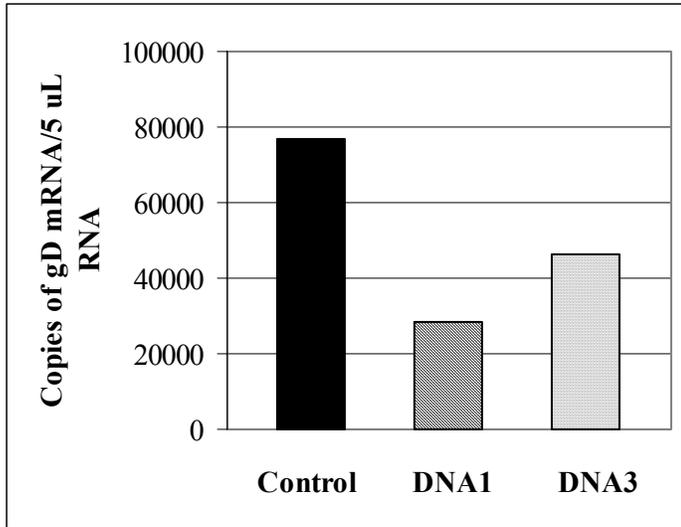


FIGURE 2.10 Decreased FHV-1 glycoprotein D mRNA by DNA polymerase specific siRNA treatment. CRFK cells were transfected with FHV-1 DNA polymerase specific siRNAs (DNA1 or DNA3) or negative control siRNA (C) 24 hours prior to infection with FHV-1 (MOI= 0.1). Following 48 hours incubation, total RNA was extracted from cells and tested by real-time RT-PCR with primers specific for FHV-1 glycoprotein D mRNA (see Part 3). These results are from a single representative experiment.

Discussion

We used RNAi to target the DNA polymerase gene of feline herpesvirus 1 and demonstrated significant suppression of viral replication by knockdown of the DNA polymerase mRNA. Based on the results of this study, the FHV-1 DNA polymerase gene is an excellent target for RNAi anti-viral treatment.

FHV-1 is a suitable virus for targeting by RNAi. The virus, in the vast majority of cases, produces a localized respiratory infection (9). Therefore, no systemic administration of siRNAs is necessary, eliminating the challenge of targeted delivery. *In vivo* studies have already demonstrated the effective uptake of siRNAs from mucosal surfaces (29), including effective intranasal delivery of siRNAs for treatment of respiratory viruses (5, 21). RNAi has also been shown to be effective in treatment of ocular disease (18).

A potential benefit of using RNAi therapy for treatment of chronic FHV-1 ocular infections is the relatively long-term silencing obtained with RNAi. In this study, we showed knockdown that lasted at least 72 hours after a single transfection. Therefore, unlike the current herpesvirus antiviral treatments which must be applied 4-6 times per day to be effective (35), siRNAs could be used much less frequently.

Silencing can be enhanced by consecutive applications of siRNAs into virus infected cells; however, this can lead to selection of mutants that have changes in the target region (39), especially in chronic infections with RNA viruses that produce quasispecies populations (19). FHV-1 is a DNA virus and due to lower mutation frequencies of DNA viruses, escape mutation is not expected to be a significant problem

(12). Targeting conserved regions in essential genes is also less likely to lead to mutational escape, and in targeting these genes, replication of the virus can be inhibited (29).

Targeting conserved regions also mitigates the problem of sequence diversity between viral isolates (29). Fortunately, FHV-1 has an apparently low rate of genetic variability between isolates (13). The region of the DNA polymerase gene targeted in this study codes for highly conserved amino acid motifs of herpesvirus DNA polymerase genes, is unique to each viral species, and lacks DNA sequence drift for alphaherpesviruses, including herpes simplex virus type 2 (36) and feline herpesvirus, as determined in this study.

Development of escape mutants has been shown to be prevented or decreased by using a combination siRNAs targeting different parts of the viral genome (39). Also, targeting multiple genes has been shown to be more effective than targeting a single gene (29). Therefore, a better method of FHV-1 RNAi would also incorporate siRNAs that target another essential gene. One candidate is FHV-1 glycoprotein D (see Part 3).

This study lays the groundwork for potential *in vivo* investigations in cats, the natural host of this virus, and a model that more closely resembles the HSV-1 infection in humans, compared to rabbit or mouse HSV-1 models (27). Such studies may provide unique insights into the prevention/treatment of herpesvirus infections by RNAi.

Acknowledgements

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Part 3 Feline herpesvirus 1 glycoprotein D is essential for in vitro replication and is a potential target for antiviral therapy

Abstract

RNA interference (RNAi), a gene regulatory mechanism conserved in eukaryotes, has been successfully reprogrammed in mammalian cells to inhibit expression of viral genes, functioning in anti-viral defense, similar to its natural biological role in plants. Inhibiting viral genes in infected cells has allowed determination of the role of those genes in infection. In this study, we used RNAi to examine the function of feline herpesvirus 1 (FHV-1) glycoprotein D in the infection of feline cells. We determined that glycoprotein D is necessary for production of infective virions, suggesting FHV-1 glycoprotein D is necessary for viral attachment and/or penetration of host cells, similar to its counterpart in the related virus, herpes simplex type 1. Two chemically produced siRNAs targeting the glycoprotein D gene significantly reduced virus titers in treated cells; therefore, this gene is a potential target for RNAi anti-viral therapy.

Introduction

RNA interference (RNAi) is a RNA-guided gene regulatory mechanism that is found in a variety of eukaryotic organisms, including yeast, plants, and mammals. This cellular machinery is known to be activated by double-stranded RNA (9), which is chopped into duplexes of approximately 21 nucleotides called small interfering RNAs (siRNAs) (8) by a member of the RNase III family of ATP-dependent ribonucleases called Dicer (3). The siRNA duplexes are incorporated into the RNA induced silencing complex (RISC). RISC then targets homologous transcripts by base pairing and cleaves

the mRNA (11). The mRNA is further degraded by exonucleases (for a review, see reference 10).

siRNAs can be produced and delivered to cells to silence specific genes of interest, and chemically produced siRNAs of 21 nucleotides have been used routinely for RNAi experiments since the time they were discovered to initiate RNAi in mammalian cells (7). RNAi can also be activated in mammalian cells by longer siRNAs, 27-mers, which were shown to be more potent inducers of RNAi than 21-mers. These longer siRNAs are processed by Dicer and therefore, the resultant siRNAs are more efficiently incorporated into RISC (17).

RNA interference, initiated by exogenous siRNAs, has been used successfully in previous experiments to determine the function of viral genes (26, 27), and also used therapeutically for mammalian viral infections (reviewed in (16). One method of suppressing viral replication in cells is by silencing viral attachment proteins (1) (30).

