REVERSE GENETICS SYSTEM FOR MOUSE HEPATITIS VIRUS STRAIN 1

An Honors Fellows Thesis

by

KRISTEN TAYLOR CARTER

Submitted to the Honors Programs Office
Texas A&M University
in partial fulfillment of the requirements for the designation as

HONORS UNDERGRADUATE RESEARCH FELLOW

April 2011

Major: Biochemistry

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Approved by:

Research Advisor: Julian Leibowitz
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ABSTRACT

Reverse Genetic System for Mouse Hepatitis Virus Strain 1. (April 2011)

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MHV strains are routinely used as models for various diseases. MHV-1, which has been known for some time to be highly pneumotropic, has been shown to reproduce the clinical and pathological symptoms seen in humans infected with SARS in A/J mice, providing a convenient animal model that can be investigated without the restrictions necessary to work with the SARS-coronavirus. A reverse genetic cDNA assembly system was developed for the betacoronavirus mouse hepatitis virus strain A59 (MHV-A59), in 2002. A similar approach was used to assemble a full-length infectious cDNA of MHV-1. Our system was designed so that it would be compatible with the previously-developed MHV-A59 system in order to allow for the creation of chimeric viruses to identify virulence factors in MHV-1. Seven cDNA fragments representing the entire MHV-1 genome will be generated by RT-PCR, long-accurate PCR, and cloning. These fragments are designed such that the restriction site recognition sequences are removed during restriction enzyme digestion, which creates unique 4 base overhangs to allow for sequential ligation of the cDNAs. Mutations that will not affect the coding sequence

were introduced into these plasmid cDNA overhangs to make them compatible with MHV-A59 fragments, which had been previously generated by the Baric lab. Once all seven genomic fragments are cloned, this work will allow for the genetic modification of the entire genome and will make it possible to identify and study the genes that have been hypothesized to be responsible for pneumovirulence in MHV-1 and may lead to more insight to the pathogenesis of SARS-CoV.

DEDICATION

This thesis is dedicated to my friends and family.

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NOMENCLATURE

BAC	Bacterial Artificial Chromosome
CMV	Cytomegalovirus
CoV	Coronavirus
DI	Defective Interfering
FIPV	Feline Infectious Peritonitis Virus
gRNA	Genomic RNA
MHV	Mouse Hepatitis Virus
mRNA	Messenger RNA
ORF	Open Reading Frame
RNA	Ribonucleic Acid
RdRp	RNA-dependent RNA Polymerase
SARS	Severe Acute Respiratory Syndrome
sgRNA	Subgenomic RNA
TGEV	Transmissible Gastroenteritis Virus
UTR	Untranslated Region
WHO	World Health Organization

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CHAPTER I

INTRODUCTION

Severe acute respiratory syndrome (SARS) was first identified in China in November 2002 and quickly spread to other parts of the world including Hong Kong, Vietnam, Taiwan and Canada (28). Although the outbreak was quickly contained due to aggressive measures taken by the WHO, over 8,000 people were ultimately infected with the virus, with almost 800 deaths reported, giving the disease a mortality rate of approximately 10% (63, 130). After investigation by a number of laboratories, it was reported that the etiological agent responsible for the outbreak was a novel coronavirus, SARS coronavirus (SARS-CoV) (63). SARS-CoV is a zoonotic virus that was first transmitted to the human population from infected animals that were present in the live animal markets of Southern China (63). Though there have not been additional human outbreaks since 2004, in 2005 a SARS-like CoV was isolated from bats, demonstrating that the animal reservoir continues to exist (28). In addition, bat feces is traditionally used in Chinese medicine and bat meat is considered a delicacy in the Chinese culture, which means that it is possible that additional human outbreaks may occur in the future as infected bats transmit the virus to civets in wet markets, and people continue in live animal market practices (28).

This thesis follows the style of Journal of Virology

Coronaviruses are large, enveloped, positive-strand RNA viruses that have nonsegmented genomes ranging in length from 26-32 kb (9, 10, 23, 24, 45, 61, 75, 80, 138, 144, 162). Coronaviruses have been isolated from numerous animal species and are responsible for a broad spectrum of diseases (28). Though coronaviruses are prevalent in animals, there are very few animal models which have been able to reproduce the clinical features of SARS (63). Additionally, the few animal-adapted strains of the SARS virus that have been produced require a biosafety level three facility, which hampers their usefulness (63).

Mouse Hepatitis Virus (MHV) is routinely used as a model for human diseases and has the advantage that it can be studied in biosafety level two facilities (28). There are currently no effective therapeutic strategies that have been developed for SARS, which is concerning given the high mortality and morbidity of SARS-CoV infection in humans. This lack in treatment has been attributed to the scarcity of animal models that are able to reproduce the clinical symptoms (63). Recently, De Albuquerque et al reported that MHV-1 reproduces a clinically relevant model of SARS in A/J mice (28). Leibowitz et al subsequently reported a similarity between pathology, cytokine and chemokine responses of SARS-CoV and MHV-1, giving more credence to the possibility of using MHV-1 as an animal model for SARS (82). Phylogenetic analysis has placed SARS-CoV in the betacoronavirus genus, which also include the Mouse Hepatitis viruses (28). Recently the group II coronaviruses, which include MHV, have been renamed betacoronaviruses and are now considered to be a separate genus (29).

Mouse Hepatitis virus is a 31kb coronavirus (11). There are several strains of MHV, all with different tropisms and pathogenicity. As previously mentioned, MHV-1 is highly pneumotropic while MHV-A59 is highly hepatotropic and neurotropic but produces only a modest degree of pulmonary pathology (82). Other strains are predominantly neurotopic (i.e. MHV-JHM), or some combination of the above.

Knowing the structure and gene function of MHV is key to understanding how the virus replicates in its host and can provide insights into many diseases such as SARS, viral hepatitis and demyelinating diseases like multiple sclerosis, depending on the strain of MHV that is being used (119)

Before the advent of reverse genetic systems, many labs utilized DI RNAs as model replicons to investigate the effect of mutation on virus replication. DI RNAs are RNA molecules with extensively deleted sections of the viral genome that replicate in vivo with the assistance of a helper virus (154). One disadvantage of using DI RNAs is the associated risk of homologous recombination between the DI RNAs and the helper virus, which can eliminate the desired mutation (154). An advantage of the reverse genetic approach is that no helper virus is necessary for replication, which minimizes the chance that recombination will occur, resulting in viruses that lack the desired mutation and/or sequence deletion (172). There are three types of reverse genetic approaches that have been developed in the last decade or so: 1) targeted recombination 2) full length infectious cDNA stably maintained in host-vector systems (BAC and Vaccinia) 3)

infectious clones maintained as multicomponent cDNAs. There are advantages and disadvantages to all three systems, however it is generally easier and faster to introduce mutations using the multicomponent system. In this approach, full-length infectious cDNA is created by in vitro ligation of seven component fragments which each terminate in a unique restriction site (172). Once the desired mutations have been made in the specific fragment(s), they are assembled and then transcribed in vitro and the product RNA is electroporated into cells permissive for viral replication (172). Recently Baric and co-workers successfully created a reverse genetic multicomponent model for MHV-A59, a strain of MHV (172). The Weiss Lab has also successfully produced a multicomponent system model for MHV-JHM, which has not yet been published [Personal Communication with Dr. Susan Weiss, University of Pennsylvania].

