SYSTEMATIC ASSEMBLY OF AN INFECTIOUS cDNA CLONE OF MHV-1 AND OPTIMIZATION OF VIRAL RESCUE

An Undergraduate Research Scholars Thesis by

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ABSTRACT

Systematic Assembly of an Infectious cDNA Clone of MHV-1 and Optimization of Viral Rescue. (May 2013)

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The Sudden Acute Respiratory Syndrome Coronavirus (SARS-CoV) caused a severe atypical pneumonia in humans with close to a 10% mortality rate, and caused a global outbreak in 2003. To better understand the pathogenesis of coronavirus pneumovirulence, a related coronavirus model is being developed, Murine Hepatitis Virus 1 (MHV-1). A complete reverse genetic system for MHV-1 is in the process of being completed. The reverse genetic system uses 7 cDNA fragments that cover the entire MHV-1 genome. These fragments are recovered from reverse transcribed viral RNA, and unique restriction sites were added to flank each fragment in order to allow systematic assembly of the complete genomic cDNA. The genomic cDNA can be transcribed, and the recovered RNA can be transfected into susceptible mammalian cells to rescue virus. The transfection process is currently being optimized by investigating the benefits of different electroporation techniques and different overlay cell confluences on amount of infectious centers produced from each attempt of viral recovery. Once the reverse genetic system is complete and tested the system will be used to generate mutant virus, impossible to make without the reverse genetic system.

NOMENCLATURE

MHV-1	Murine Hepatitis Virus Strain 1
MHV-A59	Murine Hepatitis Virus Strain A59
SARS-CoV	Sudden Acute Respiratory Syndrome Coronavirus
cDNA	Complementary Deoxyribose Nucleic Acid
RNA	Ribonucleic Acid
Kb	kilobase pairs

CHAPTER I

INTRODUCTION

Sudden Acute Respiratory Syndrome was initially identified in Southeast Asia following a wave of patients presenting with a severe atypical pneumonia. This infectious syndrome quickly spread to more than two-dozen countries, including a suspected case in the USA. The causative agent was confirmed to be a novel group II coronavirus. Sudden Acute Respiratory cornavirus (SARS-CoV) infected at least 8,098 people, killing 774 before the epidemic was contained (1). This disease has a mortality rate of close to 10%, and has an incredible potential to cause human suffering and death and could cause a drastic economic impact. With four more non-laboratory associated cases of SARS occurring after the initial outbreak, the potential for future outbreaks is not negligible (2). SARS- CoV is a zoonotic disease, transmitted from animals to humans. The natural reservoir is thought to be in bats, as SARS like cornaviruses were identified in Chinese horseshoe bats and several other bat species were discovered to harbor other cornaviruses. (3) This reservoir poses a constant threat of reemergence of the disease, as bats and humans are often in close contact or bats contact other animals used for human consumption. In order to protect against a recurring outbreak of SARS-CoV, coronavirus biology must be studied more in-depth and strategies for treatment and prevention must be developed, or the risk of more severe epidemics looms.

SARS-CoV belongs to the family *Coronaviridae,* in the order Nidovirales. Members of the *Coronaviridae* derive their name from their appearance under electron microscopy,

relatively large circular structure with spike proteins protruding from the envelope resembling a crown. All viruses in this family have large positive sense RNA genomes, the largest of any RNA virus. These viruses enter the cell through a membrane fusion event, releasing the viral RNA into the cytoplasm of the cell. The viral RNA is used to translate the replicase-transcriptase enzyme, which replicates the viral genome in preparation for further translation and eventual viral replication. (4)

Murine Hepatitis Virus (MHV) is also a group II coronavirus and serves as an excellent and relatively safe model for coronavirus biology. MHV pathogenesis varies widely depending upon the specific strain of the virus. MHV-JHM typically causes an acute panencephalitis with demyleination of the nervous tissue in mice. MHV-A59 is more hepatotropic and neurotrophic while MHV-1 is more pneumotropic than the other strains when inoculated in mice intranasally. The MHV RNA genome is 31.5 kilobase (kb), contains 9-10 open reading frames (ORFs), and association with the viral nucleocapsid protein condenses and protects the viral RNA. (5)

In order to easily manipulate any gene of the virus for research a reverse genetic system is required. A reverse genetic system would store all of the genetic information of the RNA virus in the more stable cDNA form, which can be much more conducive to common laboratory techniques for genetic manipulation. The MHV-1's 31.5 kb genome is too large to be stored on one plasmid, and although a bacterial artificial chromosome could possibly be used it would be extremely to difficult to manipulates the genes stored in that type of vector. Therefore the most practical means for a vector will require

segmenting the genome and cloning the segments into several plasmids. This system is modeled after the reverse genetic system for MHV-A59, which was successfully developed and is frequently used currently.