Feline herpesvirus 1 (FHV-1) glycoprotein D, an envelope protein, is the viral hemagglutinin and an inducer of virus-neutralizing antibodies (22, 23). This gene also appears to be highly conserved (36). FHV-1 glycoprotein D may play an important role in the restriction of the host range of the virus to feline cells (24). It is unknown if FHV-1 glycoprotein D is a viral attachment protein, but in the related virus, herpes simplex virus type 1, glycoprotein D is an attachment protein and is essential for entry of the virus into cells (20, 21, 29).

We hypothesized that FHV-1 glycoprotein D, like its homolog in herpes simplex virus type 1, is necessary for viral infection. We used both 21-mer and 27-mer siRNAs to knockdown the expression of this gene and have shown that it is likely involved with

viral attachment and/or penetration and is an appropriate target for RNAi anti-viral treatment.

Materials and Methods

Cells and viruses: Crandell-Reese feline kidney (CRFK) cells (ATCC, Manassas, VA) were propagated and maintained in Dulbecco's minimal essential medium (DMEM) (Cambrex, Charles City, IA) supplemented with 5% heat-inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and standard concentrations of penicillin, streptomycin and Fungizone (Cambrex) at 37°C in a 5% CO₂ incubator. The FHV-1 strain used was the prototype strain C-27 (ATCC). A calicivirus wild-type strain used in the study was a generous gift from the Clinical Virology Laboratory, University of Tennessee College of Veterinary Medicine.

siRNAs and transfection: Both 21-mer (Ambion- Applied Biosystems, Austin, TX) and 27-mer (IDT, Coralville, IA) siRNAs were used to target the glycoprotein D gene of FHV-1 (Table 3.1). The 21-mers were designed by Ambion, and the 27-mers were designed with the IDT siRNA design tool. These sequences were compared with known sequences in the GenBank database to decrease off-target effects by avoiding similar sequences in the feline genome. The portion of the FHV-1 glycoprotein D coding sequence targeted by the siRNAs is identical in each of the isolates in the GenBank database, in the prototype strain used in the study, and in an additional 10 isolates (2003-2006) obtained from the Clinical Virology Laboratory, University of Tennessee College of Veterinary Medicine. Negative control 21-mer and 27-mer siRNAs were purchased from Ambion and IDT, respectively. A Cy-5 labeled control siRNA (IDT) was used as a

TABLE 3.1 siRNA designed to target FHV-1 glycoprotein D mRNA.

| siRNA | Antisense sequence (5'→3') | Location targeted |
|---------------------------|---|--------------------------|
| FHV glycoprotein D | Based on GenBank accession D30767 (23) | |
| gD 1 | UGGUUAUA ACUCUUCGAUAUUGUCC uu ^a | 1397-1379 |
| gD 2 | CAUCAUAA UCAAAACCAGUUCAUCG uc | 1351-1333 |
| gD 3 | UUGAUGUU ACAUAACGUACUUCAGC ug | 1076-1058 |
| G1 | AAUCAAA ACCAGUUCAUCG tc ^b | 1351-1333 |
| G2 | AAUAGUGG UAAUCACGAGC tc | 1556-1538 |
| G3 | UUUAUGG UGAGGUUGUGGG tt | 1591-1573 |

- a. Bold letters represent guide sequence subsequent to processing by Dicer, lower case letters represent 3' overhangs
- b. Upper case letters represent guide sequence, lower case letters represent 3' overhangs

transfection control. Transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in Opti-MEM (Gibco-Invitrogen), according to manufacturer's protocol. Five μL of Lipofectamine 2000 was used to transfect 100 nM 21-mer siRNAs per well and 50 nM 27-mer siRNAs per well in six well plates. Approximately 5×10^4 CRFK cells diluted in DMEM, supplemented with 10% FBS, were added to each well containing the transfection mixtures. Plates were incubated for 24 hours prior to infection with FHV-1. Each siRNA was tested in duplicate, and functional siRNAs were retested for a total of three experiments per siRNA. Additional controls used for each experiment included uninfected/non-transfected CRFK cells, infected/non-transfected cells, and an interferon β control, which consisted of non-transfected CRFK cells infected with feline calicivirus, an RNA virus that activates interferon β in CRFK cells.

Plaque assays: FHV-1 strain C-27 was grown in CRFK cells until 50% cytopathic effect was produced. The titer of the virus was determined by plaque assay as previously described (5), with the exception of using an agarose overlay. Instead, serum from a cat with an IFA titer of $>1:2560$ (a gift from the University of Tennessee Clinical Virology Lab) was used at a dilution of 1:50.

Transfected CRFK cells were infected with FHV-1 at a MOI of 0.1. One hour after incubation, the cells were washed with DMEM and fresh DMEM supplemented with 10% FBS was added to each well. Infected cells were incubated for a total of 48 hours. 500 μL of cell culture medium was removed from each well after 48 hours and stored at -80°C for plaque assay. Infective virus titers (PFU) were determined for each well by plaque assay.

Flow cytometry: Prior to infection with FHV-1, (24 hours after transfection) the transfection control well was tested to determine transfection efficiency. The cells were trypsinized and washed in phosphate buffered saline (PBS) (Gibco- Invitrogen) and flow buffer (60 mL 0.5% sodium azide solution, 87 mL PBS, and 3 mL FBS), and resuspended in 1 mL PBS. Transfection efficiency was determined by analyzing intracellular Cy-5 fluorescence with a Beckman Coulter Epics XL flow cytometer (Fullerton, CA). Following FHV-1 infection and 48 hours incubation, the cells in each test and control well were trypsinized, washed with PBS, and resuspended in 1 mL of PBS per sample. 100 μ L of each suspension was removed and placed on ice for RNA extraction, and the rest of each sample was processed for flow cytometry. The flow cytometry samples were washed in flow buffer, divided in half, and the cells were pelleted. Half of each sample was stained with 200 μ L fluorescein isothiocyanate-labeled anti-FHV-1 polyclonal antibody (Accurate Chemical and Scientific Corp., Westbury, NY) and the remainder was stained with the primary monoclonal antibody FHV 7-5, a generous gift from Dr. Chris Grant (Custom Monoclonals International, Sacramento, CA) at a dilution of 1:50 in PBS for 1 hour on ice. The cells were then washed and the cells previously treated with the primary antibody were treated with 2 μ L of the secondary antibody, fluorescein isothiocyanate-labeled F(ab')₂ rabbit anti-mouse immunoglobulin G (Southern Biotech, Birmingham, AL) for one hour on ice. The cells treated with the polyclonal antibody were resuspended in 1 mL PBS per sample and stored at 4°C until analyzed. After a final wash for the remaining cells, they were resuspended in 1mL PBS per sample, and all the samples were analyzed by flow cytometry.