CHAPTER II

LITERARY REVIEW

Introduction to coronaviruses

Coronaviruses are single-stranded positive-sense RNA viruses that range from 26-32 kb in length (9, 10, 23, 24, 45, 61, 75, 80, 138, 144, 162). They have been isolated from numerous species and are responsible for acute and chronic respiratory, enteric and central nervous system diseases in many species of animals (73, 100). They have also been shown to infect humans and are responsible for 10%-20% of all common colds (1, 155, 164). The name coronavirus is derived from the crown-like appearance of the virus particles in negatively stained electron micrographs (in Latin the word corona=crown) (3).

Coronaviruses have a nonsegmented genome and resemble typical eukaryotic mRNAs as they have both 5' caps and 3' poly a tails (61, 76, 77, 97, 138, 144, 162).

The coronavirus genome contains a variable number of ORFs depending on the species and strain of the virus. The two largest and most 5' ORFSs have been designated OFR1a and ORF1b and occupy up to two-thirds of the genome (19). OFR1a and ORF1a/b

encode proteins that comprise the replicase machinery, which has been hypothesized to play a role in virulence (97, 177).

All coronaviruses have a similar genetic organization in which ORF1a and ORF1b encoded proteins required for RNA replication are 5' to the genes encoding the viral structural proteins (137, 177). The genes for the structural proteins are always arranged in the order S-E-M-N, although they often have so-called accessory genes that are not essential for viral replication in cell culture interspersed amongst them (1).

The 5' UTR is between 200 and 800 nucleotides long for coronaviruses while the 3' UTR is between 200 and 500 nucleotides long. Both the 3' and 5' UTRs have been shown to play a role in viral replication. Coronaviruses are enveloped viruses that are roughly spherical in shape and are approximately 120nm in diameter with 20nm long surface projections or spikes. These spikes, which are made of spike protein, a heavily glycosylated type 1 glycoprotein, give the virus its characteristic appearance, which is illustrated in Figure 1 (6, 100).

The nucleoprotein (N) is a multifunctional phosphoprotein that encapsidates the genomic RNA into a helical nucleocapsid within the mature virion (53, 101). The N protein also plays important roles in virus assembly and is involved in viral RNA transcription as well as viral replication (16, 21, 33, 38, 58, 103, 104, 126, 156, 160). The membrane (M) protein is the most abundant glycoprotein both in the viral envelope and the entire

virus (59). The envelope (E) protein is a minor structural protein and is only present in small quantities in infected cells and the virus envelope, yet it plays an important role in virus production, specifically in envelope formation (25, 40, 70, 90, 112, 174). The spike (S) protein, which is the determinant in host/cell specificity, is also responsible for the attachment of coronaviruses to the target cell, which triggers the fusion of the virus particle with the cell membrane of the host cell, starting the infection (20, 30, 42, 67, 120, 145, 168).

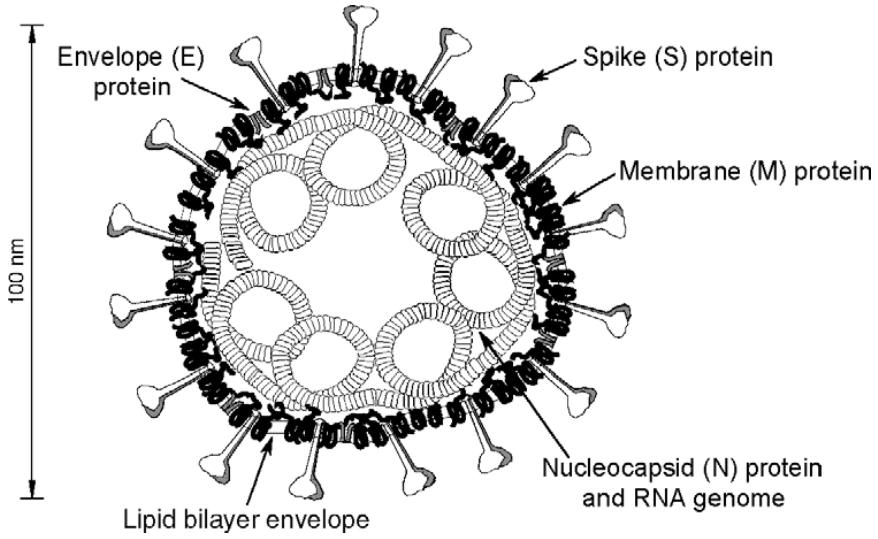

Figure 1 Schematic structure of coronavirus virion (97).

There are also a variable number of other nonstructural and nonessential genes that are found amongst the different species.

The host range of most coronaviruses is very narrow, which is mostly a result of the specificity of the spike (S) protein (55).The viruses usually infect only their natural hosts

and/or closely related animal species, however it has been shown that it is possible to perform cross-species infection in a laboratory setting and the SARS-CoV was able to spread from its presumed natural host in bats to palm civets and a few other species and subsequently to humans (5).

Taxonomy of coronaviruses

The *Coronaviridae* family is a member of the order *Nidovirales*, along with the *arteriviridae* and *roniviridae* families (23, 37). These three families have been placed in the order *Nidovirales*, because they share several common characteristics: 1) gene expression through transcription of a set of multiple 3'nested subgenomic RNAs 2) expression of the replicase polyprotein through ribosomal frameshifting (37, 97). The name nidovirus actually refers to the nested replication as the word nido is Latin for nest.

Coronaviruses can be found, along with *torovirinae or* toroviruses, in the *coronaviridae* family (14, 15, 37). The most distinctive features of this family are: 1) the genome size, coronaviruses have the largest genomes among all RNA viruses, including RNA viruses that have segmented genomes, and 2) the high rate of recombination (97). Due to their high rate of mutation, coronaviruses are considered a significant threat as emerging pathogens because it is easy for them to adapt to new hosts and new niches through mutation of the spike, S, protein as well as undergoing recombination events with other strains (32). To put it into context, coronaviruses have a mutation rate of approximately two mutations per human passage or 0.17 mutations per genome per day (22, 159, 167).

Initially, coronaviruses were divided into three antigenic groups based on serological analysis (54). However, later analysis of phylogenetic relationships and genome organization resulted in the restructuring of the different species within the three original groups (46, 136). Recently the group II coronaviruses, which include MHV, have been renamed betacoronaviruses and are now considered to be a separate genus (29). MHV is therefore a species of betacoronavirus, as is SARS-CoV, and the different isolates of MHV (i.e.MHV-1, MHV-4) are categorized as different strains.

