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EFFECTS OF MUMPS MATRIX PROTEIN MUTATIONS ON EFFICIENCY OF PARTICLE
PRODUCTION AND INTERACTION WITH VIRAL NUCLEOCAPSID PROTEIN

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ABSTRACT

The mumps virus is a paramyxovirus that infects humans and can cause disease, with possible lethal or long-lasting side effects. Although a vaccine for this virus was developed, it has recently become relevant again with outbreaks occurring even in countries with comprehensive vaccinations. The matrix protein encoded in the MuV genome is thought to organize viral assembly and budding by its interaction with viral nucleocapsid protein and glycoproteins. The mumps matrix protein along with nucleocapsid protein and a glycoprotein, fusion protein, were previously found to be sufficient for particle production in vitro. This thesis shows that upon certain sequence insertions into the MuV M protein, efficient particle production can still be seen, but the dependence on NP protein for particle production varies. The amount of charged residues found in the inserted sequence, along with the length of insertion, both seem to play a role in mutants ability to produce virus-like particles in the absence of NP protein. The mutant M proteins were found to have a slower rate of degradation in cells when compared to wildtype M, which could provide an explanation for this varied phenotype. This slower degradation rate was also seen in cells when both wildtype M and NP were expressed, indicating that interaction between NP and M stabilizes M in cells similar to the stabilization of mutant M constructs. These findings suggest different roles of the matrix protein, that when varied by mutation, shows a NP independent phenotype for particle production.

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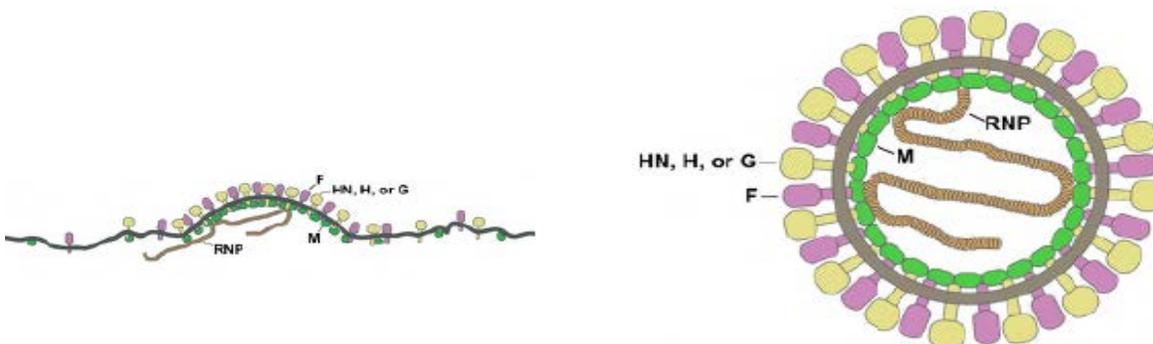
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1. Introduction

1.1 Paramyxoviridae

Paramyxoviruses are negative-sense single-stranded RNA viruses of the paramyxoviridae family that are able to infect a wide range of hosts such as birds, fish, reptiles, and mammals. Some of these viruses are asymptomatic upon infection but others cause serious diseases in humans such as mumps, measles, bronchiolitis, pneumonia, and respiratory tract diseases, which makes them important in biomedical research.¹ The paramyxoviridae is split into two sub-families, paramyxovirinae and pneumovirinae. The paramyxovirinae is then divided into five genera: rubulavirus, respirovirus, mobilivirus, avulavirus, and henipavirus. The henipavirus genera includes newly emergent Nipah virus (NiV) and Hendra virus (HeV) that are among the mostly deadly pathogens. The natural hosts for these viruses are pteropid fruit bats (flying foxes), but transmission to humans has caused deathly outbreaks with no current vaccines or approved therapeutics to treat infections.^{2,3} Twelve reported outbreaks of Nipah virus infection in South Asia and 13 known outbreaks of Hendra virus in Australia have occurred, and these outbreaks will continue as animals and humans move into locations populated with flying foxes.^{4,5} Paramyxoviruses are also of agricultural concern with viruses such as Newcastle disease virus (NDV) causing lethal disease in birds, including chickens, and the recently globally eradicated Rinderpest virus that had a long history in Europe and Asia of lethal infection in cattle.^{6,7} There is a long list of identified paramyxoviruses with new viruses continuously being isolated from different animals.

The structure of paramyxoviruses is an approximately spherical enveloped virus about 150-200nm in diameter that comes from the host cell membrane during budding. The genome consists of one continuous strand of single-stranded RNA in a helical nucleocapsid.¹ Some virions have been found to have filamentous forms and larger size up to 1 μ m.⁸ The budding mechanism of paramyxoviruses is similar between viruses in the family in that they are all enveloped viruses that form by budding from cellular membranes where viral proteins have assembled together. It has been found that the matrix protein (M) in these viruses plays a central role in organizing all of the structural components necessary for the virus to bud from a cell. The main proteins encoded by the viral genome include glycoproteins F and HN (fusion and hemagglutinin-neuraminidase), that are inserted into the viral envelopes and facilitate budding of the virion. These glycoproteins can be seen as spikes around the budding virion by electron microscopy. The HN protein is specifically responsible for attaching a virion to cells because of its sialic acid receptor. Another protein is the nucleocapsid (NP) protein that binds the RNA genomes of paramyxoviruses. The M protein interacts both with the NP protein and the cytoplasmic tails of the viral glycoproteins and is necessary for virion formation.⁹ The illustration below shows the interaction between the glycoproteins, NP, and M proteins in budding paramyxoviruses.



Budding of paramyxovirus particles. Illustration of paramyxovirus budding with M protein interacting with NP protein surrounding viral RNA and cytoplasmic tails of glycoproteins (modified from Harrison 2011(9)).

1.2 Mumps Virus

The paramyxovirus studied in this thesis is the mumps virus (MuV) in the genera rubulavirus, which has recently become relevant again with controversy over vaccination and sporadic outbreaks appearing globally. The disease is characterized by symptoms including headache, malaise, fever, and painful salivary gland inflammation and was a common childhood illness before vaccination that was not generally fatal.¹⁰ The disease can result in a wide array of inflammatory reactions including orchitis, encephalitis, and meningitis as well as long-term effects including paralysis, seizures, and deafness with severity of the disease increasing with age.¹¹ Infection only occurs naturally in humans and much of the pathogenesis of the disease remains poorly understood.

A vaccine for mumps was introduced in the USA in 1967 and disease incidence immediately began to decline. By 2001 the disease was on the verge of elimination with less than 0.1 cases per 100,000 people reported.¹² Within a few years of this report, outbreaks of mumps were seen globally even in countries where comprehensive vaccination exists. The reason for these outbreaks includes possible primary or secondary vaccine failure, unvaccinated populations, and the controversial idea that virus strains are emerging that are capable of overcoming the immunity generated from vaccines.¹³ Safety concerns over certain vaccine strains has led to their withdrawal in countries such as Japan. With no national immunization program for mumps vaccination, Japan now has one of the highest rates of mumps among developed countries.¹⁴ In the U.S. the largest mumps epidemic in 20 years occurred in 2006 and involved 6584 patients mainly in Midwestern states.¹⁵ The mumps virus in this outbreak was the same genotype G that circulated in the United Kingdom and was seen in outbreaks in Canada.¹⁶

The viral genome of MuV is a negative strand RNA molecule of 15,384 nucleotides that consists of seven transcription units: nucleo- (NP), phospho- (P), matrix (M), fusion (F), small hydrophobic (SH), haemagglutinin-neuraminidase (HN), and large polymerase (L) proteins.¹⁸ A complex of the L and P proteins forms the RNA-dependent RNA polymerase that copies the negative sense RNA to positive sense RNA and transcribes the negative sense RNA to generate mRNAs.¹³ The role of the M, NP, and HN/F proteins were previously described and the SH protein has been hypothesized to help the virus evade a host's antiviral response.¹⁹

1.3 Mumps Virus-Like Particles

In vitro studies of many viruses have achieved budding of particles from cells transfected with certain viral proteins without infecting the cells with live virus. These particles are called virus-like particles (VLPs) and lack the DNA or RNA genome of the virus but have been shown through microscopy to resemble the shape of authentic viruses.²⁰ VLPs have been engineered as vaccines, such as the human papillomavirus (HPV) vaccine, and have been useful in determining the roles of different viral proteins in viral budding.²¹ The VLP system is especially convenient when working in the laboratory because pathogenic viruses are able to be studied without risk of infection.