RNA extraction and real-time RT-PCR: Total RNA was extracted from 100 μ L aliquots of each test and control sample with the RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's protocol. The RNA samples were treated with two on-column DNase digestions (Qiagen) and diluted 1:1000 to reduce DNA contamination. Purified RNA samples were stored at -80°C until tested by real-time RT-PCR. Primers and probes for real-time RT-PCR were developed with Primer3 (32) to detect FHV-1 glycoprotein D and interferon β mRNA (Table 3.2). 28S rRNA was used as a control to standardize RNA concentration (Table 3.2). Real-time RT-PCR was performed for each transcript using the SuperScript III Platinum One-Step qRT-PCR kit (Invitrogen) in a SmartCycler[®] II (Cepheid, Sunnyvale, CA). Five μ L of diluted RNA was used in 25 μ L total volume reactions, which contained 200 nM of each probe and 300 nM of each primer. The reaction conditions for glycoprotein D and interferon β were reverse transcription at 42°C for 30 minutes, an initial heat step of 95°C for two minutes to activate the hot start Taq polymerase, followed by 45 cycles of 95°C for 15 seconds, 50°C (glycoprotein D) or 60°C (interferon β) for 60 seconds, and 72°C for 30 seconds. The reaction conditions for 28S rRNA were reverse transcription at 50°C for 30 minutes, 95°C for two minutes, and 45 cycles of 95°C for 15 seconds, 62°C for 60 seconds, and 72°C for 30 seconds. RTase negative controls were run using Platinum Taq (Invitrogen), replacing SuperScript III, to rule out excessive DNA contamination. Each set of samples was run with mRNA standard dilutions to validate mRNA quantitation.

mRNA standards and standard curve production: mRNA standards were produced for glycoprotein D and interferon β by cloning the PCR products into plasmid vectors and transducing *Escherichia coli* (TA Cloning Kit, Invitrogen). Recombinant

TABLE 3.2 Primers and probes used to detect mRNA.

| Primer/Probe | Sequence (5'→3') | Location |
|--------------------------------------|--|-----------------|
| Glycoprotein D | Based on GenBank accession D30767 (23) | |
| Forward | CCTTGATGGAGCTCGTGATT | 1528-1547 |
| Reverse | TCGAATCCTCACTCCCAGAC | 1564-1573 |
| Probe | ACCCTATAACCCACAACCTCACCATAAAGC | 1700-1681 |
| Interferon β | Based on GenBank accession AB021707 | |
| Forward | ATTGCCTCAAGGACAGGATG | 237-256 |
| Reverse | CAGGATCGTTTCCAGGTGTT | 454-435 |
| Probe | TTTTCAGTAGAAGCACCTCTAGCACGGGAT | 354-383 |
| 28S rRNA | Based on GenBank accession AF353617 (12) | |
| Forward (12) | CGCTAATAGGGAATGTGAGCTAGG | 663-686 |
| Reverse (12) | TGTCTGAACCTCCAGTTTCTCTGG | 783-760 |
| Probe ^a | AGACCGTCGTGAGACAGGTTAGTTTTACCC | 690-719 |

- a. Probe designed from sequence obtained from real-time RT-PCR product from CRFK cells

plasmids were isolated (SNAP MiniPrep Kit, Invitrogen), sequenced (Molecular Biology Resources Service, University of Tennessee, Knoxville, TN), linearized (Hind III, Fisher Scientific), and used for in vitro transcription (AmpliCap T7 High Yield Message Maker Kit-Epicentre Biotechnologies, Madison, WI). mRNA transcripts were treated with DNase (Qiagen) and purified (RNeasy Mini Kit, Qiagen). These standards were used to produce standard curves for absolute quantitation of glycoprotein D and interferon β mRNA transcripts isolated from the samples. The RNA concentration and purity of the mRNA standards was determined with a BioPhotometer 6131 spectrophotometer (Eppendorf, Westbury, NY). The numbers of RNA copies were estimated based on the molecular weights of the RNA standards and the RNA concentrations. Ten-fold serial dilutions were prepared, and aliquots of each dilution were stored at -80° C and used only once.

Standard curves were generated by testing dilutions of the standard RNAs by real-time RT-PCR and the standard curves were generated by the SmartCycler[®] II software (Cepheid). The intra-assay and inter-assay coefficients of variation of the reactions were determined using dilutions of the standard RNAs as previously described (34). To ensure the standard RNAs and the target RNAs were amplified with similar efficiencies (based on the calculation: Efficiency = $[10^{-1/\text{slope}}] - 1$) (38), four 10-fold serial dilutions of RNA extracted from an FHV infected control well and RNA extracted from a Calici infected interferon β control well were also prepared and tested by real-time RT-PCR.

Results

FHV-1 glycoprotein D specific siRNAs inhibit glycoprotein D mRNA and protein expression: To examine if glycoprotein D expression could be knocked down by RNA interference, three 27-mer and three 21-mer siRNAs were designed to target five different areas of glycoprotein D mRNA. The siRNAs were transfected into cells prior to infection with FHV-1. CRFK cells were effectively transfected with Lipofectamine 2000, with efficiencies of 95% and greater, based on results from a transfection control siRNA (data not shown).

Quantitative real time RT-PCR was used to determine glycoprotein D mRNA knockdown 48 hours after infection. mRNA copy numbers for each sample were estimated from a standard curve generated by dilutions of standard glycoprotein D RNA. The standard curve spanned five orders of magnitude, and the curve showed linearity over the entire range used for quantitation of mRNA (Figure 3.1). The results were reproducible, with an intra-assay coefficient of variation based on copy numbers of 10-21% (0.5-1.24% based on Ct values) and an inter-assay coefficient of variation of 34-52% based on copy numbers (1.33-3.40% based on Ct values). The theoretical limit of detection was 20 copies of glycoprotein D mRNA.

