GENERATION OF RECOMBINANT MAREK'S DISEASE VIRUSES *IN VIVO*

by

Dawn Lenihan

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Approved: _____

Mark S. Parcells, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____

Carl J. Schmidt, Ph.D.
Committee member from the Department of Animal and Food Science

Approved: _____

Jung-Youn Lee, Ph.D.
Committee member from the Board of Senior Thesis Readers

Approved: _____

Alan Fox, Ph.D.
Director, University Honors Program

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ABSTRACT

Marek's Disease Virus is a highly contagious herpesvirus that causes T-cell lymphomas in chickens. Although there are vaccines which prevent lymphoma formation, MDV has increased in virulence as new strains evolve in the field. Mutations have been identified in the *meq* gene of MDV strains that appear to directly correspond to the virulence level of the virus. The first part of this project will attempt to create recombinant viruses using MD5Δ*meq* in which the *meq* genes have been deleted from the MD5 strain of the virus. MD5Δ*meq* replicates in chickens, but will not cause disease. The *meq* gene from three different strains; JM, RB1B and MK will then be combined with MD5 through co-transfection of the MD5Δ*meq* with the corresponding *meq* locus from each strain, creating viruses that differ only in the *meq* gene. For the second part of this project, an MD5-based construct was made that had *lox*P recombination sites flanking the *meq* loci. This insert was also co-transfected with the MD5Δ*meq* virus in order to generate recombinant viruses with *meq* regions that can be interchanged using Cre recombinase. Since viruses that have recombined the *meq* gene into their genome cannot be selected in cell culture, chickens were inoculated with co-transfected cells for the selection of recombinants through tumor formation. Using this *in vivo* selection method, we have previously generated an MD5:RB1B Meq virus. Any recombinants isolated during this experiment will be used to evaluate the effect of *meq* mutations on changes in MDV virulence.

Chapter 1

INTRODUCTION

1.1 Marek's Disease

Marek's Disease (MD) is a T-cell lymphoma of chickens caused by a herpesvirus known as Marek's Disease Virus (MDV) (16). MD is a significant concern in commercial poultry production due to its highly contagious nature and prevalence in the field. Marek's Disease was first described by Jozsef Marek in 1907 as a disease causing polyneuritis or inflammation of the major nerves, specifically the sciatic nerve and certain areas of the spinal cord, in chickens (16). This description remained consistent through the early 1950s. Although there is no isolated virus from this time period, based on descriptions it is thought to be a mild form of the virus (mMDV) (21). Then, from the late 1950s through the 1960s an acute form of MD was discovered that caused explosive outbreaks and led to very high mortality (2). This virus was described as having 40% mortality in layers, and caused lymphomas of visceral organs in addition to the nerve lesions previously encountered (21). Unfortunately, through the early 1960s Marek's Disease was often confused with lymphoid lukosis, a disease with very similar symptoms to MD (2). This confusion prevented significant progress from being made in the study of the disease.

In the late 1960s, Marek's Disease was finally identified as a herpesvirus, and vaccines to prevent the disease were quickly developed (16). The first vaccine was attenuated HPRS-16, an acute MDV-1 strain. However, turkey herpesvirus 1 (HVT), a live vaccine which was very similar to MDV, became the preferred vaccine because it

did not cause the disease in chickens (16). This vaccine initially reduced the incidence of Marek's Disease by 99% (16). Although vaccination against MD is highly effective, it has apparently contributed to the evolution of field strains of increased virulence. Another form of the virus was discovered in the late 1970s that infected vaccinated chickens at a frequency higher than would be expected (21). Viruses isolated from this period were found to be more virulent than previously isolated viruses (21). This increase in virulence continued into the early 1980s when vvMDVs were first discovered. Due to the increasing virulence of the virus, combination vaccines have been developed using combinations of HVT and MDV-2 (16). However, strains are continuing to develop that are resistant to even the best vaccines and combinations of vaccines (16). Therefore, it is important to determine the mechanism of increased virulence in order to create more effective vaccination programs.

1.2 Marek's Disease Virus

Marek's Disease is caused by Marek's Disease Virus (MDV), an oncogenic herpesvirus (16). MDV causes acute lymphoproliferative disease in chickens, which leads to T-cell lymphomas in both the visceral organs and peripheral nerves (16). These T-cell lymphomas, along with the slow growth of this virus in cell culture led to its initial characterization as a *Gammaherpesvirus* closely related to Epstein-Barr virus (EBV) (16). However, analysis of MDV using electron-microscopy found repeat structures in the virus that are more characteristic of an *Alphaherpesvirus* (16). This new classification was later confirmed using restriction enzyme mapping and DNA sequencing of the virus. Marek's Disease Virus belongs to the genus *Mardivirus* along with MDV-2 (GaHV-3) and Turkey herpesvirus 1 (Maleagrad

herpesvirus 1) also known as MDV-3 (16). However, MDV is the only one that is pathogenic.