For many paramyxoviruses including human parainfluenza virus type 1, Sendai virus, NDV, and NiV, the matrix protein expressed alone is sufficient to effectively produce VLPs.²² Alternatively, MuV M protein expressed alone in cells showed inefficient VLP production, although a small amount of M-alone particles were seen. The most efficient production of VLPs was observed when the M protein was co-expressed with NP and F proteins together and particle

production was comparable to quantities observed in MuV-infected cells. The morphology of these particles was also shown to be very similar to MuV virions. Although all three viral proteins were needed for efficient particle production, the M protein was necessary for any particle production as is seen with many paramyxoviruses.²³ The NP protein expressed in the cells likely encapsidated cellular RNAs to form structures that resemble viral RNPs as seen with simian virus 5 (SV5) that also requires M, NP, and a glycoprotein for efficient VLP production.²⁴ During viral infection NP protein interacts with P protein bound to the viral genome which reduces nonspecific encapsidation of cellular RNAs.²⁵

1.4 Matrix Protein

The role of the paramyxovirus matrix protein as a central organizer of viral budding has previously been described because of its ability to bind directly to cellular membranes as well as interact with the RNP complex and cytoplasmic tails of viral glycoproteins. The structure of M protein has only been elucidated from human respiratory syncytial virus (RSV) by protein crystallization. The RSV M protein is comprised of two domains with compact beta-sheets connected by an unstructured linker.²⁶ An atomic structure of the matrix protein was also determined from NDV which showed that positive charges are mainly found on the surface of the protein that could electrostatically interact with the negatively charged membrane.²⁷

The exact mechanism of M action in virus assembly and budding is not known but early studies showed that the M protein of vesicular stomatitis virus (VSV) will aggregate only in the presence of nucleation sites, and remains soluble in the cytoplasm. These nucleation sites were not positively identified but they were hypothesized to be modified M proteins or M proteins

associated with the plasma membrane. This finding could help to explain an early step in the budding process of a new virion from a cell.²⁸ Recent studies have also shown nuclear trafficking of many M proteins with a functionally conserved nuclear localization signal seen in NDV-M, NiV-M, HeV-M, SeV-M and MuV-M. This nuclear localization and export is regulated by ubiquitination of the M protein.²⁹ NiV-M mutants that were defective in nuclear import or export were not able to produce VLPs, indicating that M trafficking to the nucleus is a necessary prerequisite for particle production.³⁰ The ubiquitination of M proteins has been previously shown in the lab with PIV5. Monoubiquitination of PIV5-M is necessary for efficient particle assembly and budding, indicating that ubiquitin may play an important role in all paramyxoviruses by regulating matrix proteins.³¹

2. Materials and Methods

2.1 Cloning Mutant Matrix Proteins

Various amino acid sequences were cloned into the Mumps virus matrix gene between amino acid positions 337-338. The matrix protein used is from the MuV Iowa strain, and the M gene was subcloned into the eukaryotic expression vector pCAGGS to generate pCAGGS-MuV M as described previously.

2.1.1 2-Step PCR Amplification of Mutants with No Restriction Sites

Polymerase chain reaction (PCR) was used to create mutants of Mumps M gene that included a sequence insertion at amino acid position 337. In order to create mutants with no restriction site sequences surrounding the added insertion sequence, a two-step PCR procedure was implemented. Each PCR reaction contained the following:

- a. 0.5ul of forward primer
- b. 0.5ul of reverse primer
- c. 100ng of template DNA
- d. 25ul Phusion polymerase Mastermix
- e. Distilled water to make final volume 50ul.

The sequences for the forward and reverse primers of all the mutants can be found in appendix A. The primers were designed and ordered. Tris (2mM, pH8.4) was added to primers to have a final concentration of 100uM. For the first step of PCR the forward primer of MuV M with a Not1 restriction site sequence, which was previously obtained, was added with the reverse

primer created for each mutant. This reverse primer contained the nucleotide sequence for amino acids upstream of MuV M along with the full 30 nucleotide sequence of the myc-tag or other specified sequence, and the reverse complement of this was used. The first step of PCR also contained samples with a previously obtained reverse primer for MuV-M that contained an Xho1 restriction site sequence. This reverse primer was combined with forward primers with the specified sequence insertion and then the nucleotide sequence for amino acids downstream of MuV M. The conditions for PCR are listed below.

- a. Initial denaturation 98C/30sec
- b. Denaturation: 98C/20sec
- c. Annealing 55C/30sec
- d. Extension 72C/1 min per kb of insert
- e. Final extension: 72C/5mins
- f. Cool to 4C

After the first step of PCR, two products for each mutant were obtained, with one containing the N-terminal sequence of MuV M and the other containing the C-terminal sequence of MuV M but both containing the desired inserted sequence. The PCR products were run on an agarose gel to check for the correct size and purified as described below. 100ng of each product was then added in the second step of PCR along with the forward and reverse primers of MuV M with the Not1 and Xho-1 restriction site sequences. The conditions for PCR remained the same as in the first step. This second step should produce a PCR product with the full MuV M sequence flanked by restriction sites and the specified insertion at position 337 that has no restriction sites added.

2.1.2 Agarose Gel Purification of PCR Product

After both PCR steps, the product was gel purified. A 0.7% agarose gel was created by combining 30ml of 1x agarose buffer (10X buffer: 0.4M trizma base, 0.05M sodium acetate,

0.009M EDTA, glacial acetic acid pH 7.8) with 0.21g of agarose in an Erlenmeyer flask. The agarose was dissolved in the solution by microwaving the flask. After microwaving, 1.5ul of ethidium bromide was added to the solution and it was poured into a gel casting setup and the appropriate size well combs are inserted.

A 1ul sample of PCR product with 5ul of 5x loading dye was run on the agarose gel along with an appropriate control and DNA ladder to check the approximate size of PCR product. The gel was run at 100V and scanned under UV illumination. If the PCR worked and the sample appears to be the right size it is then purified by running another agarose gel.

For purification 6-7ul of 10X loading dye is added to the full PCR product and a 0.7% agarose gel is prepared with larger wells. Samples are loaded and run at 100V. The bands are visualized by UV light and the desired PCR product band is cut out and transferred to 2ml Eppendorf tubes. The gel piece was then weighed and a volume of Qiagen Gel (QG) buffer equal to 2.5x the weight of the gel is added. The gel is then melted at 55C using a heat block for 5-10 minutes. After gel has completely melted the solution is transferred to a Qiagen DNA column. The samples were centrifuged at 12,000rpm for 1 min and the liquid flow-through discarded. 500ul of wash solution obtained from Genscript Corporation was added to each column and centrifuged again with flow-through discarded.

The columns were then transferred to clean Eppendorf tubes and 30ul of elution buffer (2mM Tris, pH 8.5, Genscript Corp.) was added to each and centrifuged. The flow-through was collected as the purified PCR product.

2.1.3 Digestion of PCR Product

The PCR product after the 2-step PCR procedure was digested with restriction enzymes Not1 and Xho1. Each digestion reaction contained:

- a. 30ul PCR product
- b. 0.5ul Not1
- c. 0.5ul Xho1
- d. 1X NEB buffer (stock of 10X) - 5ul
- e. 1X BSA (stock 100X) – 0.5ul
- f. Make up volume to 50ul with d/w

The digestion reaction was kept in a 37C incubator for 3 hours or overnight.

The product was then column purified as described above but with 125ul of binding solution (Genscript Corp.) added in place of the QG buffer.

2.1.4 Ligation of PCR Product

To set up ligation the concentration of each PCR product has to be quantified. 1ul of each insert was mixed with 5ul of loading dye and run on a 0.7% agarose gel with a DNA ladder to quantify bands. A ligation calculator was then used to determine the amount of insert that needed to be added for each ligation reaction. For the ligation calculator a vector:insert ratio of 1:6 was used, 50ng of vector was needed and the size of the vector pCAGGS is about 4700bp.

The ligation reactions were set up with the following:

- a. 50ng vector
- b. Xng insert (as determined by ligation calc and gel quantification)
- c. 1X T4 DNA ligase buffer – 2ul from 10X stock
- d. 0.5ul T4 DNA enzyme
- e. Add d/w to make the volume 20ul

The ligation reaction was kept overnight in a water bath at 16-22C.

2.1.5 Transformation

Cells were transformed to take up the pCAGGS vectors that contained the PCR product. 30ul of competent cells (DH5 α *E. coli*) were added to each sample and incubated on ice for 15 minutes. The samples were then heat shocked in a 42C water bath for 30 seconds and immediately placed back on ice. 1ml of LB media was then added to each sample and they were left to shake for 30-45 minutes at 250rpm. The cells were then centrifuged at 10,000 rpm for 1 min. Most of the media was then removed, leaving around 50ul. Cells were resuspended and this entire volume was plated on lysogeny broth (LB)-ampicillin (0.1mg/ml) petri dishes.

2.2 Plasmid DNA Isolation

2.2.1 Picking DNA Colony

After transforming and growing cells on a LB-amp late overnight, colonies were picked from these plates in order to obtain the plasmid DNA from the cells. Each colony represents a bacterial cell that has replicated over time. A sterile pipette tip was used to select single colonies from all the plates and then placed into a tube with 3ml of LB media with 0.1mg/ml ampicillin. These tubes were left overnight at 37C.

2.2.2 Plasmid DNA Mini-Prep

The cells were centrifuged at 6000rpm for 5 minutes and then resuspended in 120ul of cold GTE.

To lyse the cells, 240ul of lysis buffer (200mM NaOH, 1% SDS in dH₂O) was added and the mixture was placed on ice for 5 minutes. 360ul of cold 4M potassium acetate (11.5ml glacial acetic acid, 8.5ml H₂O, 80ml 5M KOAc) was then added and placed on ice for 5 minutes.

The samples were centrifuged at 14,000rpm for 15 minutes and supernatant was transferred to a new tube.

To precipitate nucleic acids, 390ul of isopropanol was added and nucleic acids were precipitated at 14,000rpm for 10 minutes. The supernatant was removed and the pellet was left to dry completely.

The nucleic acid pellet was then resuspended in 200ul of TE+RNase (10mM Tris, 1mM EDTA, 20ug/ml RNase) to remove RNA. This was incubated at 37C for 30 minutes.

To clean up the DNA 100ul of PEG solution (40% PEG-8000, 30mM MgCl₂) was added to each sample then centrifuged at 14,000rpm for 10min. The pellets were then washed twice with 1ml of cold 70% ethanol to remove residual PEG. DNA was resuspended in 30ul of 2mM Tris buffer, pH 8.4.

2.3 VLP Experiments

2.3.1 Plasmids

The MuV M, NP, and F plasmids have been described previously.²³ The mutant MuV M plasmids were obtained by PCR mutagenesis, and verified by DNA sequencing.

2.3.2 Transfection

Human 293T cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells placed in 6-cm dishes and transfected when they reach around 70 to 80% confluency. The transfections were carried out in Opti-MEM using Lipofectamine-Plus reagents with plasmid quantities per dish for optimal particle production as determined by previous titration experiments carried out in the lab. The plasmid quantities per dish were: 0.4ug pCAGGS-MuV M (including mutants), 100ng pCAGGS-MuV NP, and 100ng pCAGGS-MuV F. For VLP experiments with no NP protein transfected, an equal amount of empty vector (pCAGGS vector with no insert) was used to equalize total plasmid DNA quantities.