Of the siRNAs tested, one of the 27-mers (gD1) and one of the 21-mers (G3) were shown to be highly effective, with knockdown of glycoprotein D mRNA by 77% by gD1 and 87% by G3, whereas the rest of the siRNAs were shown to have minimal to moderate effects compared to control samples transfected with negative control siRNAs (Figure 3.2 and data not shown). The least functional siRNAs, producing approximately 30%

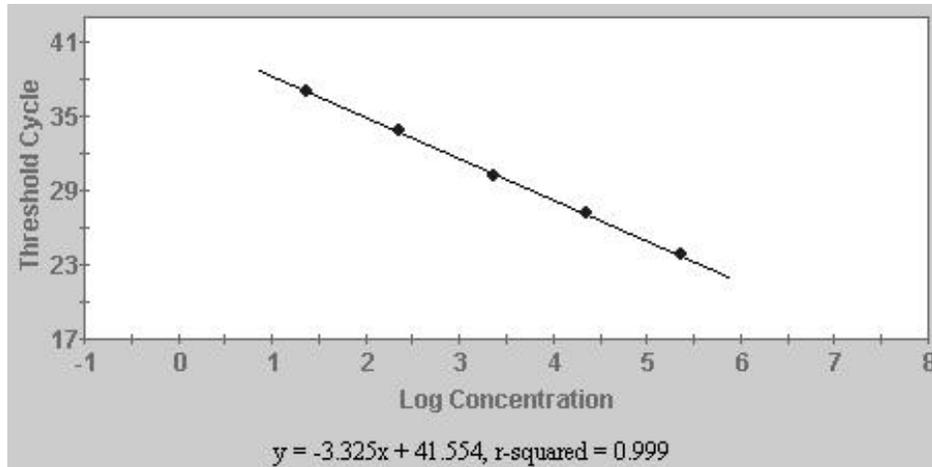


FIGURE 3.1 FHV-1 glycoprotein D standard curve. The curve was generated by testing five 10-fold serial dilutions of glycoprotein D standard RNA. The efficiency of the reaction was 100%, compared to the efficiency of 10-fold serial dilutions of glycoprotein D mRNA of 101% (not shown). Therefore, this standard curve is suitable for estimation of copy numbers of glycoprotein D mRNA.

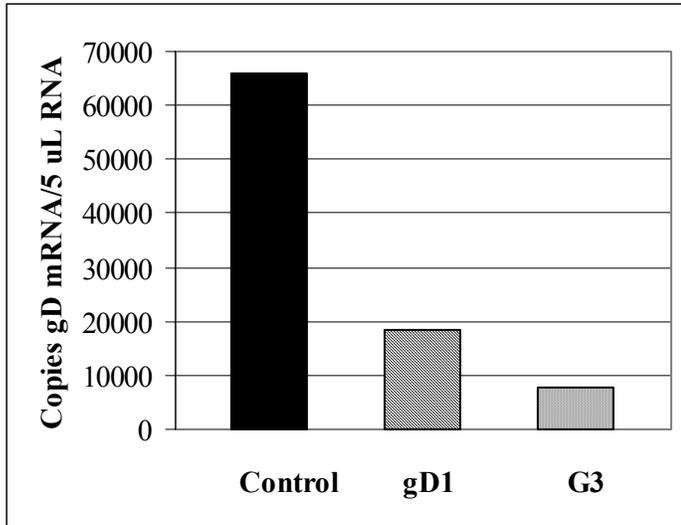


FIGURE 3.2 Knockdown of FHV-1 glycoprotein D mRNA by siRNAs. CRFK cells were transfected with gD1 siRNA, G3 siRNA, or negative control siRNA 24 hours prior to infection with FHV-1 (MOI= 0.1). Following 48 hours incubation, total RNA was extracted from cells and tested by real-time RT-PCR with primers specific for glycoprotein D or 28S rRNA. The samples were normalized with 28S rRNA and copies of glycoprotein D were estimated from the glycoprotein D standard curve. The figure shows the results from a single representative experiment. Three independent experiments were performed with similar results. Control = averaged results from 21-mer and 27-mer negative control siRNA treated FHV-1 infected cells (not statistically different, $P = 0.26$ univariate ANOVA), gD1/G3 = FHV- 1 glycoprotein D specific siRNAs

knockdown each, were the 27-mer gD2 and the 21-mer G1, which both targeted the same region of the coding sequence (data not shown). Therefore, gD1 and G3 were chosen for additional experiments.

gD1 and G3 siRNAs were also tested in cells infected with FHV-1 (MOI=1) 24 hours following transfection and tested for mRNA knockdown 24 hours following infection. G3 was effective under these reaction conditions, however; the 27mer gD1 was not effective (data not shown).

Glycoprotein D protein synthesis was assessed in glycoprotein D specific siRNA treated versus negative siRNA treated cells by flow cytometry. For this test, FHV-1 monoclonal antibodies, donated by Dr. Chris Grant (Custom Monoclonals International), were characterized by Western blot analysis, flow cytometry, virus neutralization, and hemagglutination inhibition assays. One monoclonal antibody (FHV 7-5) was shown to react with an antigen that is approximately 50-60 kDa in size (Figure 3.3), found on the surface of infected cells by flow cytometry, and inhibited hemagglutination of feline cells (Figure 3.4). These results suggested this monoclonal detects FHV-1 glycoprotein D (22, 23). However, this monoclonal did not neutralize virus infectivity in the absence of complement as expected, but this is a variable characteristic based on epitope (23). Therefore, this antibody was used to assess the amount of glycoprotein D present on the surface of FHV-1 infected cells transfected with negative control siRNA or with glycoprotein D specific siRNAs. Knockdown of glycoprotein D by gD1 and G3 siRNAs decreased glycoprotein D protein expression by 27% and 43%, respectively, compared to the negative siRNA transfected control (Figure 3.5).

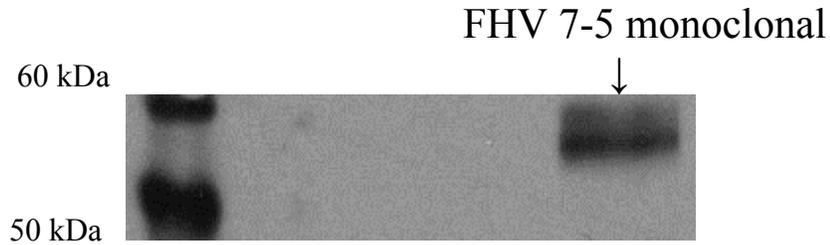


FIGURE 3.3 Detection of FHV-1 glycoprotein D with FHV 7-5 monoclonal antibody. Proteins from FHV-1 infected cells were treated with SDS sample buffer, electrophoresed on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane (Bio- Rad, Hercules, CA). The membrane was blocked overnight in 5% milk at 4°C. The membrane was probed with FHV 7-5 monoclonal antibody for 1 hour at room temperature. The membrane was washed five times with PBS containing 0.05% Tween 20 and probed with peroxidase labeled goat anti-mouse IgG (KPL, Gaithersburg, Maryland) for one hour at room temperature. Five additional washes were performed, and the protein was detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

C 4 8 16 32 64 128

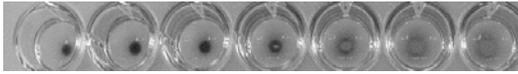


FIGURE 3.4 Inhibition of FHV-1 hemagglutination by FHV 7-5. A

hemagglutination inhibition test was performed with monoclonal FHV 7-5 and treated FHV-1 infected CRFK cells by using 0.5% (v/v) feline red blood cells as previously described (23). C represents the control well, which lacked FHV-1, and the numbers represent the inverse of two-fold serial dilutions of antibody.