Replication of coronaviruses

The spike (S) protein triggers the fusion of the viral membrane with the host cell membrane (20, 30, 42, 67, 120, 145, 168). There are some strains of MHV that enter endocytically and fuse with the endocytic vesicle to insure the virus nucleocapsid enters the cytoplasm, however MHV-1 fuses with the cell membrane rather than the endocytic vesicle. Once the virus has entered the target cell, it begins to translate viral replication proteins and then to copy the genome in order to access the structural genes needed to make progeny virus. The entire replication cycle takes place in the cytoplasm of the host cell (163). The first step involves the translation of replication proteins from the gRNA, which acts as a eukaryotic mRNA. One of these protein products is an RNA-dependent RNA polymerase essential for viral RNA transcription and replication (97). Positive-strand RNA viruses utilize the RdRp to synthesize negative-strand RNAs that are used as templates for subsequent mRNA and genomic RNA synthesis. The virus specific RdRp

assists both in the synthesis of negative-strand RNA from gRNA and in the subsequent transcription of mRNAs from the negative sgRNAs templates (75).

Coronaviruses are unique in that they use discontinuous transcription to produce the minus-strand template for sg mRNA (133). After the replicase proteins have been translated, the negative-sense RNA is produced from the positive-sense gRNA, starting from the 3' end of the gRNA. Both full and subgenomic length negative-sense mRNAs are generated during translation. As with other members of the nidovirus family, coronaviruses infected cells contain both gRNA and nested sgRNA, which are numbered 1-7 in order of decreasing size (13). The term nested is when referring to the sgRNA of members of the Nidovirus family because all of the sg RNA sequences start at the 3' end of the virus and extend varying distances towards the 5' end, which is shown in Figure 2 below (62, 76, 83).

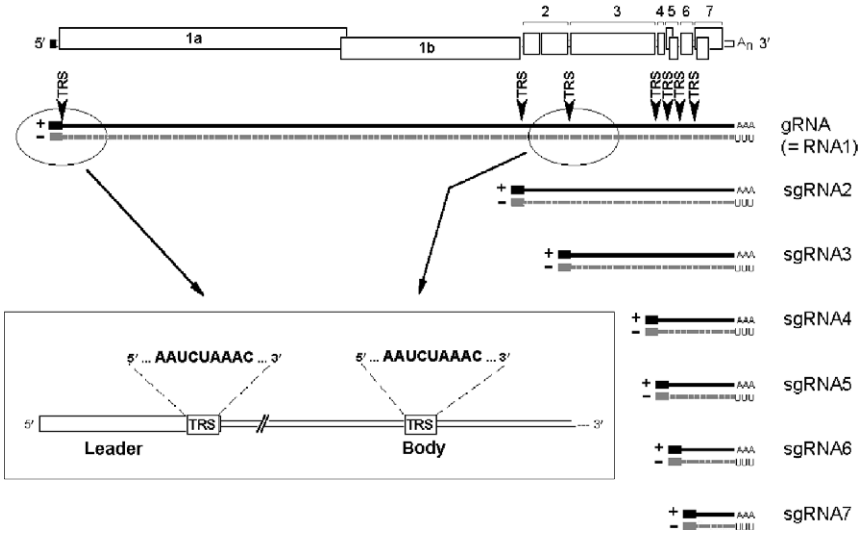

Figure 2. Coronavirus RNA synthesis as represented by MHV (97).

The virus produces the entire nested set arrangement of sg RNA during the replication cycle (4, 134, 135).The position on the genome at which the various sg RNAs end is determined by the presence of transcription regulatory sequences (TRS) at which time the polymerase pauses transcription and may subsequently translocate to the 5' end of the genome to copy a leader sequence of 65-100 nt, or the polymerase may continue to the next TRS site, which is why Nidoviruses are said to replicate by 'discontinuous' transcription (157, 158). This template switching mechanism has not been well-characterized biochemically but functionally the process joins the 5' leader RNA to the body of all 7 sg mRNAs (17, 74, 78, 143). Once the leader sequence has been added, the negative-sense sg mRNAs serve as templates for uninterrupted transcription of positive-sense sg mRNAs, which are produced in excess compared to their negative-sense counterparts (133-135, 158, 172). Although discontinuous transcription has become the accepted model for coronavirus replication, the details of exactly how the leader sequence is added and the specific mechanism by which the virus generates the negative-sense strand templates for sg mRNA synthesis are still under active investigation.

SARS-CoV

Outbreak

Severe acute respiratory syndrome (SARS) was first seen in Guangdong Province, China in November 2002, although it initially appeared as cases of "atypical pneumonia" (113). In February of 2003, the WHO received the first reports of a new respiratory

illness that had broken out in rural areas in China. The new epidemic spread quickly and by the time it was finally contained, persons from 29 countries including Hong Kong, Vietnam, Taiwan, and Canada, had been infected (28, 105, 110, 111, 151). Although the SARS outbreak was contained by July 2003 through the strict isolation of patients and other aggressive measures taken by the WHO and local governments, more than 8,000 people were infected with the virus, along with 778 reported deaths, giving the disease a mortality rate of approximately 10% (63, 111, 130). After investigation by a number of laboratories around the world, it was reported that the etiological agent responsible for the outbreak was a novel coronavirus, which was named SARS-CoV (35, 36, 63, 66, 94, 116, 117, 130, 140). From 2003-2004, there were several laboratory-associated cases of SARS in Singapore, Taiwan, and Beijing, as well as four non-laboratory associated cases that were reported in Guangdong Province in the People's Republic of China (PRC). In the four non-laboratory associated cases, patients identified as having SARS had only mild flu-like symptoms and no secondary transmission (89). The data that was collected by local CDC agencies indicated that rather than being infected by another human, these patients had direct and/or indirect contact with palm civets, which have been shown to be carriers of the virus, although it is not likely that they are the natural animal reservoir (108).

Possible hosts

Both serological and genetic evidence supported the theory of a zoonotic origin of SARS (56, 57). The initial reasoning for this hypothesis was based on epidemiological reports

that correlated SARS infection with exposure to wild game animals in live markets (60, 176).

In an effort to try and identify the host animal of this novel coronavirus, many of the domestic and wild animals in Guangdong Province, including masked palm civets, were examined and swabbed. RT-PCR detected viruses in civet cats (*Paguma larvata*) and the raccoon dog (*Nyctereutes procyonoides*) that were antigenically related to the human SARS-CoV (48). The virus strains that were isolated from these animals have 99% homology with SARS-CoV, although one significant difference between the human and animal strains is that SARS-CoV was shown to have a deletion in ORF 8 (176). At the moment it is unknown what effect, if any, the ORF 8 deletion has on the human virus.

Evidence indicates that the origin of the mild 2003 SARS epidemic was cross-species transmission from masked palm civets to humans, however it is not currently known how the masked palm civets came to be infected; Palm civets have since been shown to be highly susceptible to SARS-CoV infection (48, 60, 142, 161, 165).