At 24 h posttransfection the medium was changed to DMEM with 2% fetal calf serum (FCS). The culture media was then collected after 16-18hrs to analyze VLPs. The cells were also collected and lysed to analyze the relative amounts of protein expressed in the cells.

2.3.3 VLP Purification

The collected culture media was centrifuged at 7,500 x g for 2 min to remove cell debris. The supernatant was then layered on 20% sucrose cushions (10ml in NTE [0.1 M NaCl; 0.01 M Tris-HI, pH 7.4; 0.0001 M EDTA]). Sample were centrifuged at 140,000 x g for 1.5h in an ultracentrifuge machine with a Ti70.1 rotor. The supernatant was discarded and the pellet was resuspended in 0.9ml of NTE and mixed with 2.4ml of 80% sucrose in NTE. Layers containing 50% sucrose (3.6ml) and 10% sucrose (0.6ml) in NTE were applied to the tops of the samples and centrifuged at 140,000 x g for 3h.

A 4ml volume was collected from the top of each gradient and added to 10ml of 1X NTE. These were pelleted at 140,000 x g with a Ti50.2 rotor for 1.5 h and the pellet was resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer containing 2.5% (wt/vol) dithiothreitol and boiled for 5 min.

2.3.4 Cell Lysate Preparation

Cell lysates were prepared by scraping cells from the dishes and centrifuging at 5,000 x g for 5 min. The cells were then lysed in 1X passive lysis buffer (PLB) and passed through a Qiagen shredder. Samples were suspended in SDS-PAGE sample buffer and boiled for 5 min.

2.4 Detection and Quantification of VLPs by Western Blotting

2.4.1 SDS-PAGE Gel Electrophoresis

10% SDS-Polyacrylamide gels were made and the samples were loaded onto the gels. The gel was run at 120V in a 1X Tris-Glycine buffer. The gel was run until the loading dye reached the bottom of the gel.

2.4.2 Transfer

Transfer buffer was prepared that contained 1X Tris-glycine buffer and 20% methanol in distilled water and chilled at 4C. One PVDF membrane and four Whatman filter papers were cut for each gel to fit the size of the gel. The PVDF membrane was fixed by dipping it into methanol then washing it with water for 5 min and finally transfer buffer for 15 min. When the gel was finished running it was also fixed in transfer buffer for 15 min. The transfer was set up by placing a sponge, two Whatman papers, the gel, the

PVDF membrane, two Whatman papers, and another sponge in that order onto the transfer apparatus. The transfer apparatus was secured and placed into the transfer box, which was then filled with transfer buffer. The transfer was run at 80V for 45 min.

After the transfer, the membrane was removed and placed in blocking buffer (5% dry milk in 0.3% PBS-Tween) for 1 hour. The primary antibody was then added and incubated overnight. The primary antibody was washed off using 0.3% PBS-Tween wash buffer and the secondary antibody was added and incubated for 1 hour then washed.

2.4.3 Antibodies

Peptide antibodies specific to MuV M, NP, and F proteins were made previously.²³ The secondary antibody used was a goat anti-rabbit antibody. In most of the VLP experiments only the M protein antibody was used for detection. This peptide antibody also cross-reacts with the NP protein, so a weaker signal band is seen corresponding to NP without NP antibody being used.

2.4.4 Detection and Quantification

ECF chemiluminescent substrate (300ul) was added to each membrane and incubated for 1-2 minutes to detect protein on the membrane. The blot was then scanned on a FLA-7000 Scanner.

2.5 Pulse-Chase Experiments

2.5.1 Radioactive Labeling and Chase

293T cells were grown on 10-cm plates to reach 70-80% confluency and transfected with plasmid of the specified MuV M protein and either NP or empty vector. At 24 h posttransfection the cells were starved with DMEM medium lacking cysteine and methionine for 30 min. The cells were then labeled by incubation with medium containing ³⁵S-Promix (100uCi/ml; Amersham Pharmacia Biotech, Piscataway, NJ) for 30 min at 37C. After labeling the medium was replaced with nonradioactive chase medium (DMEM with FCS and penicillin/streptomycin), and the cells were incubated at 37C for distinct time periods of 0, 0.5, 1, and 3 hours.

2.5.2 Immunoprecipitation

After the specified chase time period the cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (10mM Tris, pH7.4; 1% deoxycholate; 1% Triton X-100; 0.1% SDS) containing 150mM NaCl, 100mM iodoacetamide, and 1mM phenylmethanesulfonyl fluoride [PMSF] for 5 min at room temperature. The lysate was then clarified by centrifugation at 16,000 x g for 30 min. The clarified lysate was placed in new tubes and 15ul of the MuV M antibody was added for 4 hours at 4C. The antibody complexes were then absorbed to 30ul of protein A-Sepharose beads for 30 min at 4C. The beads were then washed three times. First with RIPA buffer containing 0.3 M NaCl, then RIPA buffer of 0.15 M NaCl, and last with 50mM Tris (pH 7.4)-0.25 mM EDTA-0.15 M NaCl. The beads were all boiled for 5 min in SDS-PAGE sample buffer containing 2.5% (wt/vol) dithiothreitol and fractionated on % SDS-PAGE gels.

2.5.3 Protein Detection and Quantification

The samples were run on a 10% SDS-PAGE gel overnight. The gel was placed in a fixing buffer (10% methanol, 7% glacial acetic acid in dH₂O) for 15 min then dried for 2 hours before exposing it to a phosphor screen for 3 days to detect radioactive activity on the gel. Protein detection and quantification were performed using a Fuji FLA-7000 laser scanner (FujiFILM Medical Systems, Stamford, CT). Protein stability was calculated as the specific counts of protein at each time point divided by the value obtained at the 0 h time point.

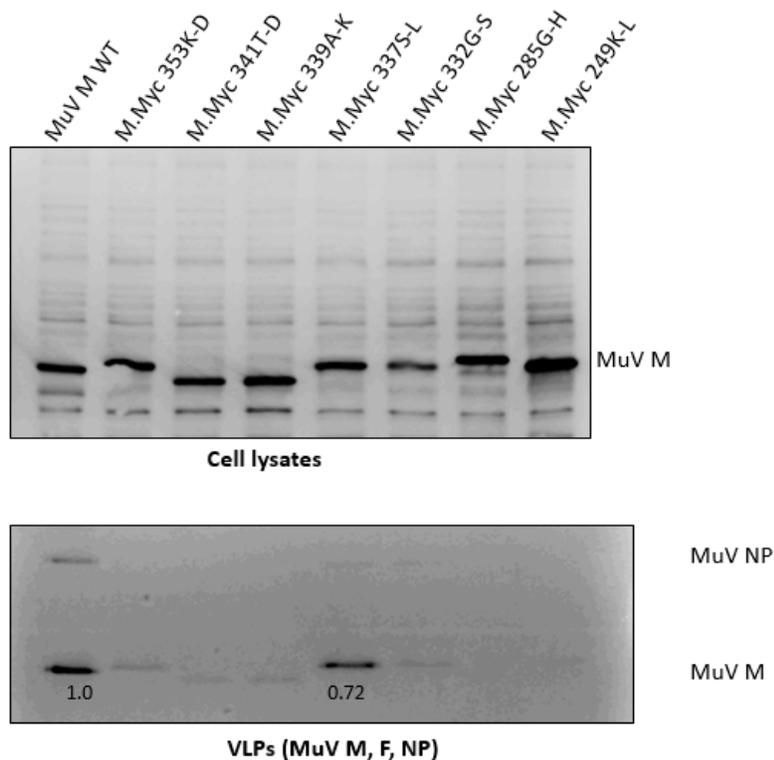
3. Results

3.1 Particle Formation Not Effected with Insertion at Amino Acid Position 337 on Matrix Protein

Previous work done in the lab found that lacking placing a tag on either the N-terminus or C-terminus end of the MuV M protein disrupted its budding function. Therefore, to investigate the possibility of adding a sequence into the matrix protein of the Mumps virus without disrupting normal viral budding, myc tag sequences were added in various internal locations on the M protein. The amino acid locations were determined based on linker regions found in the crystallized structure of Newcastle Disease Virus (NDV) matrix protein. NDV is a paramyxovirus in the genus avulavirus, which is most closely related rubulaviruses.³² Sequence alignment between these two matrix proteins could provide amino acid positions in MuV M, which correspond to linker regions. The linker regions are identified as sequences between sections of the protein that have highly evident secondary and tertiary structures. It was

hypothesized that mutations in this region would be least likely to affect normal protein folding and function.

Seven different M mutants were made with myc sequences inserted at distinctive positions on the protein. These mutants were tested for normal viral budding by performing a virus-like particle experiment with 293T cells transfected with Mumps M, F, and NP vectors. The VLP experiment showed that the only mutant protein that produced virus-like particles comparable to the wild type protein was the mutant with myc inserted at amino acid position 337 (figure 1). The mutants are named as the amino acid position that the myc sequence is inserted at, with the two amino acids that flank the inserted sequence. If a myc-tagged mutant M protein was able to behave like the wildtype M protein, this mutant could be used in experiments so that the M protein could be detected by an anti-myc antibody instead of the less specific polyclonal antibody that is currently used. This work was done prior to my work for this thesis, but serves as a basis for the experiments performed.



293T cells were transfected to produce Mumps NP and F proteins together with the indicated M mutant with sequence insertion at different positions. VLPs from culture supernatants were purified by centrifugation through sucrose cushions followed by flotation on sucrose gradients. The purified VLPs were loaded onto SDS gels and proteins were visualized using a phosphorimager. The VLP efficiency was calculated as the amount of M protein detected in the culture medium divided by the amount of M protein detected in the corresponding cell lysate and was normalized relative to the value obtained with wildtype M.