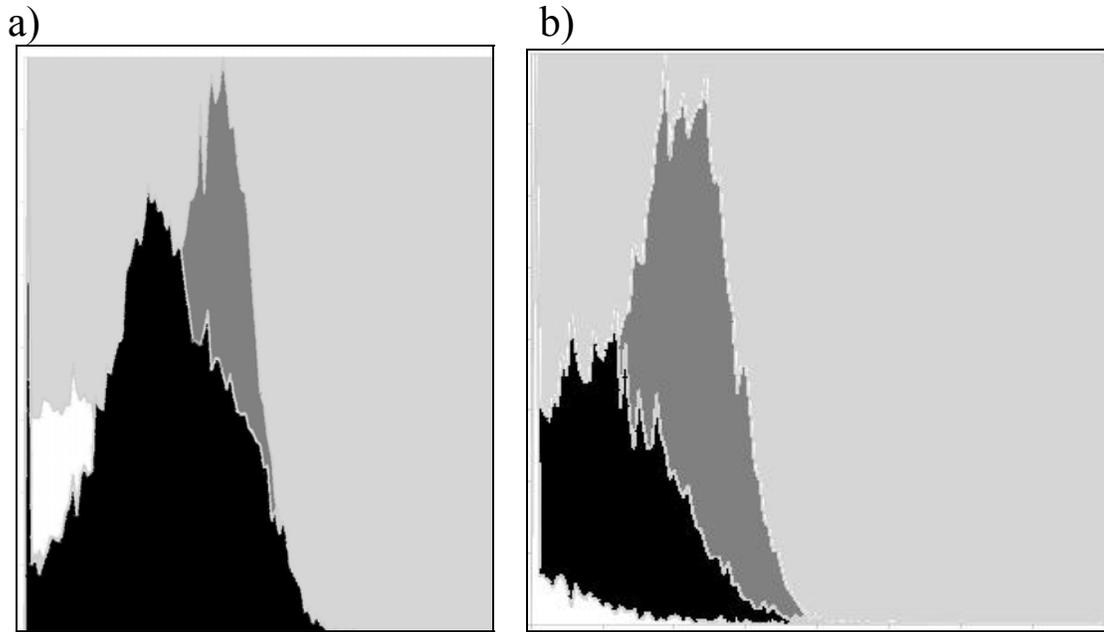


FIGURE 3.5 FHV-1 glycoprotein D specific siRNAs decrease expression of gD protein on the surface of infected CRFK cells. Cells were transfected with negative control siRNA, gD1 (a) or G3 (b) 24 hours prior to FHV-1 infection (MOI = 0.1). 48 hours following infection, cells were incubated with FHV 7-5 monoclonal antibody (gD specific monoclonal) and FITC labeled (ab')₂ rabbit anti-mouse immunoglobulin G. Color key: white- uninfected CRFK cells, black- gD1/G3 treated FHV-1 infected cells, gray- negative control siRNA treated FHV-1 infected cells. Fluorescence intensity increases from left to right, and 10⁴ cells were analyzed/sample. This experiment was repeated three times with similar results.

FHV-1 glycoprotein D specific siRNAs inhibit virus replication: To determine if knockdown of glycoprotein D mRNA affects virus replication, plaque assays were performed to quantify the amount of infective virus released into cell culture supernatants. gD1 was shown to inhibit viral replication by 84%, and G3 inhibited viral replication by 77%, compared to non-transfected and negative control siRNA transfected, FHV-1 infected cells (Figure 3.6)

Glycoprotein D specific siRNAs inhibit FHV-1 DNA polymerase mRNA: Based on the results from the replication study, we expected that interference of glycoprotein D mRNA should also result in an indirect decrease in all viral mRNAs due to replication inhibition. So, the effect of gD1 and G3 treatment on FHV-1 DNA polymerase mRNA copy numbers was determined. The RNA extracted from the cells was tested for FHV-1 DNA polymerase mRNA by real-time RT-PCR with primers specific for FHV-1 DNA polymerase mRNA (see Part 2). Though no significant decline in the DNA polymerase mRNA was detected from samples treated individually with gD1 or G3, when the siRNAs were used together (25 nM gD1, 75 nM G3) to transfect cells 24 hours prior to FHV-1 infection (MOI = 0.1), a decrease in the DNA polymerase mRNA was detected (Figure 3.7 and data not shown).

Glycoprotein D specific siRNAs inhibit expression of additional cell surface proteins: Knockdown of additional FHV-1 proteins by glycoprotein D specific siRNAs was also evaluated. Known glycoproteins on the surface of FHV-1 infected cells in addition to glycoprotein D include glycoproteins B, C, and G, and the complex formed by glycoproteins E and I. Polyclonal antibodies of feline origin were used to evaluate the potential knockdown of additional glycoproteins in flow cytometry experiments. gD1

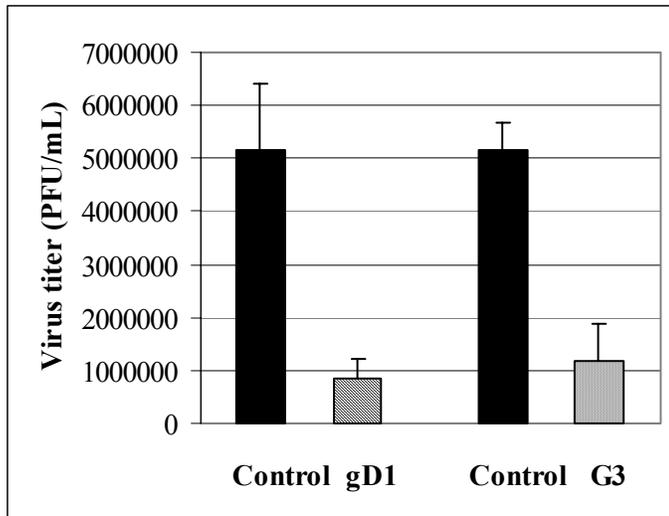


FIGURE 3.6 FHV-1 glycoprotein D specific siRNAs inhibit FHV-1 replication in CRFK cells. Cells were transfected with negative control siRNA, gD1, or G3 24 hours prior to FHV-1 infection (MOI= 0.1). 48 hours following infection, cell culture supernatant was collected and tested in duplicate by plaque assay. The experiment was repeated three times, and the results are shown as averages from the three experiments, with error bars representing 1 standard deviation from the means. The titers from the gD1 and G3 treated samples are statistically different from the titers for the negative siRNA treated and untransfected control samples (Control), with P values of 0.005 and 0.003, respectively (univariate ANOVA).