Bats have been looked at as a possible host for SARS-CoV because several different species of bats have already been identified as reservoirs for other zoonotic viruses (51, 84, 91, 92). Initially researchers were unsuccessful in finding a SARS-CoV-like viruses in bats, but two independent groups have recently detected SARS-CoV-like virus in Chinese horseshoe bats, making these animals the most likely reservoir for SARS-CoV

(79, 87). Although three different bat species from the same genera were found to be a reservoir of a novel SARS-like coronavirus, Bat-CoV, RNA fragments isolated from this virus only had 41%-62% sequence identities to SARS-CoV (123). Scientists now consider it likely that the emerging SARS-CoV epidemic strain evolved from the zoonotic strain that is naturally occurring and/or maintained in Chinese horseshoe bats and palm civets (35, 48, 66, 79, 87, 116, 123). It is thought that this virus then crossed into humans via an intermediate host species (ex. palm civets and/or raccoon dogs), which were infected while living in close contact with infected bats in live animal markets (48, 88, 142, 153). Though there have not been any more human outbreaks since 2004, SARS-CoV-like virus was isolated from bats in 2005, demonstrating that the animal reservoir continues to exist (28). In addition, a large number of people specializing in the sale of palm civets (72.7%) or wild animals (13.02%) tested positive in serological surveys for anti-SARS-CoV antibodies without showing symptoms, implicating that exposure to the animal precursor of SARS-CoV resulted in asymptomatic infection (48, 52, 166, 173). After a ban on the wild-game animal markets, there were no new naturally acquired human cases of SARS-CoV and there was a reduced detection rate of anti-SARS-CoV antibodies in sera of people in the wild animal business, which gives credence to the hypothesis that if the virus does not successfully adapt to human-human transmission then only a mild or asymptomatic infection will occur as a result of SARS-CoV exposure (85). After observing that 78% palm civets in the Quangzhou wild animal market had positive serum samples for anti-SARS-CoV antibodies, a ban was put on the sale of civets and civet meat, which also led

to a decrease in cases and the theory that caged palm civets served as an amplification host for inter-species transmission of a SARS-CoV-like virus (175).

Reemergence of SARS is a concern to many scientists because of the continued presence of related viruses in bats and palm civets and because of some of the cultural practices prevalent in China; bat feces is traditionally used in Chinese medicine and both bat and palm civet meat are considered to be delicacies in the Chinese culture (28, 108, 109, 175).

Symptoms, vaccines and treatments

While the etiological agent responsible for SARS has been isolated, scientists still are not certain what the mechanism of injury is, although it is considered by many to be a viral pneumonia (34). In addition to the respiratory symptoms, from which the virus derives its name, patients can also experience gastrointestinal symptoms, with 30%-40% suffering from diarrhea as well as splenic atrophy and lymphadenopathy (7, 34, 81, 86, 116, 150). Other clinical features of SARS include fever, dysprea, lymphopenia and a lower respiratory tract infection (107, 152).

Labs around the world are working to develop a vaccine for SARS-CoV, however until an accurate animal model is found progress towards an effective vaccine will be slow. Currently, there are no effective treatments for people infected with SARS-CoV, which is another reason why developing an animal model is crucial (65, 81, 152). So far steroids, antibacterials, ribavirin, herbal medicine, and various other drugs have been

used to treat individuals infected with SARS-CoV, however no drug or drug combination has been successful in stopping the progression of the disease (71). Many times patients must let the infection run its course and simply hope for the best (71).

SARS models

SARS-CoV is a 29.7 kb positive-strand RNA virus and is a member of the family *Coronaviridae* (97).

How to classify SARS-CoV within the context of the coronavirius family has been a source of contention among scientists. After its initial discovery, scientists suggested that SARS be placed in a new group, IV (66, 82, 94, 130). However, based on sequence comparisons and the presence of a domain unique to betacoronaviruses, it was decided that SARS should be placed with the betacoronaviruses, in subgroup b, since it appeared to be related more directly to this group than any other (46, 140).

Currently, there is a dearth of clinical models that accurately (and robustly) reproduce the clinical features/manifestations of SARS (115, 128). In an effort to identify a viable animal model for SARS-CoV pathology, scientists have subjected various animals including cynomologous macaques, African green monkeys, rhesus monkeys, marmosets, domestic cats, and ferrets to experimental infection with SARS CoV. While many of the animals supported SARS-CoV replication, no widely accepted animal model was discovered. The cynomolgus macaque (*Macaca fasciculani*) was one of the

first animals to be experimentally infected with SARS-CoV and was able to satisfy Koch's postulates to a related host, leading to the development of a disease comparable to that in humans (41, 68). Although labs initially reported that the macaques reproduced clinical symptoms that were similar to those seen in humans, several labs have recently shown that the infection seen in the macaques is extremely mild, and produces different symptoms than those commonly seen in human patients (131).

With the uncertainty concerning the viability of the macaque model, scientists began looking at other old world monkeys, such as African green monkeys and rhesus monkeys in addition to macaques as potential animal models (99, 124). However, these labs soon found that although the all of the monkeys could be infected with SARS-CoV, the clinical observations indicated that they did not develop sufficiently severe disease to be any use in evaluating pathogenesis or assessing therapeutic efficacy. Other models have been explored such as the marmoset, the domestic cat, the ferret and the Syrian hamster, however even though infections can be experimentally induced in these animals, none of these models accurately reproduces the clinical features seen in humans infected with SARS (47, 95, 124, 129, 131).

Mice have been considered as a possible model, since they would be less expensive than primate models and can be genetically engineered, which makes it possible to study different aspects of the host immune response (12). However, even though mice could be infected with SARS-CoV, as with other tested animal models they do not exhibit the

clinical symptoms frequently seen in humans (102). Subsequently several mouse-adapted strains of SARS-CoV were developed that produce severe disease in mice (27). Although these mouse adapted strains are being used to study host-pathogen interactions, it requires the use of BSL-3 conditions, which makes it difficult to rapidly and easily observe these interactions due to the necessary biocontainment protocols (102, 127). A phylogenetically similar virus that can faithfully reproduce the clinical symptoms of SARS in a mouse model, that would only require Biosafety level 2 containment facilities and protective measures, would provide significant advantages for studying this disease.

Mouse hepatitis virus (MHV)

Overview

Mouse hepatitis virus (MHV), one of the most extensively studied coronaviruses, is a 32 kb betacoronavirus (11). As a coronavirus, MHV is also a member of the nidovirus order and produces the characteristic 3' nested mRNAs during viral replication (45, 114, 141). There are several different strains of MHV, all of which have different tropisms.