Figure 1: Testing Various Positions of Myc Sequence Insertion for VLP Production

3.2 Matrix protein insertions at position 337 produce VLPs but show varied dependence on Nucleocapsid Protein

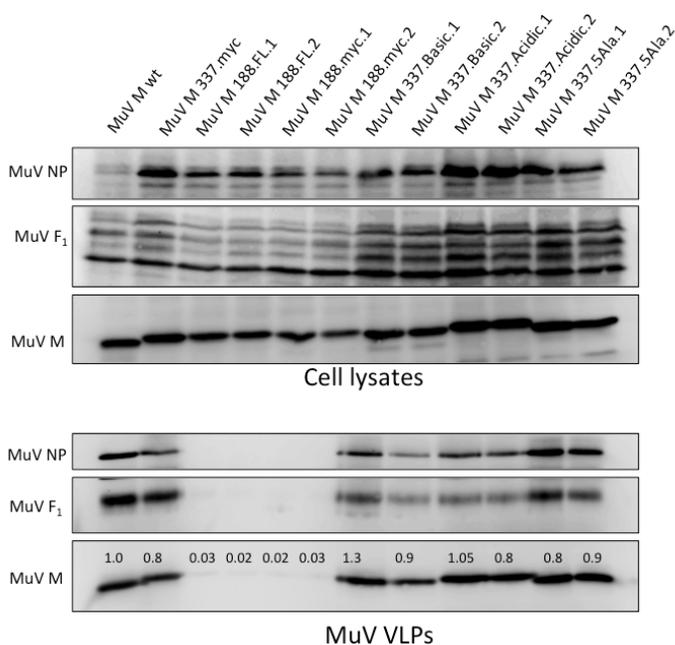
In order to test if various insertions at amino acid position 337 were able to maintain M protein function as seen with the inserted myc sequence, various M protein mutants were made. The mutant M proteins were expressed together with Mumps NP and F proteins in transfected 293T cells. The amount of plasmid DNA used for transfection was selected to maximize particle production, as was previously determined by titration experiments. VLPs were collected from the culture supernatants and purified by flotation on sucrose gradients, then analyzed on SDS gels.

We found that all sequences inserted at amino acid position 337 produced VLPs consistent to VLP production with the wild-type M protein (figure 2).

In previous work it had been determined that in order for Mumps VLPs to be produced, a minimal expression of M, NP, and F proteins was necessary. To test that this dependence was maintained for the mutant M proteins, a VLP experiment was conducted with samples that contained the mutant M protein, F protein, and the NP protein or empty vector. The results were surprising in that only the myc sequence insertion showed a phenotype that VLP production is independent of NP protein (figure 3). Mumps VLPS have been seen when matrix protein is expressed alone in a cell, but nowhere near as efficient as the VLPs seen with M, NP, and F proteins expressed.²³ This result was the first example ever reported of a MuV M protein that produces VLPs efficiently in the absence of NP.

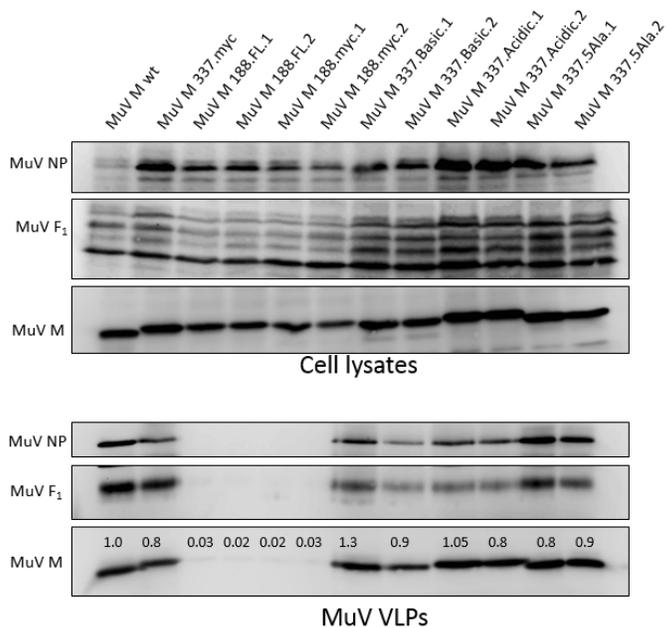
To test the dependence of the M mutant on F protein, another VLP experiment was done that tested the myc-tagged M expressed with either NP and empty vector or F and empty vector (figure 4). The results of this experiment showed that efficient particle formation was found when myc-tagged M was expressed with only the F protein and empty vector but myc-M with only NP and empty vector, hardly any VLPs were detected. Hence, we could conclude that F protein was still essential for efficient particle production but NP protein was not when the myc-tagged M was used. The MuV NP.D4 mutant has a deletion in the NP sequence and fails to bind to MuV M. When wildtype M was expressed with this mutant and F, almost no particle formation was seen but when the myc-M was expressed with this mutant and F there was particle formation as would be expected. It was hypothesized that a reason for the NP independent phenotype of the myc-tagged M was that the insertion caused a conformational change in M that somehow led to increased efficiency of VLP production in the absence of NP protein. This thesis

shows experiments that test what minimal component of the myc sequence could have caused this altered phenotype as well as the rates of degradation of M proteins in different conditions in the cell.



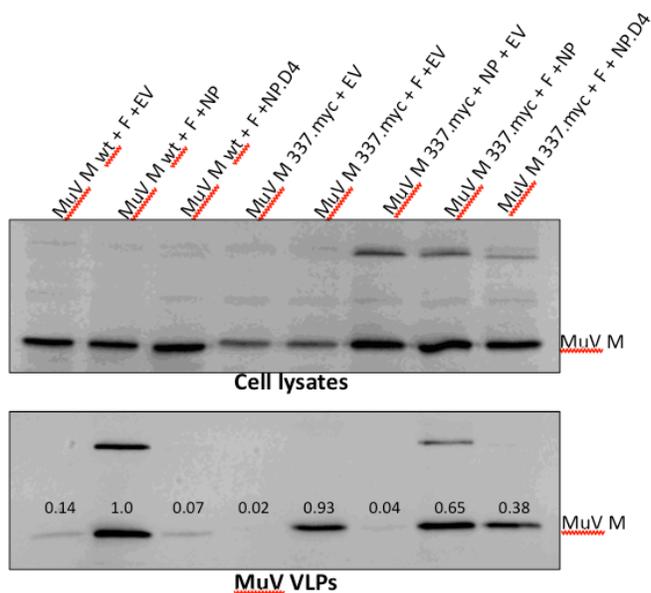
This figure shows the efficiency of particle production when using different M protein mutants. The VLPs were collected and quantified as previously described. The sequences inserted for each mutant can be seen in table 1. The mutants with sequence insertions at position 188 had been previously made but did not produce VLPs and were not used in subsequent experiments.

Figure 2: Flag-tag and charged or uncharged insertions at position 337 of MuV M maintain efficient particle production



VLPs were collected and quantified as previously described. Each M mutant was tested for VLP production with NP protein and without NP protein. For samples with no NP protein an equal amount of empty vector was transfected. The top numbers show the M protein detected normalized relative to the value obtained by the same mutant with NP transfection. The bottom numbers show the M protein detected normalized relative to the value obtained by the wildtype M protein with NP transfection.

Figure 3: Altered MuV M protein functions for VLP production in the absence of NP expression



The VLPs were collected and quantified as previously described. The figure shows relative particle production efficiencies compared to the sample with MuV M wildtype co-expressed with F and NP.

Figure 4: Altered MuV M proteins still require F expression for VLP production

3.3 Restriction Site Sequences Flanking Insertion could be Altering M Conformation

The mutant M proteins tested to this point were made previously by PCR reactions and ligation into a pCAGGS expression vector. The mutants were designed in such a way that an EcoR1 and Mlu1 restriction site was added to either end of the insertion at position 337. These restriction sites have sequences of GAATTC and ACGCGT respectively, both of which contain charged amino acid residues that could have potentially altered M protein conformation. To test whether these restriction sites were involved in the observed phenotype of efficient NP independent VLP production, mutants were made with just the two restriction site sequences inserted into the M protein. We found that these mutants showed VLP production in the absence of NP that was more similar to the wildtype M protein, and with the addition of NP the production of VLPs was significantly increased (figure 5). However, the mutants with restriction sites showed VLP production that was slightly more efficient than the wildtype in the absence of NP, so for future experiments the design of mutants was altered in order to insert a sequence without restriction sites.