The various strains of MDV have been placed in three different levels of virulence; virulent MDVs (vMDVs), very virulent MDVs (vvMDVs) and very virulent plus MDVs (vv+MDVs) (21). Classification of viruses into these different levels is based on their response to vaccination with various commercial vaccines and combinations of vaccines (21). The increasing virulence of the virus has been coupled with a change in the symptoms associated with MDV. Although signs have remained largely neurological, they have increased in severity from the polyneuritis originally described. Visceral lymphomas were added to the list of symptoms in 1925 (16). More recently, tumors from more aggressive viruses were isolated that develop more quickly that previously observed. Currently, very virulent and very virulent plus strains of MDV are in the field. They are associated with paralysis in chickens and very virulent plus strains can even cause massive brain lesions and death in vaccinated chickens (16). Marek's Disease Virus continuously evolves toward greater virulence and in turn greater resistance to vaccines.

1.2.1 The MDV Genome

Marek's Disease Virus is a double stranded DNA virus (16). The genome of the virus contains a unique long (U_L) and unique short (U_S) segment flanked by inverted repeats: terminal and internal repeats long (TR_L and IR_L respectively), and terminal and internal repeats short (TR_S and IR_S respectively) (16). The TR_L and IR_L regions of the genome contain genes unique to MDV such as viral interleukin 8 (vIL-8), a chemokine homolog that may affect recruitment of target cells, Meq and vTR, that are involved in the formation of tumors in the chicken (16).

MDV is genetically very similar to human herpesvirus 1 (Herpes simplex virus-1, HSV-1) and human herpesvirus 3 also known as the varicella-zoster virus (VZV) (16). VZV is the most closely related to MDV, with only 5 genes from VZV absent in the MDV genome (16). Most genes in the U_L and U_S segments of MDV are homologous to HSV-1 and VZV. The genes that are unique to MDV are found mostly in the TR_L and IR_L regions of the genome and in the unique short open reading frames (SORFs 1-4) (16). The main distinction between VZV and MDV are the large inverted repeats surrounding the U_L region in MDV (16).

1.3 MDV Pathogenesis

Marek's Disease virus has the ability to exist for long periods of time in the environment (16). Therefore, most chickens will come into contact with the virus at some point, making vaccination against MDV an important part of any poultry operation. The pathogenesis of MDV is of particular interest because it serves as a good model for some human diseases. The early stages of the virus are very closely related to chickenpox in humans, which is the result of VZV infection (16). MDV enters the host through inhalation. Once within the host, there are four main stages of the virus replication: early cytolytic infection, latency, secondary cytolytic infection, and transformation (16).

1.3.1 Early Cytolytic Infection

Once inhaled by the host, MDV enters the respiratory tract. There, the macrophages within the respiratory tract become infected, either immediately or directly after the virus has replicated in epithelial cells (16). The virus then spreads

from the respiratory tract throughout the body. Within 24 hours, MDV can be isolated from the spleen, thymus and bursa of Fabricious (16).

In the first phase of cytolytic infection, B cells become infected. MDV then infects $CD4^+$ T cells that have been activated (16). The peak of virus replication within these cells occurs between 3-7 days post infection (p.i.) (16). Infected $CD4^+$ T cells allow the virus to spread within the animal, and to the skin, eventually leading to horizontal transmission of the virus.

Another important component of early cytolytic infection is the downregulation of major histocompatibility complex (MHC) class I molecules from the surface of cells that have been infected with the virus (7). MHC class I molecules have binding affinities with CD8+ CTL receptors which allows them to detect and destroy infectious agents (10). Downregulation of these molecules is mediated by an MDV immediate early or early gene product and is maintained during the entire lytic cycle (16). This essentially makes the infected cells invisible to the CD8+ cytotoxic T-cells (CTLs). Downregulation of MHC class I molecules is thought to be important in the secondary cytolytic stage of infection.

1.3.2 Latency

MDV enters the latent phase at around 7 days post infection. Latency is defined as the "presence and maintenance of viral genomes without production of infectious progeny virus" (16). $CD4^+$ T cells are the main reservoir for latent MDV, although latent forms of the virus have been isolated in B cells, $CD4^-CD8^-$ T cells and $CD8^+$ T cells (15). Transcription of the viral genome during latency is limited to the repeated regions of the virus (16).

Very little is known about the actual mechanisms that lead to latency. This is partially due to the fact that it is hard to distinguish between latently infected cells and transformed cells and the transition from a latent to a transformed state is difficult to determine (16). In addition, the exact number of MDV genes involved in establishing latency is difficult to determine. This is mainly because the experiment to determine the number of latent phase MDV genes used lymphoblastoid cell lines that had been infected with MDV, which are known to spontaneously reactivate (16). This most likely led to an underestimation of the number of MDV genes in the latent stage of infection. Further research is needed to better understand the mechanisms involved in latency.

1.3.3 Secondary Cytolytic Infection

During the secondary cytolytic infection, MDV is reactivated from the latency and infects peripheral lymphocytic and epithelioid tissues (Peyer's patches, Schwann cells, and the Feather Follicle Epithelium [FFE]). Downregulation of MHC class I molecules occurs during reactivation and may allow a level of infection even in the presence of MDV-restricted CTL. MDV then spreads to the visceral organs and epithelia, establishing peripheral sites of infection in the host (16). Although this phase occurs after the latent phase, it is not clear if secondary cytolytic infection is initiated by latently infected T cells or lytically infected T cells (16).