MHV-A59 is highly hepatotropic and neurotropic. MHV-JHM (aka MHV-4) is strongly neurotropic, minimally hepatotropic and poorly pneumotropic. MHV-1 is highly pneumotropic and MHV's 2 and 3 are both highly hepatotropic. Many of the differences

between the pathogenesis and tropisms of these strains can be attributed to the differences in the viral spike (S) protein (26, 105, 106, 121, 122).

It is important to note that several of these viruses are already being used as rodent models for human diseases such as encephalitis, viral hepatitis, and demyelinating diseases like multiple sclerosis (119).

MHV-1

Even though coronaviruses are prevalent in animals, there are very few animal models that have been able to reproduce the clinical features of SARS-CoV infection (63). Additionally, the few animal-adapted strains of the SARS-CoV virus that have been produced require a biosafety level three facility, which hampers their usefulness (63). In addition, adaptation of the SARS-CoV to novel hosts (rodents) results in multiple mutations and these mutations may alter the pathogenesis of the disease. Each animal-adapted strain also has different mutations to make it compatible with a specific animal (i.e. mice, primates, etc.) so even among the adapted strains they may have altered pathogenesis compared to the wild type which is a disadvantage when attempting to use that particular strain as an animal model.

As previously mentioned, there are currently no effective therapeutic strategies that have been developed for SARS-CoV, which is concerning given the potentially lethal effects of virus infection. This lack in treatment has been attributed to a scarcity of accurate

animal models (63). MHV-1, is highly pneumotropic and that is able to accurately reproduces the lung pathology that is seen in humans infected with SARS-CoV (28). This discovery is significant because it indicates that MHV-1 may be able to serve as a model for SARS. Using MHV-1 as a model of infection would also be advantageous because MHV can be studied safely in biosafety level two facilities.

MHV-1 and SARS-CoV are close phylogenetically, which is one reason why SARS-CoV was classified as a betacoronavirus, although it has been placed in subgroup b while MHV is in subgroup a (140). The viruses are also similar in both their genome organization and their individual mechanisms of replication (140, 147). Due to these similarities and the similar pulmonary pathology caused by infection with these two viruses it is likely that many of the viral genes associated with inducing severe lung disease in MHV-1 infected mice will also be associated with severe lung damage in SARS-CoV infection in humans.

The strain of mice used when looking at MHV-1 infection is significant, because as Khanolkar et al demonstrated, the adaptive immune response to MHV-1 acts as a double-edged sword: in resistant strains of mice, it helps mediate protection while in susceptible mouse strains it will contribute to the observed pathology (63). It has been shown that intranasal infection of A/J mice with MHV-1 induces clinical features and pathology that are comparable to what is seen in humans infected with SARS-CoV (28, 82).

Reverse genetic systems

The first reverse genetic system for a positive strand RNA virus was developed for the Polio virus in 1981 and provided the basis for the present day reverse genetic systems for coronaviruses and other viruses (125). There were however, several obstacles that needed to be overcome before these systems could be developed for the coronavirus family.

Obstacles

The two main barriers that needed to be overcome were: 1) the large sizes of coronaviruses and 2) the high instabilities of various regions of the replicase gene when cDNAs containing these genes were cloned into plasmid vectors and propagated in *E. coli* (98).

Background

Reverse genetic systems provide many advantages for studying coronavirus genetics, beyond just their ability to elucidate the function and/or importance of various genes. They have helped to advance our understanding of coronaviruses. Reverse genetic systems have been used to: 1) investigate the structure/function relationship of the UTR's at both the 3' and 5' ends of the genome, 2) discover the roles of enzymatic activities encoded in the replicase gene, and 3) they have been useful in work centered toward creating attenuated vaccines for a multitude of viruses (8, 18, 31, 39, 43, 49, 93, 132). Reverse genetic systems are also convenient because they allow the viral genome

to be directly manipulated and linked to a resulting phenotype. Reverse genetic systems can be broken down into three categories: 1) targeted recombination 2) full length infectious cDNA expressed in stable host-vector systems and 3) infectious clones amplified as multicomponent cDNAs (32).

Targeted recombination

Targeted RNA recombination, first developed in MHV, takes advantage of the recombination rate in coronaviruses (72, 96). Synthetic donor RNA, that contains the mutation of interest and a selectable marker, is introduced into cells that have previously been infected with a recipient parent virus (96).

One of the earliest uses of targeted RNA recombination involved a recipient parent virus MHV mutant that was thermolabile due to an internal deletion in the N gene (64, 118). Cells infected with the parental virus were transfected with donor RNA that did not contain the deletion in the N gene and recombinant viruses that arose were selected at elevated temperature. Viruses that formed large plaque at the nonpermissive temperature, had restored the deletion in the N gene.

Since this novel discovery, various elements have been added to the MHV recombination system to increase the efficiency. The alteration that had the greatest impact on the strength and efficacy of the system was the construction of the interspecies coronavirus mutant, fMHV, Figure 3 (69). In this model, the efficiency limitations of the

original system were overcome by taking advantage of the high degree of species specificity conferred by the ectodomain of the S protein (69). In order to create the fMHV chimera, the S protein ectodomain of MHV was replaced with the S protein ectodomain of FIPV, feline infectious peritonitis virus, which is a feline coronavirus (69). The targeted recombination of the S protein ectodomains conferred a selective advantage and provided a way to screen for viruses containing the desired sequence, as these viruses could only grow on feline cells after the spike protein domain exchange. Once the feline version of MHV, fMHV, was generated then the reverse process can then be employed whereby the mouse S protein, and any desired mutation, would be recombined into progeny viruses. The progeny are then screened for the ability to grow on mouse cells, which indicates that these viruses have correctly recombined with the mouse spike and any mutation downstream of S. This system was revolutionary in that it allowed for the selection of recombinants having almost any nonlethal MHV mutation, as long at the mutation was contained in the 3' most 10 kb of the genome (32).

Although targeted RNA recombination has been successful in isolating nonlethal MHV mutants in genes extending from the S gene to the 3' UTR of MHV, it does not provide access to genes upstream of the S gene, leaving only 1/3 of the genome available for analysis (50). The other two thirds of the genome consists of ORF1a and ORF1b, which contain the replicase genes. These genes have been hypothesized to contain sequences responsible for the viruses' virulence factors, so it is of great interest to researchers to be able to look at the entire MHV genome (97, 177).