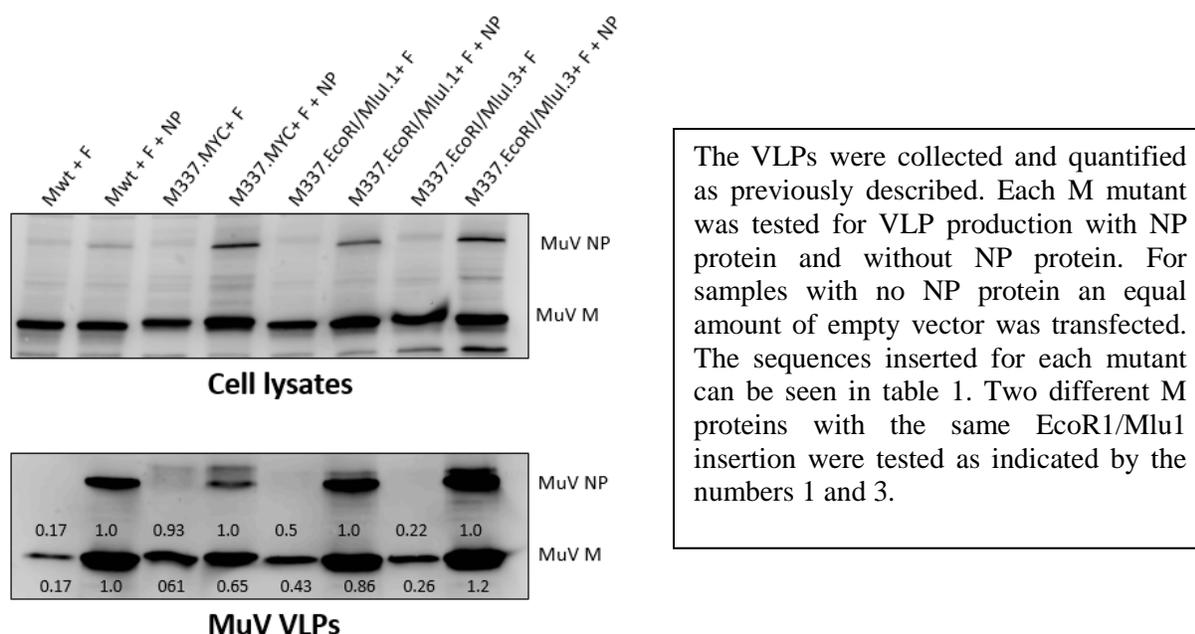


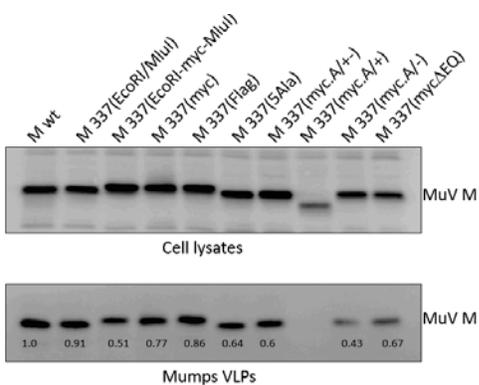
Figure 5: Mutant M with only EcoRI/MluI restriction site sequences inserted shows an increased particle production in the absence of NP

3.4 Testing Various M Mutants for NP-independent Phenotype

The next set of mutants were all made by 2-step PCR and tested in VLP experiments. The new myc and flag tagged mutants were both made without restriction sites. The other mutants were designed to test if the charged amino acids present in the myc sequence were affecting M conformation and causing the phenotype or if the length of sequence insertion was disrupting normal M behavior. Mutants with 5 alanines and 10 alanines inserted were made in order to test if just the length of the insertion, and not the charge, could have caused the NP independent phenotype. Mutants were also made that interchanged all of the charged amino acids in the myc sequence with alanine, or just the positive or negative charges with alanine, to test the effect of

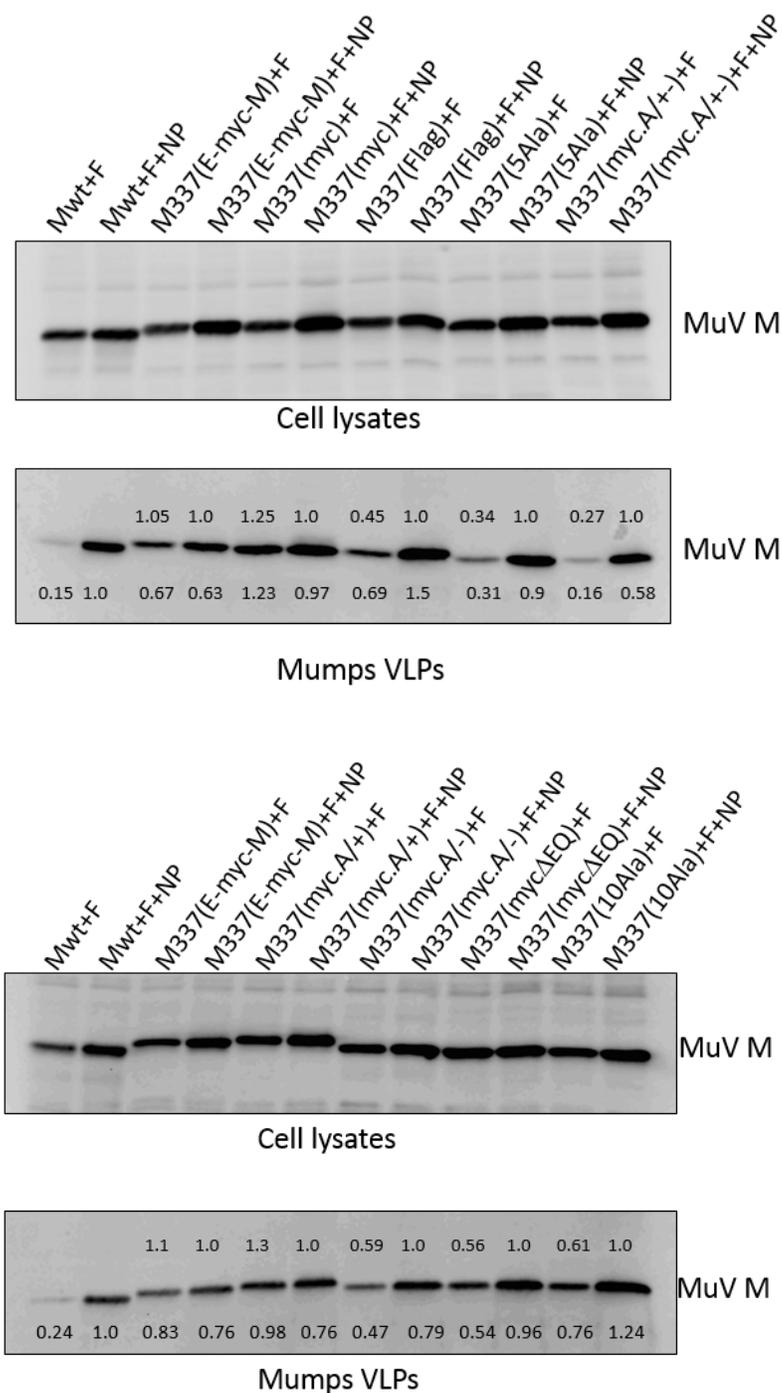
charge in the added sequences for NP dependence in VLPs. The new mutants were all able to produce VLPs when expressed with Mumps NP and F (figure 6).

The next step in the process was to test these mutants for NP dependence as before. The next two figures show all of the new mutants compared with wildtype M in the presence and absence of NP (figure 7). A summary of these results can be represented in a bar graph with the bar indicating the VLP efficiency of each mutant with no NP expressed when the efficiency of each M protein with NP is normalized to 1 (figure 8). The results of these experiments do not show a definitive answer of whether charge or length completely causes the NP-independent phenotype but the mutant that showed NP-dependence closest to wildtype M was the mutant with all charged residues of myc changed to alanine. The mutant with only 5 alanines inserted also showed a phenotype very similar to wildtype M, with the dependence on NP decreasing when the length of insertion is raised to 10 alanines. The mutant with the myc Δ EQ insertion was made because the remaining sequence should be sufficient for the antibody against the myc-epitope to bind. This mutant showed promise because it showed a much greater NP dependence than the full myc-tag sequence but particle production was still greater than the wildtype M in the absence of NP.



The VLPs were collected and quantified as previously described. The sequences inserted for each mutant can be seen in table 1. The mutants with myc sequences where the charged residues are switched to alanines are named according to whether the positively charged residues are replaced with alanines (myc.A/+), the negatively charged residues are replaced (myc.A/-), or both (myc.A/+). The mutant with positive residues changed to alanine was not the right size and a different mutant was used in subsequent experiments that was shown to efficiently produce VLPs (data not shown).

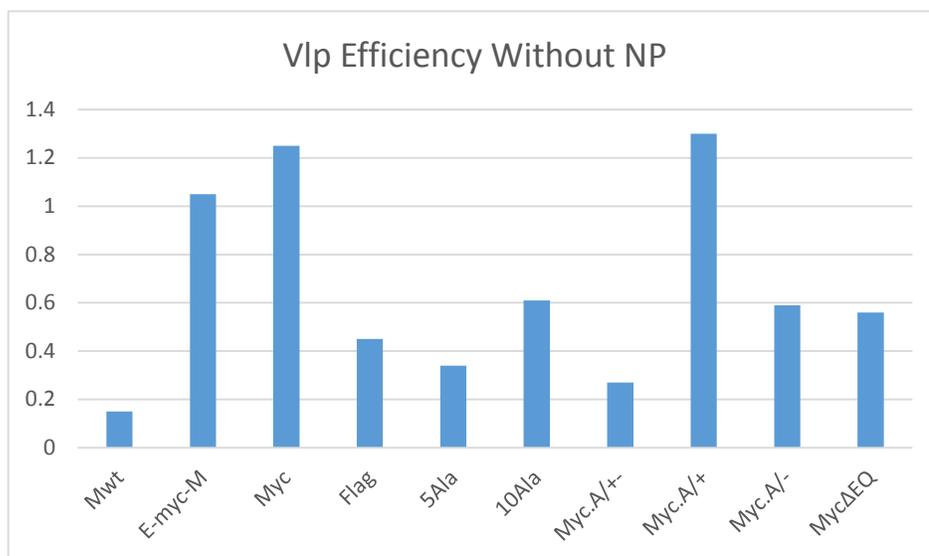
Figure 6: M protein mutants with inserted sequences of various charges and length all maintain efficient particle production



The VLPs were collected and quantified as previously described. Each M mutant was tested for VLP production with NP protein and without NP protein. For samples with no NP protein an equal amount of empty vector was transfected. The top numbers show the amount of M protein detected and normalized relative to the value obtained by the same mutant with NP transfection. These numbers are summarized in figure 8.