1.3.4 Transformation

Transformation of infected $CD4^+$ T cells is the final stage of MDV infection. This occurs within 2-6 weeks p.i. and is followed by lymphoma formation (16). Cells that have been transformed by the virus are very similar to latently infected

cells, indicating that latent infection may be a necessary precursor to transformation (16). Definition of the transformed component of MDV-induced lymphomas however, has shown that transformed cells show higher expression levels of Meq, surface antigen CD30 and downregulation of surface antigen CD28, a costimulatory molecule essential for T-cell activation (4). MDV does integrate into the genome of transformed cells; however, incorporation of MDV DNA into host chromosomes appears to be random (6). All cells obtained from MD lymphomas contain integrated MDV genomes, suggesting that integration of MDV genomes into the host cell may be a necessary precursor to lymphoma formation (6). The actual percentage of infected cells that will be transformed remains to be determined.

MDV is very easily transmitted between chickens that come in contact with one another through inhalation of infectious dander (16). As infected T cells transport virus to the skin, free infectious virus is produced in the terminally differentiated epithelial cells within the feather follicles (FFE) (16).The free infectious virus, as well as dander-associated virus, is then shed and can infect chickens that inhale the infected dander. Chickens can be infected with MDV either directly or indirectly, with no direct contact with the virus required for infection (2).

1.4 Meq

Marek's *Eco*RI Q-encoded protein or *Meq* is the most extensively studied MDV gene (8). *Meq* is a 339 amino acid protein encoded in the MDV *Eco*RI-Q fragment of the serotype 1 MDV strain (8). A basic leucine zipper protein, *Meq* is very structurally similar to the Jun/Fos oncoproteins and has been considered to be the main oncogene in MDV as it is consistently expressed in tumor cells induced by MDV (13). There are two copies of *meq* in the MDV genome. One copy is present in each repeat

surrounding the U_L region of the genome (8). *Meq* has many functions and contains domains for DNA binding and dimerization (11). Additionally, *meq* has transactivation and repression activities within MDV (11).

1.4.1 Discovery of Meq

In chickens infected with MDV, T cell lymphomas are present within 2-6 weeks post infection (16). This indicates that MDV most likely encodes an oncogene, prompting those studying the virus to begin searching for a possible oncogene. The transcriptional activity of the virus in tumor cells focused the search to genes that were expressed in the tumors (8). The search was further refined to repeat regions within the tumors, which were found to be highly expressed in MDV-induced tumors and cells lines established from those tumors (8). The genes within this region included *meq*, pp38 , pp14 and others (13). After further analysis of the region, it was found that *meq* was consistently expressed in all MDV tumor cells and that pp38 was lytic infection associated (8). Further evidence that *meq* was indeed the oncogene for MDV is the fact that *meq* is not present in the nononcogenic serotypes 2 and 3 of the virus.

1.4.2 Meq and Transformation

In an experiment to determine the role of the *meq* gene in tumor formation, Lupiani, et al., (2004), generated a recombinant virus rMD5Δ*meq* in which both copies of the *meq* gene had been deleted from a very virulent strain of MDV (13). From the results of this experiment, it was found that chickens infected with this recombinant virus containing *meq* deletions did not develop MDV-associated lymphomas. These data further suggested that *meq* is involved in lymphocyte transformation. Other data supporting the role of Meq in MDV-mediated

transformation came from the laboratory of Dr. Hsing-Jien Kung, whose laboratory discovered Meq (8). They showed that Meq formed heterodimers with the c-Jun protein, bound to the MDV genome as homodimers and shut down several lytic-phase genes (Levy et al 2003). Furthermore *meq* transformed fibroblasts *in vitro* by activating proliferation-associated genes, while downregulating apoptosis-associated genes (10, 11). In addition, further study of *Meq* showed that inhibition of *meq* in MDV-transformed tumor cells led to growth inhibition in the host (13). From this result it can be concluded that in addition to helping regulate latency and transformation, *meq* is also essential for transformation (13).

A possible mechanism by which *meq* transforms cells has been suggested. First, *Meq* and Jun interact to activate a Jun/AP-1 pathway (11). Activation of this pathway causes upregulation of *meq* transcription (16). Within transformed T cells, *Meq* and Jun bind to the AP-1 site of an IL-2 promoter, activating the IL-2 autocrine loop (10). *Meq* can also bind to RB, p53 and cyclin-depedant kinase 2, all factors involved in cell cycle control, as well as the transcriptional repressor complex protein CtBP (3). These interactions of meq with factors involved in cell cycle control prevent T-cells from undergoing apoptosis. T-cells can then function for longer periods of time and maintain the latent phase.

1.4.3 Meq and MDV Lytic Infection

In the experiment previously described, Lupiani *et al*, also examined the function of *meq* on virus replication. It was found that *meq* was not necessary for virus replication *in-vitro*. In addition, lack of *meq* genes in the MDV genome did not impair *in-vivo* virus replication in lymphoid organs and feather epithelium (13). These

findings suggest that, although *meq* is involved in transformation, it is not responsible for lytic infection in chickens (13).