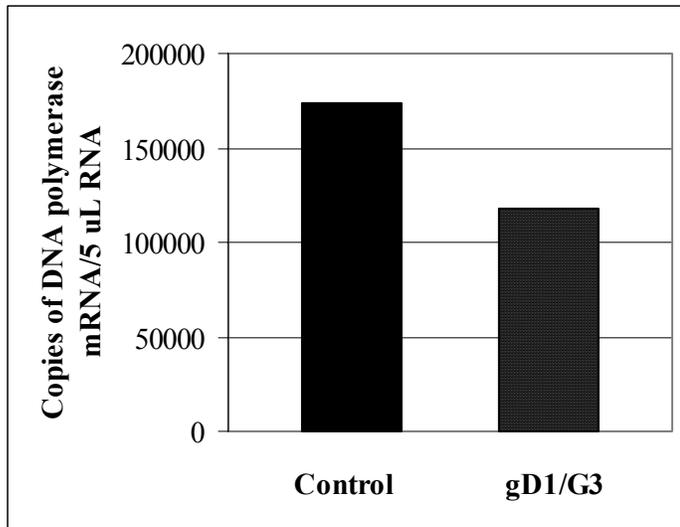


FIGURE 3.7 Indirect decrease of FHV-1 DNA polymerase mRNA by glycoprotein D specific siRNAs. CRFK cells were transfected with FHV-1 glycoprotein D specific siRNAs (25 nM gD1 siRNA and 75 nM G3 siRNA) or negative control siRNAs (25 nM 27mer control, 75 nM 21mer control) 24 hours prior to infection with FHV-1 (MOI= 0.1). Following 48 hours incubation, total RNA was extracted from cells and tested by real-time RT-PCR with primers specific for DNA polymerase mRNA (see Part 2) or 28S rRNA. The samples were normalized with 28S rRNA and copies of DNA polymerase mRNA were estimated from a DNA polymerase standard curve generated from ten-fold serial dilutions of DNA polymerase standard RNA (see Part 2).

and G3, when used independently, did not display much effect on the other glycoproteins; however, when used in combination (25 nM gD1, 75 nM G3), produced significant knockdown in surface FHV-1 glycoproteins on infected cells compared to negative control siRNA transfected, FHV-1 infected cells (Fig. 3.8 and data not shown).

The siRNA effect produced by glycoprotein D specific siRNAs is specific: The effect of virus replication suppression by knockdown of the glycoprotein D mRNA in this study had to be distinguished from non-sequence specific destruction of all viral mRNA transcripts as a result of induction of the type 1 interferon pathway (18). Interferon induction has been ruled out in previous studies in epithelial cells by interferon β detection methods (4, 6). A quantitative real-time RT-PCR assay for detection of interferon β mRNA in feline cells was designed and used to estimate copy numbers based on a standard curve produced from an RNA standard. The standard curve generated by dilutions of the interferon β standard RNA spanned six orders of magnitude, and the curve showed linearity over the entire range used for quantitation of mRNA (Figure 3.9). The results were reproducible, with 7-43% intra-assay variation based on copy numbers (0.38-2.88%, based on Ct values) and 16-41% inter-assay variation based on copy numbers (0.67-2.87% based on Ct values). The theoretical limit of detection was 25 copies of interferon β mRNA. Results from this study showed that the interferon β detected in siRNA treated cells was negligible compared to the control sample (calicivirus infected cells) and similar to the background level detected in infected cells, not transfected with siRNAs (data not shown). Therefore, the suppression of viral replication was the result of the knockdown of glycoprotein D mRNA and not due to type 1 interferon production.

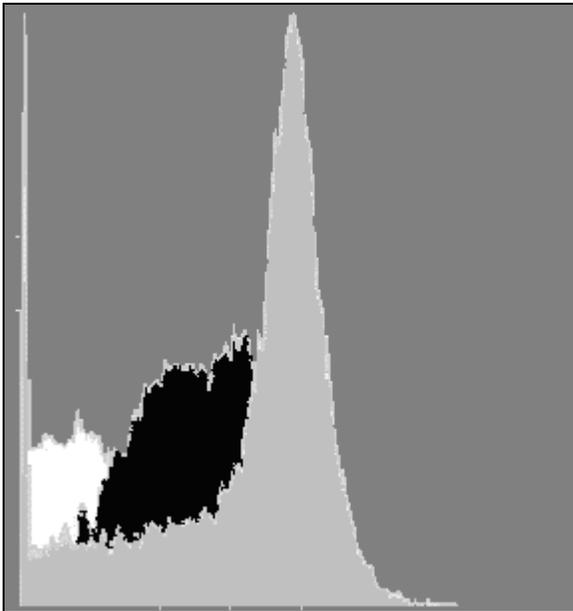


FIGURE 3.8 FHV-1 glycoprotein D specific siRNAs decrease FHV-1 proteins on the surface of infected CRFK cells. CRFK cells were transfected with FHV-1 glycoprotein D specific siRNAs (25 nM gD1 siRNA and 75 nM G3 siRNA) or negative control siRNAs (25 nM 27mer control, 75 nM 21mer control) 24 hours prior to infection with FHV-1 (MOI= 0.1). Following 48 hours incubation, cells were incubated with fluorescein isothiocyanate-labeled anti-FHV-1 polyclonal antibody (Accurate Chemical and Scientific Corp., Westbury, NY). Color key: white- uninfected CRFK cells, black- gD1/G3 siRNA treated FHV-1 infected cells, gray- negative control siRNA treated FHV-1 infected cells. Fluorescence intensity increases from left to right, and 10^4 cells were analyzed/sample.

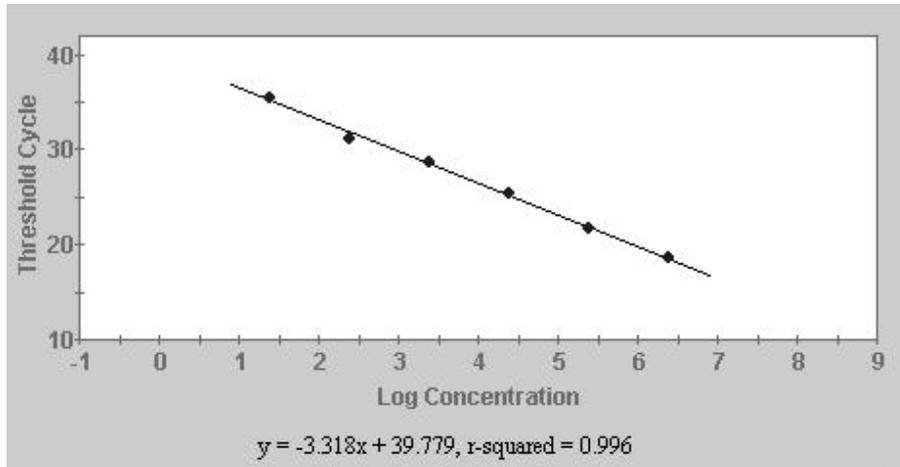


FIGURE 3.9 Interferon β standard curve. The curve was generated by testing six 10-fold serial dilutions of interferon β standard RNA. The efficiency of the reaction was 100%, compared to the efficiency of 10-fold serial dilutions of interferon β mRNA of 96% (not shown). This standard curve was used for estimation of copy numbers of interferon β mRNA, with a theoretical detection limit of 25 copies of RNA.