Figure 7: Both the length and presence of charged residues in the myc sequence inserted into M contribute to the NP independent phenotype

Figure 8: Summary of the effects of charge and length of inserted sequence in M on NP dependence



The bars show the amount of M protein detected in the culture medium divided by the amount of M protein detected in the corresponding cell lysate and are normalized relative to the value obtained upon the specified M protein + NP + F transfection. The figure shows the relative efficiencies of particle production for each of the M mutants in the absence of NP. The mutant with the myc-tagged insertion where all charged residues are replaced with alanine and the mutant with only 5 alanines inserted both show dependence on NP most similar to the wildtype M for particle production.

3.5 Testing Minimal Sequence Insertion that shows NP-independent Phenotype

To further investigate the effect of a mutation insertion in the M protein another round of mutants were made to test the minimal sequence added that could give the observed NP independent phenotype. The myc sequence was shortened from both the N and C terminal ends to make different length fragments that were inserted into the M protein. All of the mutants made expressed VLPs, besides one, which was not used in subsequent experiments. The mutant with sequence insertion EQKLIS was not the right size and consecutive tries to produce the correct mutant were not successful so this mutant was not analyzed. The mutants were all tested for NP

dependence in an initial experiment, but the wildtype M was not run in this experiment so results are hard to conclude (figure 9). In the following VLP experiment to test for NP dependence none of the mutants showed a clear phenotype, with VLP efficiencies in between the wildtype and full-length myc. The mutant with sequence insertion EQKLISEE showed a promising low efficiency of VLP production in the absence of NP in both experiments, and this mutant should also have sufficient sequence for the myc-antibody to bind.

A summary table of all the mutants tested shows a comparison of the NP-dependent phenotype of wildtype M with each mutant M (table 1).

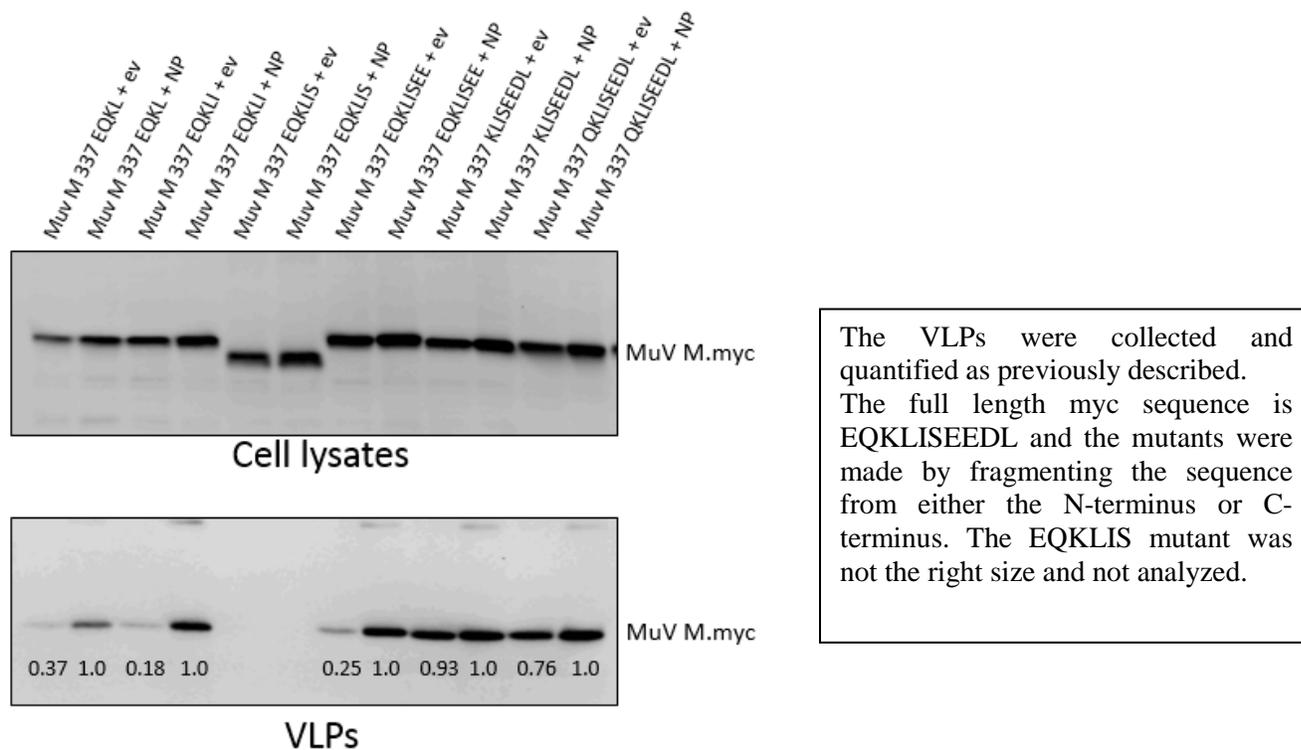
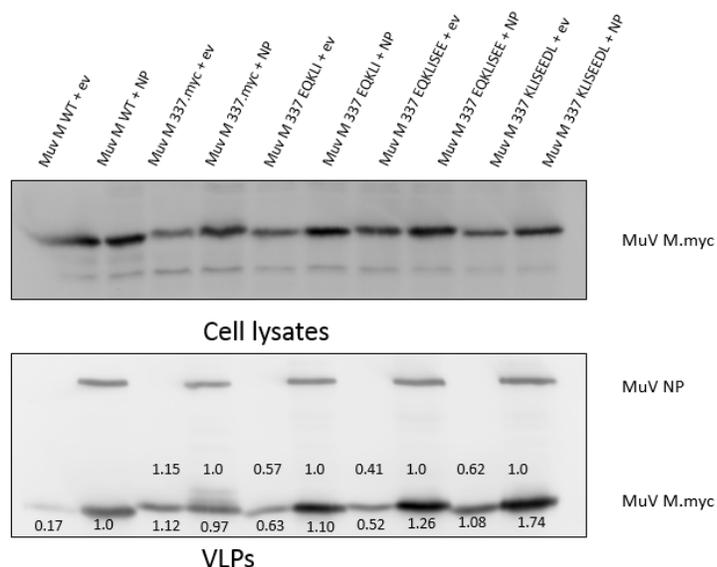


Figure 9: Mutants with fragments of the myc sequence need a critical amount of residues to show NP independence



The VLPs were collected and quantified as previously described. The lower expression of proteins from this experiment could be due to the transfection of old cells or cells that were too dense or sparse upon transfection. The results from this experiment show a more inconclusive result on which fragments of the myc sequence have a NP dependent phenotype similar to the wildtype.

Figure 10: Mutants with fragmented myc sequence insertions show varied NP dependence

Table 1: Summary of sequence insertions and comparison of mutant M NP-dependent phenotypes with wildtype M

Mutant M Protein	Particle Production Efficiency Without NP	Comparison to MuV M wildtype	Amino Acid Sequences of Insertions
E-myc-M	1.1	+	EFEQKLISEEDLTR
E-Basic-M	0.43	+/-	EFKRHRKTR
E-Acidic-M	0.33	-	EFDEEEDTR
EcoR1/Mlu1	0.36	-	EFTR
Myc	1.25	+	EQKLISEEDL
Flag	0.45	+/-	DYKDDDDK
5Ala	0.34	-	AAAAA
10Ala	0.61	+/-	AAAAAAAAAA
Myc(ala/+)	0.27	-	AQALISAAAL
Myc(ala/+)	1.3	+	EQALISEEDL
Myc(ala/-)	0.59	+/-	AQKLISAAAL
KLISEEDL	0.7	+	KLISEEDL
EQKL	0.37	-	EQKL
EQKLI	0.38	-	EQKLI
EQKLISEE	0.33	-	EQKLISEE

The particle production efficiency without NP was determined relative to that mutant's particle production efficiency with NP + F. If more than one experiment was done with each mutant, the average quantification was used. The average quantification of wildtype M without NP was found to be 0.19. Mutants with - indicate phenotype closest to the wildtype M (within 0.25). Mutants with +/- have a phenotype in between wildtype and myc (within 0.5 of wildtype). Mutants with + have a phenotype least similar to wildtype M (over 0.5).

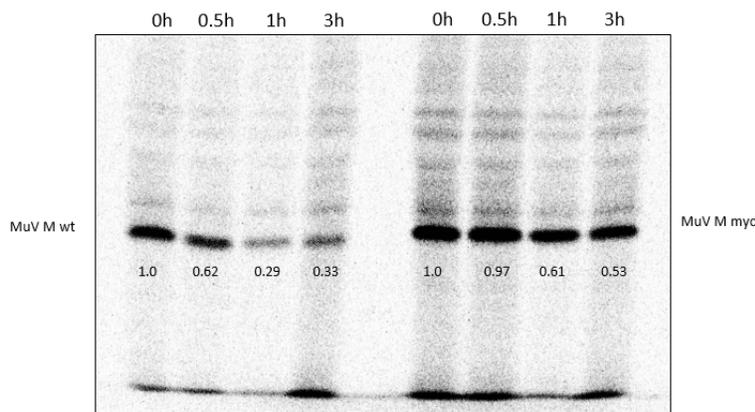
3.6 Turnover Rates of M Protein

An interesting observation was noted with the cell lysates of the VLP experiments done up to this point. The cell lysates show that M protein expression in the presence of NP appears to increase as seen in lanes 1 and 2 of figure 3. The lysates also showed the myc-tagged M expressed better in cells with no NP present than the wildtype M as seen in lanes 1 and 2 of figure 2. These observations suggest that M protein is stabilized upon either NP co-expression or myc-sequence insertion at position 337. To definitely test this possibility, a pulse-chase experiment was set up, where cells would be transfected with the plasmids specified and starved in a medium deficient in cysteine and methionine amino acids so protein synthesis is halted. The cells are then placed in a media with ^{35}S for 30 min so any proteins made during this time period will be radioactively labeled. The cells are then chased with nonradioactive media and collected at different time points to check the amount of radioactively labeled protein that degrades over time.