1.4.4 Mutations in Meq and MDV Virulence

All current research on Marek's Disease indicates that MDV continues to evolve strains of greater virulence. Shamblin, et al., (2004), analyzed strains of MDV of different levels of virulence to determine if mutations in a particular gene were responsible for the increase in virulence of the virus. Specifically, they looked at major glycoprotein genes, pp38, and *meq*. For major glycoprotein genes and pp38, no mutations were found that corresponded to virulence of the virus. However, it was found that the *meq* gene contained polymorphisms and point mutations that seemed to directly correlate with MDV virulence.

The different mutations found in *meq* were consistent with the virulence level of the virus. Lower virulence MDVs had point mutations at positions 71 and 77 in the amino terminus of the genome (20). In addition, these strains had an amplification of the proline-rich repeat region of the gene (20). These strains contained various numbers of a 21 amino acid repeat sequence that was flanked by the tandem reiteration of four prolines within the proline-rich area (16). In higher virulence MDVs, point mutations were found within these tandem reiterations of proline at position 2 (20). This second proline was often changed to either alanine (A) or glutamine (Q) (16).

The effects of these mutations on MDV virulence may correspond to the ability of *meq* to bind to DNA and transactivate or transrepress genes (20). The transactivation domain of *meq* was at the extreme C-terminus, while the repression domains were in the amino terminus and in the proline-rich repeat regions (20). When

these proline-rich regions remained uninterrupted in the lower virulence MDVs, it led to better repression of the virus. However, vv+ MDVs contained interruptions in the proline-rich repeat regions, with the most virulent viruses having the most interruptions (20). These interruptions ostensibly hinder the repression of the virus, leading to greater transactivation by the Meq protein, which may be important to virulence.

Interestingly, the *meq* mutations observed have all occurred in chickens that were vaccinated with viruses that do not encode the *meq* genes (MDV-2 and HVT) (20). Therefore, these mutations are most likely not due to selection of virus that has developed immunity to the vaccine, but rather selection by the virus may be due to the increased level of virulence and infection caused by the mutation, or some functional selection that may be mediated by changes in Meq or some other gene (20). Therefore, a better understanding of the actual mechanism by which *meq* increases the virulence of MDV is necessary in order to create better vaccines.

1.5 Hypothesis

Our current hypothesis is that *meq* influences virulence through altering the shape and/or motility of latently-infected T-cells, perhaps making them less susceptible to being killed by cytotoxic T cells (CTLs) elicited by vaccination. To test this hypothesis, we have attempted to generate recombinant MDVs containing the *meq* genes of different virulence MDVs in the context of an identical genetic background. Since an MD5-based recombinant MDV in which both copies of the *meq* gene had been deleted did not cause tumors, we hypothesized that recombinant MDVs which have incorporated *meq* could be selected on the basis of their ability to cause tumors *in vivo.*

1.5.1 Role of Meq in Increased Virulence

In a previous experiment, Abouzahr, et al. (2006), analyzed human lung cancer tumors that had become resistant to T cell lysis after undergoing treatment with specific cytoloytic T lymphocytes (CTLs) (1). These tumor cells were found to have a significantly increased resistance to being killed by CTLs. After further analysis of these resistant tumors, Abouzahr et al., (2006) discovered that the tumor cells have morphological changes mediated by changes in the actin cytoskeleton (1). Specifically, there was overexpression of the actin genes ephrin-A1 and scinderin in the resistant tumors. This overexpression may alter actin polymerization, changing the susceptibility of the tumor to CTL cells. Abouzahr et al., (2006) also discovered that when ephrin-A1 and scinderin are silenced, the resistant tumor returns to a state of normal vulnerability to CTLs, providing further evidence that these genes are important in the creation of resistant tumors (1).

Abouzahr et al., (2006) also analyzed the synapse between the tumor cells and CTLs. In normal tumors, these junctions had a very tight interaction at the synapse. However, in resistant tumors, the synapses were much more loosely formed than the normal tumor synapses. Tight synapses are required for effective CTL cytotoxicity. CTLs act by rapidly transferring granules to the tumor, which then fuse with the plasma membrane of the tumor. These loose synapses hinder the ability of the CTL to release granules into the tumor, explaining the resistance some tumors have developed to CTL killing. Resistant tumors were also observed to be a more round shape, with less extensions projecting from the cell than normal tumors. This may effect synapse formation, and the organization of the actin cytoskeleton.

We hypothesize that *meq* functions in the same way in MDV, as Meq-target genes identified by Levy et al., (2005), included a major subset of cytoskeleton

regulatory genes (11). We hypothesize that *Meq* may therefore alter the shape of the latently infected T cells, which could affect its ability to form synapses with the CTLs, making the T cells less susceptible to being killed by the CTLs. Other morphological changes to the T cells, such as in the actin cytoskeleton, may also be caused either directly or indirectly by the *meq* gene. This increased resistance of T cells to CTL killing would explain the correlation between the *meq* gene and increased virulence in MDV.