The construct being engineered breaks the genome into 7 segments inserted into 7 plasmids, each of the segments has unique restriction sites flanking the viral genome segment insert allowing for easy excision and ligation of the segments into the full length genomic cDNA. Each plasmid could be easily manipulated, and different mutations could be mixed and matched easily by switching segments.

To recover a virus from the reverse genetic system, the segments must be excised from the plasmid vector, and then *in vitro* ligation would be used to join all the segments together. The complete genomic cDNA would be transcribed, and the RNA transcript would be transfected along with the RNA template for the nucleocapsid helper gene into BHK-R cells. The RNA would interact with the N protein, and act just as if a virus inserted the RNA into the cell. Eventually viral particles would emerge from the cell, just as in a natural infection, and spread to other cells creating an infectious center from which viral particles can be recovered. See Figure 1 for an outline of the recovery of a virus from the system.

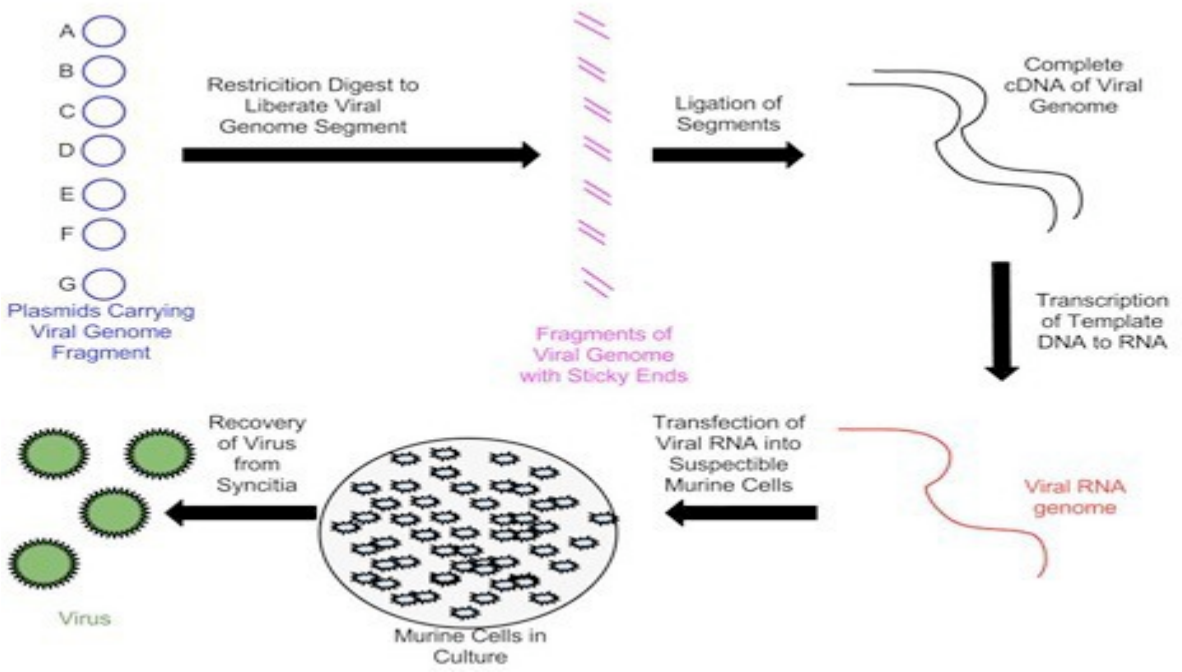

Figure 1: Schematic of Recovery of Virus from Infectious Reverse Genetic System.

The degree of success for viral rescue depends upon a number of factors throughout the entire recovery process. The efficiency of transfection dramatically affects the success of viral rescue, as the N protein must be expressed in the same cell that contains the viral RNA genome, because the viral RNA must be associated with the N protein to properly begin translation and viral infection. The N expression plasmid and viral RNA must be transfected into the same cell to produce any viral activity and eventually the formation of an infectious center. The higher the transfection efficiency the more likely the necessary cotransfection occurs, and the more infectious centers.

Two methods of transfection will be used to determine the optimal protocol for viral rescue. Electroporation uses an electrical pulse to disturb the phospholipid bilayer and

introduce polar molecules like DNA or RNA into the cell. Nuceleofection uses electrical impulses and special buffers to introduce the substrate into the nucleus and cytoplasm. Both techniques can be used to introduce the viral RNA and N gene helper plasmid to the cells.