When comparing wildtype M to the myc-tagged M a very interesting result was seen with the myc-tagged M degrading slower over the three hour time course (figure 12). The next experiment was then done to compare wildtype M with and without NP in cells. This experiment also led to very interesting results in that the wildtype M degraded more quickly in the absence of NP (figure 13). With NP present the wildtype M degraded at a rate consistent with the rate seen for myc-tagged M without NP. These stabilities could help to explain the NP-independent phenotype seen for mutant M proteins. If wildtype M degrades more rapidly in cells than the mutant M, the availability of M protein could be a factor in the NP independent phenotype that is seen with the myc-tagged M.

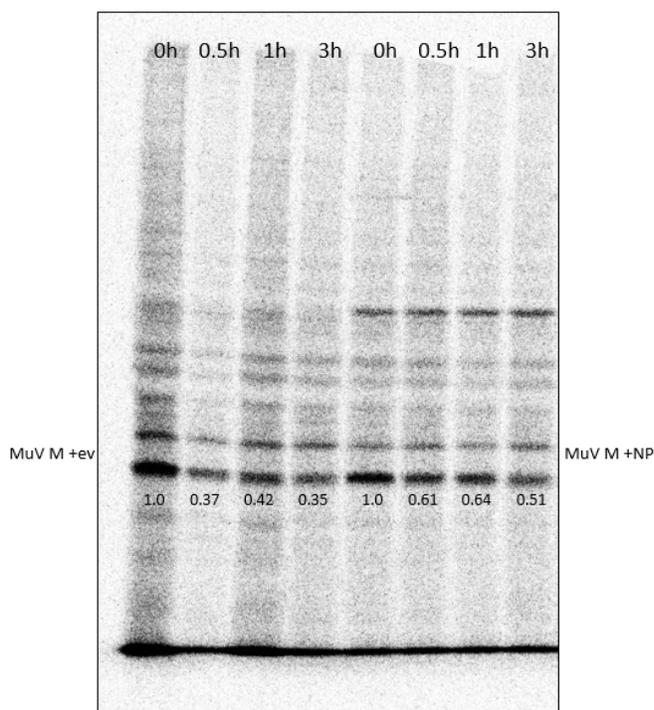
Another pulse-chase experiment was also performed to compare turnover rates of myc-tagged M in the presence and absence of NP. This experiment did not show any clear distinction between M degradation rates with or without NP (figure 14). These results help to elucidate the question of whether NP expression stabilizes M in the same way as the myc-insertion, or by a different way. If stabilization for the myc-tagged M occurred in a different way than NP stabilization, an additive effect might be seen

when the myc-tagged M is co-expressed with NP. Because no additive effect was seen from this experiment it suggests that the effects of myc-tagged M and NP expression are redundant and both stabilize M in the same way.



Cells were radiolabeled with ^{35}S -Promix for 30 min and then incubated in chase medium for the indicated times. The quantification shows relative labeled protein present compared to the 0h time point.

Figure 11: Mutant myc-tagged MuV M shows slower turnover in cells



Cells were transfected with a wildtype MuV M vector and either a NP vector or empty vector. Cells were radiolabeled with ^{35}S -Promix for 30 min and then incubated in chase medium for the indicated times. The quantification shows relative labeled protein present compared to the 0h time point.

Figure 12: Mumps M with NP shows slower turnover in cells similar to myc-tagged M

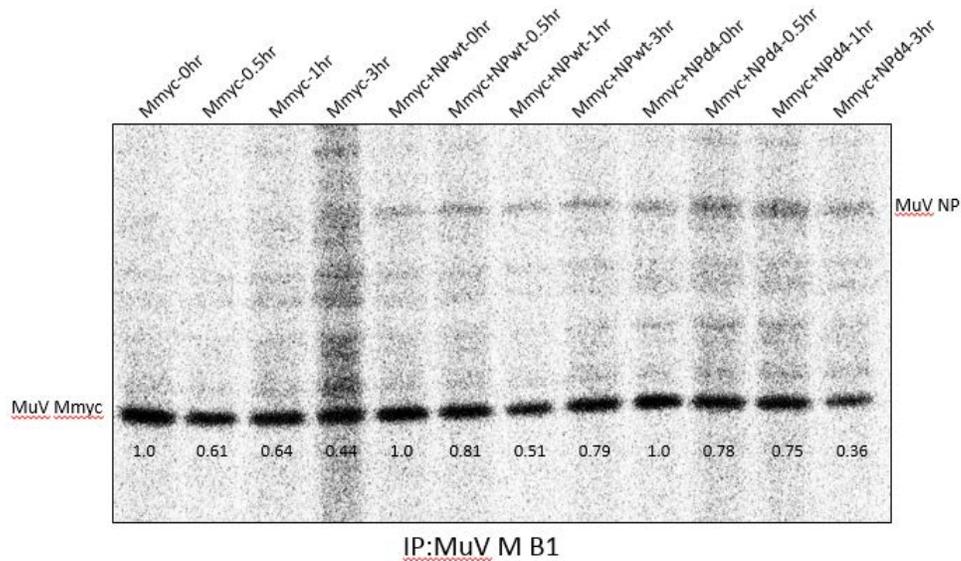


Figure 13: Myc-tagged M in presence and absence of NP show similar degradation rates

Cells were transfected with myc-tagged M and either NP, NPd4, or empty vector. The NPd4 mutant has a deletion in the NP sequence and fails to bind MuV M. Cells were radiolabeled with ^{35}S -Promix for 30 min and then incubated in chase medium for the indicated times. The quantification shows relative labeled protein present compared to the 0h time point.

4. Discussion

4.1 Summary

My original project in lab was to develop a mutant Mumps matrix protein with a myc tag insert that would behave the same as the wildtype protein but could be detected by an anti- myc antibody. Previous experiments performed showed that a sequence inserted at position 337 of the M protein did not disrupt the protein's ability to efficiently bud VLPs. Additionally, in order for

the mutated protein to be used in experiments and the results to be generalized, the mutant must act like the wildtype in VLP budding. This stipulation was broken when a VLP experiment was done that tested the mutants for NP dependence. The myc mutant showed an interesting phenotype in that it appeared to lose its dependence on NP for making efficient VLPs. This observation was novel because past results had concluded that the wildtype MuV M protein along with both F and NP viral proteins were needed for efficient particle production. This finding then spurred the subsequent experiments that tested which part of the myc sequence caused this altered phenotype and what minimum sequence could be inserted that would cause this loss of NP dependence.

Manipulation of the myc sequence to create different mutants of MuV M led to varying results when the mutants were tested for NP dependence for VLP production. The results suggested that characteristics of the myc inserted sequence, such as the length and number of charged amino acid residues, could have affected M conformation and led to its NP dependence. But another observation was made when performing the VLP experiments; the cell lysate expression of the matrix protein appeared greater in samples with NP present compared to samples where empty vector was substituted. This finding led to questions about the stability of the matrix protein in a cell and how these aspects of M could lead to the varied VLP phenotypes seen for mutated M proteins. The pulse-chase experiments were a way to quantifiably detect the amount of newly synthesized M protein that degraded over time. The results suggested that the myc-tagged M protein degraded slower in comparison to the wildtype M, indicating a greater stability or less toxicity in the cells. This degradation rate was similar to wildtype M in the presence of NP, also indicating that NP could play a role in stabilizing M protein in cells. When

looking at the myc-tagged M in the presence or absence of NP, a significant difference in degradation rates was not seen.

4.2 Analysis of NP Dependence of Mutant M Proteins

The first part of this thesis focused on making sequence insertions at amino acid position 337 of MuV M. A summary of the mutants in figure 9 showed that the sequence with all charged amino acids changed to alanines, had a phenotype most closely associated with the wildtype M protein. It also showed that a sequence insertion of only 5 alanines when compared to 10 alanines showed a phenotype more similar to the wildtype, indicating that both charges and length of the inserted sequence could play a role in the M protein losing its dependence on NP protein for particle production. An original hypothesis for the observed phenotype of the myc-tagged M was that it caused a conformational change in M that transformed M into its ‘budding-active’ state. This ‘budding-active’ state of M could be induced by NP interaction in the case of wildtype M to lead to particle production. Therefore, if the mutants caused the M to fold into budding-active conformation, it would explain the gain of function of efficient particle production in the absence of NP.

This hypothesis for a conformational change in MuV M protein for virion production can be further investigated based on the results of this thesis. The observation of many mutants that had a phenotype in between wildtype M and myc-tagged M led to a consideration that something else must be playing a factor. The manipulation of host machinery in many enveloped viruses has been found to be necessary for the viral assembly and budding. In many retroviruses a protein-protein interaction between viral protein and host proteins can be made through

interaction sequences, called late domains, which recruit host factors to virus assembly sites.³³

The M protein of paramyxoviruses, including MuV, do not have the typical late domains of other retroviral proteins but alternative sequences have been found in many of the M proteins that suggest host protein recruitment. Another rubulavirus, PIV5, was found to interact directly with host factor angiomin-like 1 (AmotL1).³⁴ These results suggest that MuV M also interacts with some host factors that are necessary in the progression of viral budding.

The knowledge that MuV M interaction with host proteins in cells is very probable can lead to further suggestions for the results found in this thesis. The variable phenotypes of the different mutants could be explained by the hypothesis that a critical amount of M protein available at the cell membranes is needed for particle production. If MuV M protein interacts with a host protein this could sequester the matrix protein, resulting in the close to zero particle production seen for wildtype M protein in the absence of NP. The NP protein in cells could then have a higher affinity for M protein and competitively bind M, allowing it to locate to the cell membrane and interact with glycoproteins for viral budding. The varying NP dependence of the different mutants could then be explained based on varying affinities of the mutant to the host protein as well as expression in the cells. The amount of M protein available because of both or one of these factors could directly result in the efficiency of VLP production without NP.