1.5.2 Results from Prior *In Vivo* Selection Experiment

A previous experiment in this lab used an MD5-based strain of MDV from which both copies of the *meq* gene were deleted. This strain was then co-transfected with *meq* loci from different virulence MDVs which were amplified from vMDV (JM102), vvMDV (RB1B) and vv+MDV (MK) strains of the virus. These passaged transfected cells, which ostensibly contained some recombinant viruses were then inoculated into chickens to select for recombinant viruses through Meq expression. Using this method, one recombinant virus was isolated that had the *meq* gene of the vvMDV, RB1B strain inserted into the MD5 genome. Isolations of recombinant viruses with *meq* genes from JM102, a lower virulence strain, or MK, a higher virulence strain, were not recovered. In order to actually compare different strains of *meq* and determine patterns associated with MDV virulence, recombinant viruses must be constructed with all different strains of *meq* in one identical genetic background.

1.5.3 General Plan of Research, Rationale, and Modifications to Previous Work

The first part of this project will repeat the previously described experiment to determine if recombinant viruses can in fact be isolated from the other

two strains, or if the vvMDV from RB1B is again the most lymphomagenic form of Meq. The second part of this project provides an alternative way in which the *meq* gene from various strains can be compared. By adding loxP sites on either side of the *meq* gene in the MD5 strain of MDV we hope to generate a recombinant virus that will allow the *meq* loci to be removed and *meq* genes from other strains to be inserted directly into the genome using the Cre recombinase – loxP system. Cre is the 38-kDa product of the cre gene of bacteriophage P1 (19). LoxP, which stands for locus of X-over P1, is a 34-bp site on the P1 genome that can be recognized by Cre (19). Cre can then catalyze a DNA recombination between two *lox*P sites, which causes the DNA between these two sites to be excised from the genome (19). In this experiment, placing *lox*P sites on either side of the *meq* gene will result in the excision of the *meq* gene from the MDV genome after treatment with Cre. Other forms of the *meq* gene can then be inserted directly into the genome. In this way, the *meq* gene from each strain can be compared without isolating a recombinant virus from each individual strain of MDV.

Chapter 2

MATERIALS AND METHODS

2.1 Cells and Viruses

All MDV strains used were propagated in secondary chicken embryo fibroblasts (CEF) using standard methods(14). The cosmid clone-derived MD5 and *meq*-deletion virus, MD5ΔMeq were described previously(13), and were the generous gifts of Dr. Sanjay Reddy, Texas A&M University. These strains were generated using a series of overlapping cosmid clones, comprising the entire MDV genome. The JM102 strain used for PCR amplification of the *meq* locus was that found in the cell line CU210, provided Dr. Karel A. Schat, Cornell University. Similarly, the RB1B strain used for amplification of the *meq* locus was at passage 12 and was obtained from Dr. Robin W. Morgan, University of Delaware. The MK, a.k.a N strain, was obtained from Dr. John K. Rosenberger, Aviserve LLC.

2.2 Transfer Vector Construction

Meq loci from JM102, RB1B, MD5, and MK strains of MDV were amplified using primers flanking the *Eco*RI-Q fragment of each virus. PCR was performed using Platinum Taq Supermix (Invitrogen) or Acuzyme *Pfu* polymerase (Invitrogen) followed by a TA-tailing reaction of 15 minutes using Platinum Taq. Each PCR product was cloned into pCR2.1 Topo (Invitrogen) and positive clones were screened by restriction endonuclease digestion. The DNA sequence of each *Eco*R1-Q fragment was determined using a combination of primers (20). Plasmid

DNA sequencing was performed at the University of Delaware Sequencing Facility using an ABI Prism 3130XI, Genetic Analyzer (http://www.dbi.udel.edu/core/dnasequencing.html).

In order to insert *lox*P recombination sites into the MDV genome flanking the *meq* gene (floxing the *meq* loci), we designed primers to amplify the *Eco*RI-Q fragment having *lox*P sites incorporated at their 5'-ends (Table 1). This PCR reaction was performed using Platinum Taq and Pfx polymerases and employed 40 cycles with an extension time of 2.5 minutes. The amplified products (2.4 – 2.6 kbp) were cloned into pCR2.1 Topo and several clones were submitted for DNA sequence analysis. The amplified *lox*P-flanked *meq* loci have been cloned from JM102, RB1B, MD5, and MK (N) strains of MDV. To date, the *lox*P-flanked locus from MD5 has been fully sequenced and was found to contain no mutations. This MD5 fragment was used for generating the larger transfer vector.

To provide flanking sequences for the introduced *lox*P sites, a 4.4 kbp fragment from the *meq* locus was amplified and cloned into pCR2.1 Topo (Table 1). This transfer vector (TV) had *Nhe* 1 and *Hin*D III sites introduced at 5' and 3' ends respectively. TVs were generated from JM102, RB1B, MD5, and MK strains of MDV. The *meq* segment isolated and amplified from each strain was then digested using enzymes *Nhe* 1 and *Hin*D III. This insert was gel purified and ligated into the vector pUC19ΔE, a pUC19 derivative in which the EcoRI site had been destroyed, which had been digested with *Xba* I and *Hin*D III. Restriction endonucleases *Xba* I and *Nhe* I generate compatible cohesive termini and there are no additional *Nhe* I or *Hin*D III sites within the coding sequence of the *lox*P-flanked EcoRI-Q fragment of TV.