The amount of confluence of the cells that the transfected cells get overlaid upon can also influence the amount of infectious centers. Different degrees of confluence can allow more overlaid cells to attach to the well or conversely less space for cells to attach forcing more contact between cell types.

The reverse genetic system will allow for the genetic manipulation of the nonstructural genes, which currently is impossible. The ns2 protein has been shown to have phosphodiesterase activity, which blocks the RNase L pathway downstream of the interferon response (6). The RNase L pathway is activated when viral RNA is detected by cellular pattern recognition receptors, triggering the induction of interferon α/β, which stimulate a pathway producing RNase L. The ns2 protein cleaves an intermediate molecule in this response; protecting viral RNA from degradation, shielding the virus from one pathway of the host's innate immunity. Therefore a virus without the ability to down regulate RNase L response should have limited pathogenicity compared to wild type virus.

CHAPTER II

METHODS

Recovery of viral RNA:

Murine DBT (Delayed Brain Tumor) cells were infected with MHV-1 and incubated for 48 hours. The DBT cells were lysed with buffers containing RNase inhibitors and mercaptoethanol supplied by a reverse transcription kit. The lysate was then treated according to the directions in the kit. Using primers designed to segment the genome into specific fragments reverse transcription was carried out. Fragments of the genome were necessary because of the large size of the genome, and the fragments also decreased the toxicity to the cells carrying the DNA. The DNA was then amplified using PCR. The PCR products were purified and sequenced.

Insertion into plasmids:

The seven fragments were inserted into commercially available plasmids. The fragments used either naturally occurring restriction sites, or artificially created by adding on to the end of the PCR product, or by using site-directed mutagenesis. The fragments were treated according to the EZclone kit, and blunt end ligation was used to insert the fragments into the vectors. The vectors are then electroporated into electrocompetant bacteria for further amplification.

Recovery from plasmids:

The bacteria containing the fragments are first grown to high concentrations and then a MidiPrep plasmid extraction kit was used to purify the DNA. The DNA was digested sequentially with two different restriction enzymes. The fragments were separated by gel electrophoresis and gel extraction. The separated fragments were then ligated together. Then a trancription kit was used to transcribe the DNA into RNA. The viral RNA was electroporated into cells. The cells would then be overlayed onto a semi confluent layer of susceptible cells.

Optimization of recovery:

Recovery would be done the same up until the electroporation point. Two different types of transfection would be used, the normal electroporation and a nucleofection method. The cells would then be plated and infectious centers would be counted and compared.

CHAPTER III

RESLULTS

Previously a reverse genetic system for MHV-A59 was assembled from in vitro transcribed genomic cDNA, which resulted in an infectious virus. Therefore the same approach was applied to the MHV-1 genome to complete a reverse genetic system. The genome is too toxic and large to place into a single plasmid, therefore the genome was segmented into seven fragments. Currently several of the plasmids containing the genomic fragments are still under construction. The reverse transcriptase enzyme has relatively low fidelity, and several rounds of site directed mutagenesis were needed to introduce the interconnecting junctions needed for fragment assembly to produce the full length genomic cDNA and introduce silent mutations into naturally occurring *BspQI* restriction site. See Table 1 for completion status of plasmids.

Viral infection was successful and generated viral RNA that was recovered and purified. Reverse transcriptase was used to generate cDNA fragments, and primers were selected to match desired fragments sizes and locations. Then the cDNA fragments where amplified using PCR, with primers designed to add some of the interconnecting junctions and amplify specifically the desired fragments. The fragments where cloned into commercially available plasmids using blunt end ligation. This step is complete for all of the fragments currently.

Fragment	Approximate Size (Base Pairs)	Completion Status
A	4000	Needing BspQI silent mutation
B	3000	COMPLETE
C	2000	COMPLETE
D	2000	COMPLETE
E	2400	COMPLETE
F	7300	Awaiting swap of two clones
G	9400	Needing several sequence fixes

Table 1. cDNA fragments to cover entire MHV-1genome and status as of 4/5/13

CHAPTER IV

CONCLUSIONS

Currently four of the seven plasmids spanning the entire MHV-1 genome are complete. These plasmids required extensive cloning and manipulations in order to clone the genome segment into the plasmid and allow for eventual reconstruction and viral recovery. Once the final three plasmids are constructed, several things will need to be accomplished. The first of which will be test digestions and ligations to confirm that the genomic fragments will ligate together properly. After checking the ligations, the complete cDNA will be transcribed and transfected into murine cells and virus will be collected. Finally the recovered virus will have to be compared to wild type MHV-1.

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