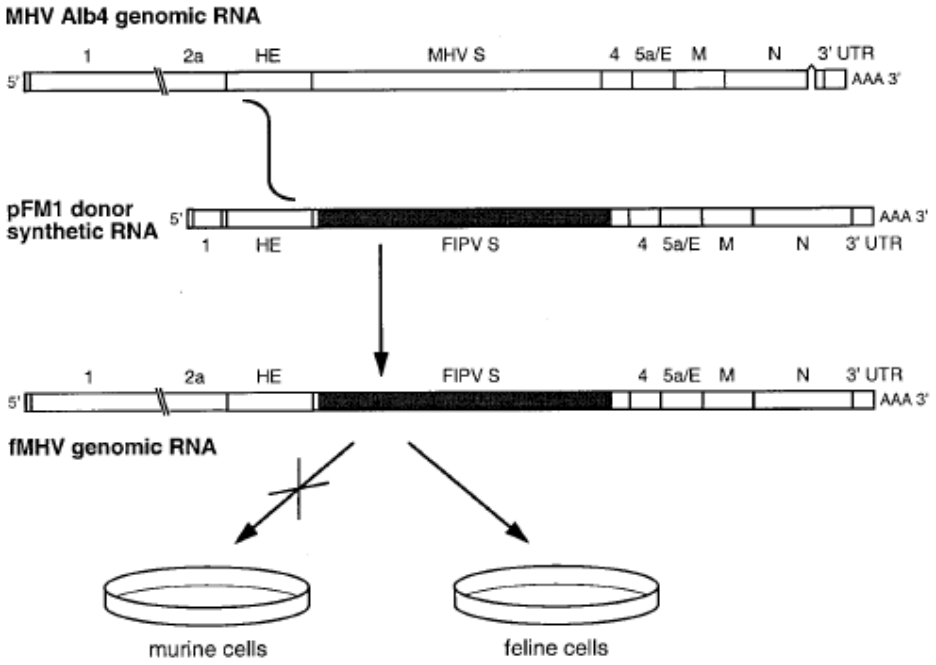

Figure 3. Schematic showing generation of fMHV (69).

Full length infectious cDNA expressed in stable amplification

Bacterial artificial chromosome (BAC)

The first full-length infectious clone of a coronavirus was created in TGEV by Almazan et al (2). The clone was generated by a stepwise reconstruction of full-length genomic cDNA from a DI minigenome, with the incorporation of the last fragment of the genome, which caused instability in the more traditional bacterial vectors (i.e. *E. coli*), occurring in the last step before transferring the entire system to a BAC (2). Viable viruses were then produced via a DNA launch. In a DNA launch, a cytomegalovirus

(CMV) promoter is placed at the 5' end of the full-length genomic cDNA. The cDNA is then transfected into the host cell, which begins to transcribe mRNA that is eventually exported to the cytoplasm. Once the mRNA is in the cytoplasm, viral replication resumes normally (32). The BAC system was very stable when it was first devised, due to the fact that it is a low copy number plasmid, but additional stabilization was obtained by inserting an intron into the regions of the ORF1 gene that are associated with the viruses' toxicity in bacteria (2, 44). The introns were devised in such as way that they keep the DNA stable in the nucleus, but are excised when the RNA transcripts are transported out of the nucleus, so that they have no effect on the translation of viral proteins. The advantages of the infectious clone method used in the BAC reverse genetic system is that it is possible to modify the entire coronavirus genome and it allows for easy selection of successful recombinants (32). The DNA launch used by the BAC system is also advantageous because it does not require expensive *in vitro* transcription reagents to produce the virus, which is a disadvantage in other reverse genetic systems that use an RNA launch (32). The disadvantage to using the BAC system is that two regions of the viral genome have to be interrupted with introns, which requires that these regions first be identified and then specifically targeted to disrupt the sequence in order to achieve long term stability (32).

Vaccinia virus

Although the BAC system was successful in generating a full-length cDNA infectious clone, there was some instability observed after extensive passage in *E. coli*, which is

why Thiel et al devised a new system in which full-length cDNA of HCoV-229E was amplified in a vaccinia virus vector (146, 149). The full-length infectious cDNAs, which were generated by long-range PCR, were inserted into the vaccinia virus genome using unique restriction sites (148, 149). The advantages of using vaccinia as a vector are that there is none of the instability of the cloned cDNA insert, as seen with more traditional vectors such as *E. coli*, and vaccinia, as a poxvirus vector, is suitable for cloning large cDNAs (139, 148). Once the cDNA has been cloned into the vaccinia vector, it is packaged into infectious virus particles, following transfection into cells that have previously been infected with a helper poxvirus. The virus particles are then isolated and screened, and once it is confirmed that they contained the coronavirus genome, the recombinant virus is amplified and the vector DNA is isolated and purified (148). The coronavirus cDNA is then excised from the vector and used as a transcription template for an RNA launch (148). An RNA launch differs from a DNA launch in that transcription of the genome occurs in vitro with the use of T7 or SP6 RNA polymerases rather than in vivo using the CMV promoter (32). In addition to having none of the cDNA instability commonly seen in *E. coli* vectors, the vaccinia virus system also has the advantage that it allows for the incorporation of mutations by homologous recombination, which eliminates the need to alter the desired fragment and *then* incorporate it into the full-length construct as is required with both the BAC system and the multicomponent system (32). However, it is time intensive to generate the desired mutations using homologous recombination. There are also safety concerns when using vaccinia virus because 1) anyone using vaccinia vectors should be vaccinated with the

vaccinia virus as per CDC recommendations and 2) the vaccinia vector contains the entire genome of the virus, is replication competent, infectious, and being actively amplified in cells so there is the potential for unexpected activation of the genome. This may not be an issue with strains that do not have the capability of infecting humans, such as MHV, but with more pathogenic strains such as SARS-CoV, this could be an issue if the DNA gets rearranged to generate a viable chimeric virus (32).

Infectious clones amplified as multicomponent cDNAs

An alternate method for producing full-length infectious clones of coronaviruses was proposed by Yount et al in 2000 (170). This method involves systematically breaking down the genome into several segments so that the toxic regions in OFR1 are disrupted with the full genomic cDNA reconstituted in vitro by ligation. This makes it possible to stably propagate the fragments in *E. coli*, making it easier to grow up and store the fragments. To ensure that the fragments will ligate together in the correct order, nuclease restriction sites (ex. BsmBI or BglI) are engineered at the end of the fragments so that after restriction fragment digest, the fragments can ligate together to generate a full-length cDNA clone. Once the full-length clone is created, viral RNA is generated through an RNA launch, as with the vaccinia virus system, and then electroporated into the target cells. The first coronavirus to be generated using this approach was transmissible gastroenteritis virus (TGEV) in 2000 by Yount et al. Since 2000, full-length infectious clones have been generated of MHV-A59, SARS-CoV and IVB (169-172).

As with the BAC system, the multicomponent system has the advantage of being able to modify the entire coronavirus genome and easily select for viruses with the desired mutations. This system has the added advantage of enabling rapid introduction of mutations and a higher degree of safety when working with infectious human agents, since the mutations can be introduced by only working with the relevant plasmid (32). The disadvantage to working with this system is the added time that it takes to generate an infectious clone for each virus and the need for extremely pure individual fragments (32).

The Baric lab, in addition to developing the multicomponent reverse genetic system for MHV-A59, also created a new technology, called No See'm, to seamlessly ligate the fragments together into a full-length genome. The basis for No See'm technology are restriction enzymes, such as BsmbI, which recognize a strand-specific sequence rather than a palindromic sequence (172). This makes it possible to engineer restriction sites at the ends of the fragment so that when the fragment is placed in a reverse orientation, the recognition site will be removed from the sequence, leaving an arbitrary overhang of viral DNA, which allows the fragments to seamlessly ligate together following the digestion (Figure 4). This will ensure that only the viral sequence is present at the junction site (172).