The rationale for subcloning the large TV into pUC19ΔE, is to have the large flanking sequence from which *Eco*RI-Q fragments could be rapidly replaced with the *lox*P-flanked *Eco*RI-Q fragments. After insertion of the *lox*P-flanked *Eco*RI-Q into the TV, the orientation of the inserted cassette was assessed by restriction endonuclease cleavage analysis. Clones having the *lox*P-flanked *Eco*RI-Q fragment in the proper orientation were then used for co-transfection with MD5ΔMeq DNA.

All *lox*P-flanked *Eco*RI-Q fragments, *meq* loci and large transfer vectors (TVs) were subjected to DNA sequence analysis. For each construction, 3-4 clones were sequenced using vector (M13 forward and M13 reverse), and MDV specific primers (Table 1). DNA sequencing was conducted by Bruce Kingham at the University of Delaware Sequencing facility in the Allen Laboratory.

2.3 Schematic Diagram of Vector Construction

The EcoR-Q region of the MDV genome, which encompasses the *meq* gene was PCR-amplified from MDV strains JM102, RB1B, MD5 and MK using primers that included *loxP* sites at their 5'- ends (Figure 3.1 A). Each of these were cloned into pCR 2.1 Topo and their DNA sequences were confirmed. The *lox*P-flanked MD5 *meq* clone #2 was found to have intact *loxP* sites and did not contain any point mutations within the Meq-coding region. This segment was then used in the further construction of the transfer vector, for the introduction of the *loxP* sites at the EcoR-Q region of the genome.

To provide flanking sequences for homologous recombination of the lox P-flanked (floxed) Meq locus, a transfer vector was then amplified and *Nhe* I and *Hin*D III restriction sites were introduced at the 5' and 3' ends of this larger fragment respectively. The EcoR-Q fragment was centrally located in the TV, so that

mutagenized EcoR-Q fragments (containing *loxP* sites) could be directly inserted. The TVs were first Topo-cloned, and then sub-cloned into pUC19ΔE, a pUC19-derivative in which the *Eco*RI site had been disrupted (Figure 3.1 B). The *lox*P-flanked MD5meq locus described above was then ligated into the TV from which the native EcoR-Q fragment had been deleted. This provided about 1 kbp flanking sequence on either side of the EcoR-Q fragment to allow for homologous recombination into the genome of the MD5ΔMeq virus.