Discussion

RNAi has been manipulated in previous experiments for the determination of viral gene function (19, 26, 27), and we have shown that RNAi can be effectively used in CRFK cells to knockdown FHV-1 glycoprotein D mRNA when transfected prior to infection, thereby allowing determination of the function of this gene. Knockdown of FHV-1 glycoprotein D mRNA resulted in a decrease in the amount of glycoprotein D and other FHV-1 glycoproteins detected on the surface of infected cells, as well as indirect decrease of FHV-1 DNA polymerase mRNA, independent of the production of type 1 interferon. FHV-1 glycoprotein D, like its counterpart in herpes simplex virus type 1, is essential for producing infectious virions *in vitro*.

Based on these results, we speculate that FHV-1 glycoprotein D is involved with viral attachment and/or penetration. Knockdown of this protein on the viral envelope appears to impair entry of the virus into cells, resulting in a reduction in the amount of infectious virus compared to control samples, but not necessarily the amount of other viral mRNAs and proteins, except when knockdown is enhanced by using a combination of two highly functional siRNAs.

Both 21-mer and 27-mer synthetic siRNAs were used in this study. The 27-mers have been shown to be more potent inducers of RNAi compared to 21-mers and potentially functional at sites refractive to RNAi by 21-mer siRNAs (17). Interestingly, Ambion designed a siRNA that targeted a site also determined suitable by the IDT design tool. However, this particular site was refractory to RNAi by both types of siRNAs. The

Mfold program (25, 39) was used to predict the glycoprotein D mRNA structure, and we discovered that despite the amenable sequence, the target site is likely self-annealed in a helix in its energetically most favorable structure (data not shown), which has been previously shown to inhibit RNAi (35).

Also of interest, the 27-mer siRNA (gD1) was not functional in knockdown of glycoprotein D mRNA 24 hours after infection of cells with a multiplicity of infection of 1, but the 21-mer (G3) was functional. We attribute this to the fact that unlike the 21-mer, the 27-mer has to be processed by Dicer. RNA silencing is generally maximal at approximately 24 hours post-transfection (14). However, in an RNAi study in rotavirus, the highest suppression was found to occur with infection of cells with rotavirus 72 hours after transfection with siRNAs, suggesting some of the elements of RNAi could be induced or activated by the presence of siRNAs, increasing their effective concentration inside the cell (2).

We also discovered the 27-mer siRNAs were toxic to cells in a concentration dependent manner, likely resulting from off-target effects with increased concentrations of siRNA leading to a stress response within the cell (31). Therefore, the 27-mer siRNA (gD1) was determined to be less toxic and functional at a concentration lower than what was used for the 21-mer siRNA (G3).

Off-target effects may include stimulation and suppression of expression of genes unrelated to the intended target (31, 33). These effects appear to be sequence-related; siRNAs may cross-react with mRNAs of limited sequence homogeneity (13). Therefore, observing the same phenotype with multiple individual siRNAs targeting the same gene increases the confidence that the knockdown of the intended gene can be attributed to the

observed phenotype (14, 15). We were able to show the same effects with two different siRNAs targeting two different areas of the glycoprotein D mRNA, strengthening our findings.

FHV-1 glycoprotein D appears to function similarly to its counterpart in herpes simplex virus type 1 and is a suitable target for suppressing FHV-1 viral infection in cells. It is therefore a potential target for anti-viral therapy; although, as a structural protein, it is not transcribed until “late” in infection, and may not be as good a target as a gene transcribed early in the infection process (16, 28), such as the FHV-1 DNA polymerase gene (see Part 2). However, use of siRNAs targeting this gene could be successfully used in combination with siRNAs targeting the DNA polymerase gene, also reducing the potential for development of escape mutants (37), and likely will be more effective than targeting a single gene (30).

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Part 4 General Summary

Feline herpesvirus 1 (FHV-1) glycoprotein D is an important contributor to the immune response to FHV-1 infection, and neutralizing antibodies against this protein are produced in cats during infection (9, 10). The glycoprotein D homolog in the related alphaherpesvirus, herpes simplex virus type 1, contributes to host cell attachment and is necessary for entry of the virus into cells (7, 8, 11). It was hypothesized that glycoprotein D has a similar role in FHV-1 infection and that this gene and the FHV-1 DNA polymerase gene would be appropriate targets to alter viral replication, by inhibiting entry of the virus into the cells (glycoprotein D) or by directly inhibiting replication of the viral genome (DNA polymerase). RNA interference was used to knockdown the expression of FHV-1 glycoprotein D and DNA polymerase genes in CRFK cells. In both experiments, knockdown of the intended target did result in suppression of virus replication *in vitro*.

Knockdown of glycoprotein D mRNA in this study significantly decreased the amount of infectious virus released into the cell culture supernatant. Knockdown of the mRNA with two highly functional siRNAs (70% knockdown or greater) transfected separately was not sufficient enough to knockdown other viral mRNAs, such as the DNA polymerase mRNA, or the expression of other viral glycoproteins. This phenomenon was also seen in a previous study with RNAi of the rotavirus attachment protein, VP4 (3). However, when silencing of the glycoprotein D mRNA was enhanced by using the two siRNAs combined, significantly detectable knockdown of DNA polymerase mRNA and FHV-1 proteins on the surface of infected cells was achieved, with an obvious decrease in the amount of cytopathic effect (CPE) produced in cell culture.

RNAi does not prevent the initial entry of virions into the cells, and by targeting the glycoprotein D gene (a structural protein, transcribed late in infection), the first round of viral replication is unaffected. Knockdown of glycoprotein D expression reduces the amount of the protein incorporated into the viral envelope, altering the infectivity of the virions and presumably inhibiting some but not all from infecting cells. Therefore, when knockdown is enhanced, seemingly more virions are inhibited from infecting cells, resulting in a detectable decrease in the amount of viral mRNAs in addition to the glycoprotein D mRNA detected inside the cells and enough decrease in the number of replicating virions to have a significant suppressive effect on the expression of additional viral glycoproteins. Unfortunately, the amount of viral DNA in the cell culture supernatants was not determined in this study, nor was the amount of infectious virus associated with the infected cells, to corroborate this idea. Based on the findings in this study, though, glycoprotein D is a potential target for RNAi anti-viral therapy.