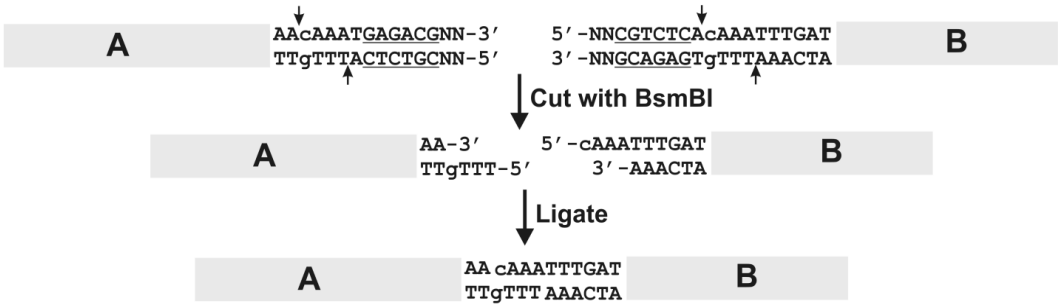

Figure 4. Scheme showing the use of No See'm technology to seamlessly join two fragments together. Using a type IIS restriction enzyme, in this case BsmBI, we can simultaneously eliminate the enzyme site and generate a unique 4 base overhang at each site. These overhangs add specificity, as they will only ligate to the overhang with their exact complement sequence and ensure that only virus DNA is present at the junction of two fragments. Figure is courtesy of

CHAPTER III

METHODS

Viruses and cells

DBT, and L2 cells were grown in Dulbecco's modified Eagle medium (DMEM)(Life Technologies) supplemented with 10%Cosmic calf serum (Hyclone), 4mM glutamine, and penicillin at 50 units/mL and streptomycin at 50 μg/ml. MHV-1 was the virus used as our wild type strain.

Generation and extraction of viral RNA

DBT cells were grown to 60-80% confluency in a T75 flask. DBT cells were infected with MHV-1 at a MOI of 1.0 or greater. RNA was extracted from the infected monolayer of DBT cells using the RNeasy RNA extraction kit from Qiagen.

Generation of fragments

Both forward and reverse primers were designed for each fragment (A, B, C, D, E). In order to make the system compatible with MHV-A59, alterations had to be made to the A and B fragments at the site of the A/B junction, D/E and E/F. These mutations were made using primer-mediated PCR mutagenesis. These non-coding mutations did not alter the coding sequence. All primers used in this experiment can be found in Appendix A. Within 0-3 hours after extraction and purification, RT-PCR was performed on the viral RNA to generate cDNA. Reverse transcription reactions were carried out using 3μl

of total RNA, 1μl of the forward primer, 1μl of the reverse primer, 1μl of dNTPs and 7 μl of Nuclease Free Water which was heated to 65°C for 5 minutes. After heating, the reaction was put on ice for ~1 minute and then 2μl of Superscript III Reverse Transcriptase (Invitrogen) was added to the tube along with 1 μl 0.1M DTT and 4 μl 5X First-Strand buffer. The RT reaction was then heat activated at 70°C for 15 minutes and the cDNA that was generated was amplified by PCR For sequence comparisons and primer design, we used the MHV-1 sequence as reported in GenBank (accession: FJ647223.1). The primers that were used for the PCR reactions were the same as those used for RT-PCR and are listed in Appendix A. Each fragment was amplified by long accurate PCR using rTth polymerase (Applied Biosystems) and Vent polymerase (New England Biolabs). Denaturation of cDNA was done at 94°C for 10 seconds followed by the annealing step, which was performed at temperatures ranging from 57-65°C depending upon the fragment, for 4 minutes. The elongation step was carried out at 68°C for one minute. Following PCR, the amplified fragment DNA was run on a gel, with an empty well left between each of the samples to ensure that no cross-contamination of the fragments occurred. The gel was visualized using a Claire Research Dark Reader light box. Bands were removed from the gel using a scalpel and the gel purification kit by 5PRIME was used to purify the DNA. Once the fragment DNA was purified, it was concentrated in a Speed Vac and then ligated into a pSMART vector (Lucigen) and transformed into E. coli per the manufacturer's instructions. Transformants were selected by spreading the bacteria on LB agar plates containing 30 μg/ml kanamycin in volumes of 50 μl, 100 μl and 200 μl. Plates were incubated for 18-24 hours at 30° C.

Colonies were picked from the plates and inoculated into 50 mL of 2XYT broth cultures and incubated for 24-30 hours at 30°C. Plasmid DNA was purified using the E.Z.N.A plasmid Midi prep kit from OMEGA BIO-TEK, following the manufacturer's protocol.

Sequencing was carried out using purified plasmid DNA from the Midi prep. Restriction digests were performed on the fragments to test for the presence of the insert.

CHAPTER IV

RESULTS AND DISCUSSION

System design

A systematic assembly approach was previously used by Yount et al to generate a full-length infectious cDNA of MHV-A59 (172). We reasoned that the same strategy used for MHV-A59 could also be applied to MHV-1. Yount et al had already accounted for the regions of sequence toxicity-instability in MHV-A59. The regions of instability in MHV-1 are highly likely to be in the same location as those for MHV-A59 since the sequences in this region are highly conserved, so we were able to use the same junction sites for the creation of the MHV-1 system. BspQ1 is the restriction enzyme that is used with the A fragment, rather than BsmBI, to create the unique overhang so that A can ligate together with B. However, there is an additional internal BspQ1 present in the A fragment which has to be removed before the A and B fragments can be ligated together. This will be done using a [company name] site directed mutagenesis kit in conjuction with a specially designed primer. The alteration will not produce a coding mutation. We plan on utilizing the No See'm technology first described by Yount et al to ensure that the fragments assemble in the correct order (172). The No See'm approach works by using restriction enzymes, in our case BsmB1 rather than Esp1 used by Yount, that cleave at a specific non-palindromic sequence, leaving highly variable 4-nucleotide ends that do not randomly assemble. These DNA fragments will only anneal with other

fragments that contain the complementary 4-nucleotide overhang generated at an identical BsmB1 site, which allows for fine control over the system. The No See'm approach is illustrated in Figure 4.

Generation of MHV-1 fragments

I was responsible for creating fragments A-E. Our lab has already created the G fragment and the F fragment, which has to be broken up into two smaller pieces, was entrusted to a graduate student.