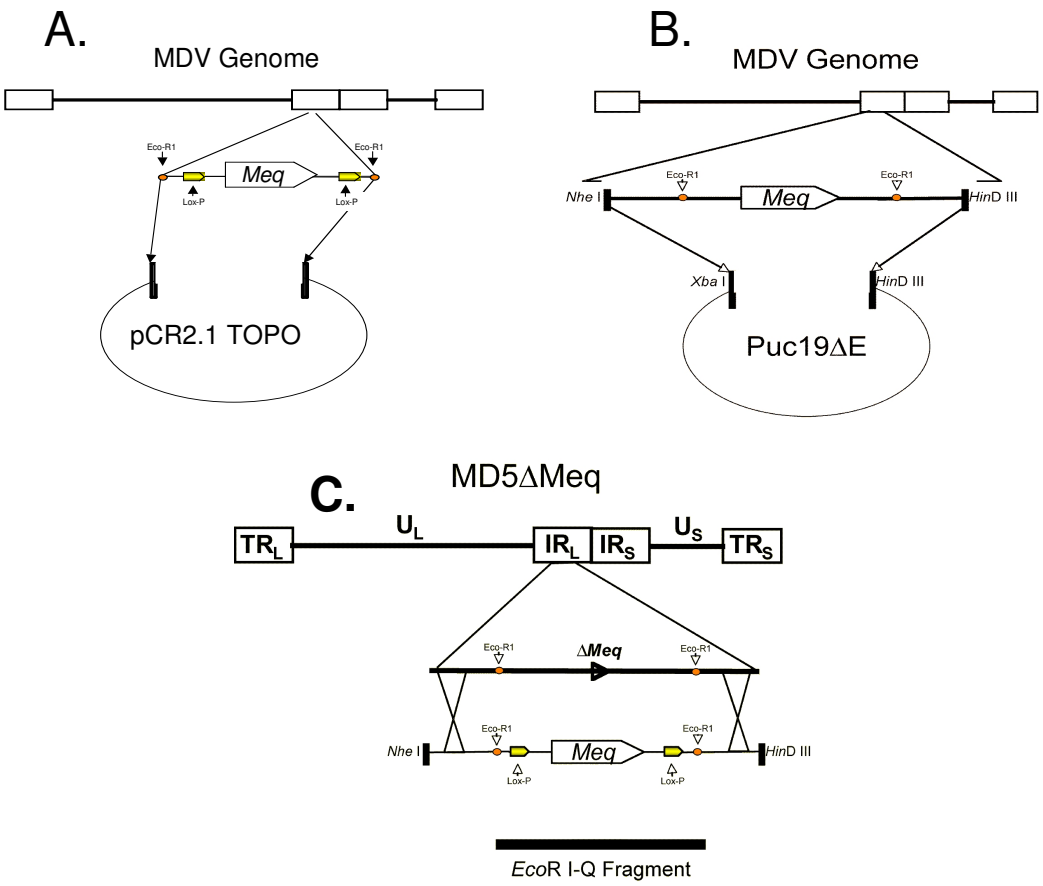


Figure 2.1. Construction of a loxP-flanked Meq locus. Panel A depicts the MDV genome (top) and the area of the genome used in the construction of the *lox*P-flanked *Eco*R-Q fragment. The *meq* gene of the MDV genome was amplified using primers that targeted the *Eco*RI sites flanking the *meq* gene. *Lox*-P sites were inserted on either side of the *meq* gene through incorporating *Eco*RI and *lox*P sites in the 5'-ends of these primers. Panel B shows the construction of the transfer vector (TV). A 4.4 kbp fragment of the meq locus, flanked by *Nhe* I and *Hin*D III sites, was amplified and subsequently subcloned into the *Xba* I and *Hin*D III sites of pUC19ΔE, a derivative of pUC19 from which the *Eco*RI site had been deleted. This created about 1 kbp of flanking sequence for the *lox*P-flanked *Eco*RI-Q segment to recombine into the MDV genome. Panel C depicts the MD5ΔMeq genome, the DNA of which was then co-transfected with the TV (panel B) with the goal of generating recombinants

2.4 Transfections

Two strategies were used to generate recombinant MD5 viruses having *meq* genes from other strains of MDV (JM102, RB1B, MK and loxP-flanked MD5). In our first method, we co-transfected the *meq* locus from each strain (cloned into pCR2.1 Topo and linearlized with *Not* I) with MD5ΔMeq virus DNA into CEF. For each co-transfection 500 ng of plasmid DNA was combined with 10 μg of MD5ΔMeq-infected CEF DNA. For transfections, the calcium phosphate method first described by (Morgan et al, 1990) was used, with modifications for CEF(14).

In order to amplify potential recombinant MDV pools, at 5-6 days post-transfection, co-transfected CEF monolayers were harvested and passaged one time, from duplicate 60mm dishes to one T75 flask/transfection with additional CEF added. After one additional week, each T75 flask was harvested and frozen in one vial of freezing medium (90% fetal bovine serum/ 10% DMSO cryopreservative). We performed each transfection/passage three times for each construct. These transfections were then combined and used to generate inocula for *in vivo* selection of recombinants.

As a second method, MD5ΔMeq-infected 6-mm dishes of CEF were transfected with 1 μg of each *meq* locus vector using Lipofectamine 2000 (Life Technologies). These dishes were likewise passaged one time into T75 flasks and frozen as described above.

2.5 *In Vivo* Selection for Recombinant MDVs

To select for recombinant MDVs based on tumor formation, specific-pathogen free (SPF) chickens were tagged, sorted into groups and inoculated at one day of age with: (1) Mock-infected CEF (control for virus infection); (2) MD5ΔMeq-

infected CEF (control for loss of Meq); (3) MD5ΔMeq + JM102 *meq*; (4) MD5ΔMeq + RB1B *meq*; (5) MD5ΔMeq + loxP-flanked MD5 EcoRI-Q fragment; (6) MD5ΔMeq + MK *meq*; and, (7) MD5 (control for a Meq+ virus).

For each recombinant group, all transfections were pooled into a single inoculum. The following numbers of chickens were used for each group: Mock (15), MD5ΔMeq (18), MD5ΔMeq + JM102 *meq* (23), MD5ΔMeq + RB1B *meq* (23), MD5ΔMeq + loxP-MD5 *meq* (27), MD5ΔMeq + MK *meq* (23), and MD5 (22). At two weeks post-inoculation, (10) additional one day old SPF birds were added to each isolator to monitor for horizontal spread of each virus.

Chickens were kept in plexiglass isolators in the Allen Laboratory and monitored daily for signs of MD (paralysis) and all chickens were subject to necropsy. Chickens were given food and water *ad libitum* and isolators were cleaned on a weekly basis.

2.5.1 Virus Reisolation

To monitor virus replication and detect any possible recombinant viruses, reisolations were performed each week for four consecutive weeks from spleen cells (SPC) and peripheral blood mononuclear cells (PBMC). Spleens were aseptically removed, washed, pooled, homogenized, washed again and plated on CEF monolayers in triplicate. PBMC were isolated from whole blood using Histopaque M1119 (Sigma Chemical Co.), washed and plated on CEF monolayers in triplicate. At 6 days post-plating, monolayers were fixed with 95% EtOH, stained with anti-MDV US1 antibodies and enumerated by immunofluorescence (Parcells et al, 1999). Virus reisolation from contact-exposed birds was performed at week 6 (4 weeks post-placement) of the experiment.