However, targeting a gene transcribed earlier in viral infection likely will have a more significant affect on virus replication (4). This was shown to be the case in this study. Targeting the DNA polymerase gene of FHV-1, an early gene, resulted in significant reduction in cytopathic effect produced in cells treated individually with two different siRNAs (DNA1 and DNA3). Compared to the controls (FHV infected/ non-transfected and FHV infected/ negative control siRNA transfected cells), which contained 3-4+ CPE, cells treated with DNA1 had 1-2+ CPE and cells treated with DNA3 had 2-3+ CPE. By targeting the DNA polymerase gene, there is a direct reduction of viral replication, potentially starting with the first round of replication. Therefore, though knockdown of the amount of DNA polymerase mRNA by DNA3 was significantly less

than what was achieved for glycoprotein D mRNA by the glycoprotein D specific siRNAs, there was better indirect knockdown of other viral mRNA (glycoprotein D) and viral proteins on the surface of infected cells with this individual DNA polymerase specific siRNA.

For anti-viral therapy, targeting multiple viral genes is likely the best method to prevent production of escape mutants (17), and has been shown to be more effective *in vivo* in a herpesvirus study than targeting a single gene (12). Thus, use of siRNAs targeting both the glycoprotein D and DNA polymerase genes is probably the best strategy for the most efficient suppression of FHV-1 replication, though this experiment was not performed in this study.

In addition to choosing the best genes to target, efficient transfection of siRNAs into cells is important for successful RNAi experiments. Efficient transfection was achieved in this study with Lipofectamine 2000; however, a suboptimal concentration of transfection reagent had to be used to reduce cellular toxicity. The reagent was toxic to cells in a concentration dependent manner (data not shown). Not all toxicity could be eliminated and cells showed mild granularity, with more dead cells in transfected samples compared to non-transfected controls.

Also, transfection was unsuccessful in cells pre-plated in medium containing antibiotics 24 hours prior to transfection (data not shown); so, reverse transfections were used in this study, with cells added to transfection mixtures. It was not determined if it was the antibiotics in the cell culture medium, or the plated cells, or a combination of both that prevented transfection of the plated cells. The antibiotics in the medium during the transfection process did not kill the cells, and addition of antibiotics to the medium

four hours after transfection had no negative effects on the transfection efficiency. However, transfection was shown to be complete after four hours incubation, so antibiotics added at that point would not have affected transfection efficiency anyway.

An additional observation concerning this transfection reagent was that concentrations of siRNA below 50 nM resulted in transfection efficiencies below 95% (data not shown). So, determining the optimal concentration of siRNAs to obtain the highest knockdown with the least amount of cellular toxicity was not possible in the cell culture system used in this study with this transfection reagent. Therefore, a different type of transfection reagent would likely be indicated for future studies.

Design of siRNAs may be more important for effective targeting than the method of delivery of siRNAs into cells (5). Chemically produced 21-mer and 27-mer siRNAs were used in this study. 27-mers were used because they had been shown to be more effective than 21-mers (6). One of six 27-mers (3 glycoprotein D specific and 3 DNA polymerase specific) tested in this study was found to be highly functional (70% or greater knockdown of intended target). Two of the 27-mers targeted the same regions as two of the 21-mers. One of these 27-mers targeted the same sequence as one of the functional DNA polymerase specific 21-mer siRNAs, DNA3. When initially tested, the 27-mer designed to target this area produced 60% knockdown of DNA polymerase mRNA, less than the amount required to be considered highly functional, so it was not tested further (data not shown). So, for this particular target area, the 21-mer seemed to function better, but optimal concentrations of the 27-mer were not determined and the increased potency of 27-mers is really only obvious at subnanomolar concentrations (6), which were not used in this study.

The sequence of each individual siRNA is obviously important to its function because it facilitates the association with RISC and subsequent degradation of target mRNA (5). The most important criterion in siRNA design appears to be asymmetry in the stability of the duplex ends. The 5' end of the antisense strand should be less thermodynamically stable than the 3' end (1, 13). This facilitates the use of the antisense strand as the guide strand by RISC (5, 14). Of the DNA polymerase specific siRNAs tested, the two most effective siRNAs contained 5' ends that were less thermodynamically stable than the 3' ends, and the least functional siRNA (DNA2) had a more stable 5' end.

Also, in this study, as has been shown previously (16), the secondary structure of the target mRNA appeared to be an important determinant of whether some siRNAs would work well. The other region targeted by both a 21-mer and 27-mer siRNA was within the glycoprotein D mRNA. Neither siRNA was functional for this target region, which is predicted to be self-annealed in its energetically most favorable state and likely not accessible to the RNAi machinery. The least functional DNA polymerase specific siRNA tested (DNA2) was also predicted to be completely self-annealed. Whereas, the two most functional DNA polymerase specific siRNAs contained several bases that were in loop structures, including the 3' end of the coding sequence targeted by DNA1, the most effective DNA polymerase specific siRNA in the study.

Despite the complications in designing functional siRNAs, RNAi has been shown to be effective in the prevention/ treatment of infections by several different viruses (4). This study examined prevention of FHV-1 infection *in vitro* and because transfection of cells with siRNAs after infection was not performed, treatment of FHV-1 by RNAi was

not addressed. FHV-1 replicates rapidly in cell culture. Infectious intracellular virus can be detected six hours post infection, and cell-to-cell spread of virus can be detected six to seven hours post infection. Extracellular spread of the virus occurs at nine to ten hours post infection, concurrent with detection of infectious extracellular virus (15). Therefore, it is probable that RNAi, by itself, may not be very effective to treat an established infection, but a combination of RNAi and nucleoside analogs might encounter a synergistic effect (4) for treatment of chronic FHV-1 ocular infections in cats. RNAi might be more useful for prevention of FHV-1 infections in situations associated with increased risk for respiratory disease, such as in breeding colonies and rescue catteries (2). Or, RNAi could potentially be used to prevent the recrudescence of latent infections in chronically infected cats. Regardless, with the current lack of effective treatments for this disease in chronically infected cats, RNAi has merit as a potential FHV-1 anti-viral therapy.

This study lays the groundwork for potential *in vivo* investigations in cats. Such studies may provide unique insights into the prevention/treatment of herpesvirus infections by RNAi.

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Vita

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