As can be seen in Figure 5 we were successful in producing fragments B, C, D, and E by long accurate PCR. Successful production of cDNA was dependent on the freshness of the extracted viral RNA, which was usually purified 0-3 hours prior to the reverse transcriptase reaction. Following confirmation that the PCR had generated fragments of the correct size, the DNA was purified and cloned into the pSMART vector (Lucigen) after which the resulting ligation was electroporated into *E. Cloni* cells and grown overnight. Colonies were selected 16-24 hours post electroporation and then grown overnight in broth. Plasmids were extracted, and test digestions were performed prior to sequencing to confirm that the plasmid contained the correct size insert. Following confirmation, the samples were sent for sequencing. As of this moment we only have a complete sequencing results for fragments B and C. For the fragments for which we have sequencing results, we have made a consensus sequence from the overlap generated by the sequencing primers. The consensus sequences were then compared to the MHV-1

sequence in GenBank to determine whether or not mutations were present in the fragment and if mutations were present, whether they were non-coding or coding mutations. Non-coding mutations can be ignored, as they do not result in an alteration in the viral proteins produced, however coding mutations need to be excised and replaced with sequences that do not contain the coding mutation. This can be done using restriction fragment exchange from another isolate. The sequence for C, contains no coding mutations, but does have a non-coding mutation at base 190, which can be seen in Figure 6. The sequence for B also contains no coding mutations, but similar to fragment C does have one non-coding mutation at base 2126 (Figure 6). Fragment G, which was previously created by our lab, contains one coding mutation in 5a, and several other non-coding mutations in E, N, and 4 in spike protein respectively.

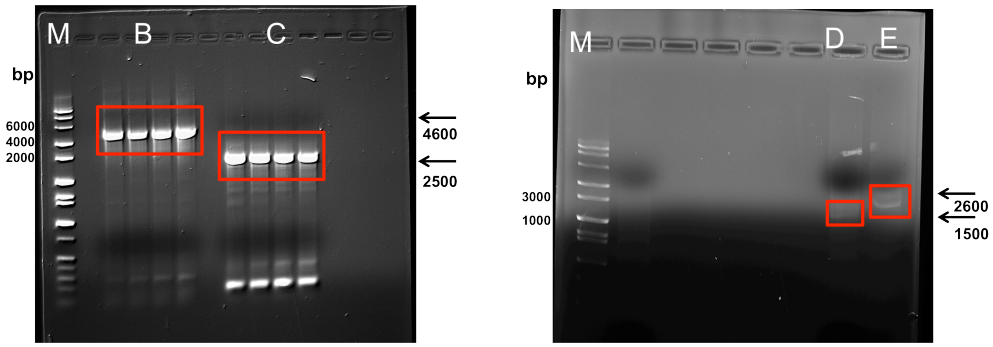

Figure 5. Gels showing successful PCR amplification of B, C, D and E fragments.

Discussion

In this study we report the successful generation of the component parts of a reverse genetic system for MHV-1. This approach was similar to the one employed by Yount et

al to generate full-length infectious cDNA of MHV-A59, although slight modifications did have to be made to account for differences in the MHV-1 genome. As shown in Figure 6 we were able to generate 2 of the seven total cDNA fragments.

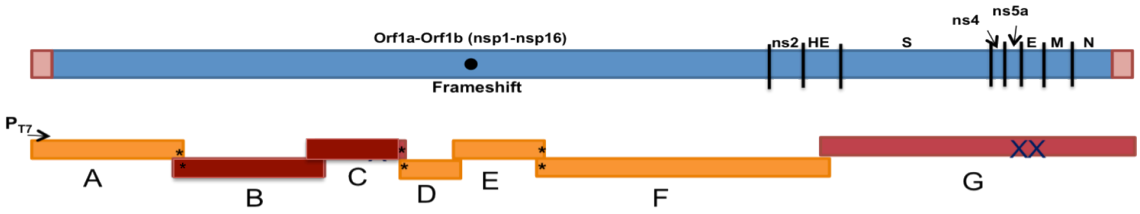

Figure 6. Schematic showing the MHV-1 genome broken down into 7 individual plasmids and what segments of the genome will be located in each piece. The maroon fragments are those that have been generated and fully sequenced. The orange pieces need to be generated and/or sequenced. The big X's on the fragments indicate the approximate positions of coding mutations. The smaller * indicate the positions where it was necessary to make nucleotide changes in order to make our system compatible with previously designed systems (MHV-A59 and MHV-JHM). None of these alterations resulted in a coding mutation.

Although we were able to generate a portion of the raw materials for the reverse genetic system, more work will need to be done to the system before it is complete, but these experiments were successful in showing proof of principle. Before the system can be used, we will have to finish sequencing fragments A-E to verify that the fragments are free of coding mutations. Mutations in the sequence are acceptable if they are non-coding and they do not use a rare codon, however if coding mutations are present they will need to be corrected before the system can be used to generate recombinant virus. Non-coding mutations can actually be beneficial, as they can serve are unique marker mutations in the genome that, once the system is completed, will allow us to ensure that the virus we are using is the modified version rather than one that has been altered by wild-type recombination or contamination. The next step, once sequencing is complete,

is to design and implement a strategy for restriction fragment exchange to generate fragments, A-E, which contain the correct sequences. (This will only be employed for those fragments that are shown to have deleterious mutations after sequencing is complete). In addition, the F fragment will need to be created, sequenced, and possibly corrected (if coding mutations are present) before the seven fragments can be ligated into a full-length clone. Before the newly developed reverse genetic system can be used, experiments must be performed to compare the viability of the recombinant virus to that of the wild type virus.

In designing the system, the compatibility to the MHV-A59 system was an important element because, once the system is finished, it will enable researchers to create chimeric viruses that contain genes from both MHV-1- and MHV-A59. Previous work in the lab has suggested that pneumovirulence factors in MHV-1 lie upstream of the S protein, in nsp 2 and/or nsp 3 [Leibowitz 2010]. Using the newly created reverse genetic system, it will be possible to examine this hypothesis by replacing the fragments containing nsp2 and nsp3 of MHV-1 with the corresponding fragments from MHV-A59. Because MHV-A59 is highly heptatrophic while MHV-1 is primarily pneumotropic, the chimeric virus will allow us to see the effect that removal of nsp2 and nsp3 have on the pneumovirulence of MHV-1 as seen in intranasally inoculated A/J mice.

This system will also allow for the rapid creation of mutations into regions of the genome that were previously inaccessible by targeted RNA recombination.

De Albuquerque et al has suggested that MHV-1 could be used as a mouse model for SARS (28). The generation of the reverse genetic system for MHV-1 will be instrumental in analyzing the host-pathogen interactions and in identifying which regions of the virus are responsible for its virulence, both in MHV-1 and hypothetically SARS. In the future, this system can also be used to test the efficacy of potential vaccines and/or antiviral therapies for SARS.

CHAPTER V

CONCLUSION

In summary, we have established several of the components required for a reverse genetic system for the betacoronavirus MHV-1. Although the raw materials have been generated, more work needs to be done to confirm and possibly correct the sequences before this system can be used to increase our understanding and MHV-1 and SARS.

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