4.3 Analysis of M Protein Degradation Rates

The pulse-chase experiments were performed in order to analyze the differences in M protein degradation between the wildtype and myc-tagged M as well as differences when NP protein was present. These results showed a slower degradation when either the myc-tagged M

was expressed alone or the wildtype M was expressed with NP. This led to the hypothesis that NP binding to wildtype M could stabilize the protein, which alone is relatively unstable and quickly degraded by host proteasomes. If the myc-tagged M changed the conformation of M to make it more stable, it could no longer need NP interaction because enough M would be able to aggregate at the cell membranes for particle production. Another observation made while performing these experiments was that cells transfected with wildtype M protein alone appeared very unhealthy with a higher cell death seen 24 hours after transfection. This could indicate that M protein alone in a cell is toxic to the cell, and either a mutation in M or its interaction with NP could decrease the toxic effects of M.

The results of the pulse-chase experiments link together the hypothesis that a budding-active state of M protein can be achieved by either co-expression of NP protein or by insertion of a myc sequence at position 337 and this conformational change in M is what causes the altered stability.

4.4 Future Experiments

Possible future directions based on the results found in this thesis are extensive because of the numerous questions and hypothesis brought up. The results that wildtype MuV M protein could have a lower stability and therefore be more quickly degraded by host proteasomes, or that M protein alone in cells is very toxic to the cell, is a very significant finding that should be further investigated. A pulse-chase experiment performed with MG-132, a potent proteasome inhibitor, could be conducted to see if the lower expression of M over time is because of degradation by a host proteasome. The toxicity of M protein in cells could also be tested by

counting the number of cells before and after a 24-hour transfection with either the myc-tagged M or wildtype M. If a difference was seen, markers of apoptosis could be tested for by RT-PCR or flow cytometry.

The observation of a lower rate of degradation for the myc-tagged M compared to wildtype M was an intriguing observation, but based on experiments in the lab this finding is not the sole reason for the NP dependent phenotype observed with myc-M. VLP experiments performed have shown that under many different conditions, including the amount of M transfected or the time points when MuV VLPs were collected, have always shown that particle production is close to zero if wildtype M is present without NP or F. If the amount of M available in the cell because of its toxicity or stability was the only factor effecting the efficiency of particle production, then production of VLPs with wildtype M in the absence of NP would be seen to vary with different experimental conditions or expression of M in the cells. The mutants made for this thesis showed this variability in particle production without NP, which indicates there is more to the story.

Future experiments to test the reasons for the varying particle production of the M mutants could include VLP experiments where differing amounts of M are expressed with or without NP. If these experiments are conducted for both the wildtype M protein and mutant M proteins, it could give insight into the hypothesis that a critical amount of M is necessary to bud mumps VLPs. The results would also show whether this critical amount is shared for both wildtype M and mutant M proteins because of possible host protein interactions.

The results of this thesis along with future work to be conducted could give more insight into the role of the MuV M protein and its interactions with other viral proteins or host factors in

the cell that leads to viral budding. The knowledge of viral protein interactions and assembly mechanisms is important for potential antiviral targeting.

Appendix A

Primer Sequences for MuV M Mutants

The MuV M forward and reverse primers with restriction site sequences were previously obtained and named:

MuVM5F(not1)

MuVM.Xho1.AS

The following are all primers designed for MuV M mutants:

MuV M Myc

1) MuV M Myc-338.S

5'-ACTGATCTCTGAAGAAGACCTGCTCGCAAAAACCTGATGAC – 3'

2) MuV M 337-Myc.AS

5'-GGCTCAGACTATGCATCAGAACAGAACTGATCTCTGAAGAAGACCTG-3'

Reverse Complement

5'-CAGGTCTTCTTCAGAGATCAGTTTCTGTTCTGATGCATAGTCTGAGCC-3'

MuV M Flag

3) MuV M Flag-338.S

5'-CTACAAAGACGATGACGACAAGCTCGCAAAAACCTGATGAC-3'

4) MuV M 337-Flag.AS

5'-GGCTCAGACTATGCATCAGACTACAAAGACGATGACGACA – 3'

Reverse Complement

5'- TGTCGTCATCGTCTTTGTAGTCTGATGCATAGTCTGAGCC – 3'

MuV M 10Ala

5) MuV M 10Ala-338.S

5'-TGCTGCAGCTGCTGCTGCTGCACTCGCAAAAACCTGATGAC -3'

6) MuV M 337-10Ala.AS

5'- GGCTCAGACTATGCATCAGCTGCAGCTGCTGCAGCTGCTGCTGCTGCA - 3'

Reverse Complement

5'- TGCAGCAGCAGCAGCTGCAGCAGCTGCAGCTGATGCATAGTCTGAGCC– 3'

MuV M 5 Ala

7) MuV M 5Ala-338.S

5'-GCATCAGCTGCAGCTGCTGCACTCGCAAAAACCTGATGACATAATAT-3'

8) MuV M 337-5Ala.AS

5'-CCTCCAGGGCTCAGACTATGCATCAGCTGCAGCTGCTGCACTCGCA – 3'

Reverse complement

5'- TGCGAGTGCAGCAGCTGCAGCTGATGCATAGTCTGAGCCCTGGAGG – 3'

MuV M Myc(Ala/+)

9) MuV M Myc (Ala/+)-338.S (Ala in place of charged residues)

5'- TCTGATCTCTGCTGCTGCTGCTGCTCGCAAAAAGTATGATGAC – 3'

10) MuV M 337-Myc (Ala/+). AS (Ala in place of charged residues)

5'- GGCTCAGACTATGCATCAGCTCAGGCTCTGATCTCTGCTGCTGCTCTG – 3'

Reverse Complement

5'- CAGAGCAGCAGCAGAGATCAGAGCCTGAGCTGATGCATAGTCTGAGCC – 3'

MuV M Myc(Ala/+)

11) MuV M Myc (Ala/+)-338.S

5' – TCTGATCTCTGAAGAAGACCTGCTCGCAAAAAGTATGATGAC – 3'

12) MuV M 337-Myc (Ala/+).AS

5'- GGCTCAGACTATGCATCAGAACAGGCTCTGATCTCTGAAGAAGACCTG – 3'

Reverse Complement

5'- CAGGTCTTCTTCAGAGATCAGAGCCTGTTCTGATGCATAGTCTGAGCC – 3'

MuV M Myc(Ala/-)

13) MuV M Myc (Ala/-)-338.S

5'- ACTGATCTCTGCTGCTGCTGCTGCTCGCAAAAAGTATGATGAC – 3'

14) MuV M 337-Myc (Ala/-).AS

5'- GGCTCAGACTATGCATCAGCTCAGAAAGTATGATCTCTGCTGCTGCTCTG – 3'

Reverse complement

5'-CAGAGCAGCAGCAGAGATCAGTTTCTGAGCTGATGCATAGTCTGAGCC -3'

MuV M KLISEEDL

15) MuV M Myc (KLISEEDL)-338.S

5'- ATCTCTGAAGAAGACCTGCTCGCAAAAAGTATGATGAC-3'

16) MuV M 337-Myc (KLISEEDL).AS

5'- GGCTCAGACTATGCATCAAAAGTATGATCTCTGAAGAAGACCTG-3'

Reverse complement

5'- CAGGTCTTCTTCAGAGATCAGTTTTGATGCATAGTCTGAGCC – 3'

MuV M EQKLISEE

17) MuV M EQKLISEE-338.S

5'-GAACAGAAAGTATGATCTCTGAAGAACTCGCAAAAAGTATGATGAC – 3'

18) MuV M 337-EQKLISEE.AS

5'-GGCTCAGACTATGCATCAGAACAGAAAGTATGATCTCTGAAGAA-3'

Reverse Complement

5'- TTCTTCAGAGATCAGTTTCTGTTCTGATGCATAGTCTGAGCC -3'

MuV M EQKLIS

19) MuV M EQKLIS-338.S

5'-GAACAGAAAGTATGATCTCTCGCAAAAAGTATGATGAC – 3'

20) MuV M 337-EQKLIS.AS

5'-GGCTCAGACTATGCATCAGAACAGAAACTGATCTCT-3'

Reverse Complement

5'- AGAGATCAGTTTCTGTTCTGATGCATAGTCTGAGCC -3'

MuV M EQKLI

21) MuV M EQKLI-338.S

5'-GAACAGAAACTGATCCTCGCAAAAAGTATGAC – 3'

22) MuV M 337-EQKLI.AS

5'-GGCTCAGACTATGCATCAGAACAGAAACTGATC-3'

Reverse Complement

5'- GATCAGTTTCTGTTCTGATGCATAGTCTGAGCC -3'

MuV M EQKL

23) MuV M EQKL-338.S

5'-GCATCAGAACAGAAACTGCTCGCAAAAAGTATGAC – 3'

24) MuV M 337-EQKL.AS

5'-GGCTCAGACTATGCATCAGAACAGAAACTGCTCGCA-3'

Reverse Complement

5'- TGCAGCAGTTTCTGTTCTGATGCATAGTCTGAGCC -3'

MuV M QKLISEEDL

25) MuV M QKLISEEDL-338.S

5'-CAGAAACTGATCTCTGAAGAAGACCTGCTCGCAAAAAGTATGAC – 3'

26) MuV M 337-QKLISEEDL.AS

5'-GGCTCAGACTATGCATCACAGAAACTGATCTCTGAAGAAGACCTG-3'

Reverse Complement

5'- CAGGTCTTCTTCAGAGATCAGTTTCTGTGATGCATAGTCTGAGCC -3'

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