2.5.2 DNA Extraction Protocol

DNA was extracted and purified from the spleen cells and PBMC isolated from each group at every virus reisolation. For DNA purification, 100 µl of cell suspensions (2 X 10^6 cells) were added to 500 µl of PK solution (0.4 mg/ml proteinase K, 10 mM Tris, pH 7.5, 2 mM EDTA, 150 mM NaCl) and incubated for 1hr at 37˚C. To extract the nucleic acids, the samples were then mixed with 300 µl Phenol, mixed by inversion, and 300 µl of $CHCl_3$ (96% $CHCl_3$/ 4% isoamyl alcohol), mixed by inversion and the phases were separated via centrifugation at 14,000 rpm for 5 min at RT. The aqueous layer of the sample was then isolated, mixed with 600 µl $CHCl_3$/ isoamyl alcohol and the phases were separated via centrifugation as above. The aqueous layer was again isolated and mixed with 700 µl isopropanol to precipitate nucleic acids. The sample was then subject to centrifugation for 10 min at RT, and the pellet of DNA was washed with 70% ETOH to remove excess salt. The DNA was again pelleted via centrifugation, air dried and resuspended in 100 µl TE buffer (10 mM Tris pH 7,5, 1 mM EDTA). This DNA was then used for PCR analysis of the *meq* loci to test for recombination.

2.5.3 PCR Analysis for Recombinant MDVs

DNA extracted each week from the samples of SPC and PBMCs (2 X 10^6) were used for PCR analysis for recombination of the *meq* gene. PCR was done using Meq 5' forward and Meq 3' reverse primers (Table 1). In addition, tumors isolated at the termination of the study were cultured and samples taken for DNA purification and PCR analysis.

2.5.4 Necropsy

For each week of the experiment, (3) chickens were removed from each isolator, bled via cardiac puncture, and euthanized via cervical dislocation. Throughout the experiment, birds were monitored and any birds that died, or showed signs of MDV were necropsied. Any lesions or tumors associated with MDV were recorded and samples were taken for testing. In addition, at the end of the experiment (week 7), all remaining birds were euthanized and necropsied. Tumor samples were taken for cell line establishment, DNA purification for PCR analysis and for reisolation of recombinant viruses.

Table 2.1 DNA Sequences of PCR Primers

Primer Name	Primer Sequence[1]	Amplicon Size[2]
EcoRQ-loxP for	5' – aac c**ga att c**gg *ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA T*tg ata taa aga cga tag tca – 3'	2,537 bp
EcoRQ-loxP rev	5' – a**ga att c**aa a*ATA ACT TCG TAT AAT GTA TGC TAT ACG AAG TTA T*ct att ctt gta atg tcg tac gag – 3'	
Nhe I– TV for	5' - AA **GCT AGC** TGT ATG TGT GTGA GCA GTC GGT TG - 3'	4,510 bp
Hind III – TV rev	5' - AA **AAG CTT** TGC CTC GGG CGA TTT CCC TGT TAT TG - 3'	
EcoRQ – for	5' – GGT GAT ATA AAG ACG ATA GTC ATG –3'	Sequencing primer
Meq 5' for	5' – GTA AAG AGA TGT CTC AGG AGC CA – 3'	Sequencing primer
Meq +54 for	5' – GTC CCC CCT CGA TCT TTC TCT – 3'	Sequencing primer
Meq +342 for	5' – TGT ACA GTT GGC TTG TCA TGA G – 3'	Sequencing primer
Meq -979 rev	5' – GAG TAT CCG AGG GAA ACT GAA – 3'	Sequencing primer
Meq 3' rev	5' – GAC GAT GTG CTG CTG AGA GTC – 3'	Sequencing primer
EcoRQ-rev	5' – CTC ATA CTT CGG AAC TCC TGG AG – 3'	Sequencing primer

[1] – Sequences in bold are added restriction sites. Sequences in italics denote added loxP sites.
[2] – Amplicon size based on published sequence of the MD5 genome (Tulman, *et al.,* 2001)

Chapter 3

RESULTS

3.1 Construction of Transfer Vectors

3.1.1 Agarose Gel Analysis of Construction Intermediates

Lox-P sites were first introduced into the MD5 *Eco*RI-Q fragment (which contains the *meq* gene) via PCR (Figure 3.1 A, left panel). Lox-P sites were then also inserted into the *meq* loci of JM102, RB1B, and MK (Figure 3.1 A, right panel). These products were Topo cloned and screened by *Eco*RI digestion (Figure 3.1 B).

The large TV sequences were amplified from the four strains of MDV: JM102, RB1B, MK, and MD5. The results of this amplification are seen in Figure 3.2. These Transfer Vectors were initially Topo-cloned and were then sub-cloned into pUC19ΔE for insertion of the *lox*P-flanked *meq* loci.

From the four loxP-flanked MD5Δmeq clones sequenced, only clone 2 was found to have intact loxP sites and did not have any point mutations within the Meq coding region. This clone was then digested with *Eco*RI to isolate the fragment. This loxP-EcoQ-loxP fragment was then gel extracted and purified. This fragment was then used to generate the TV.

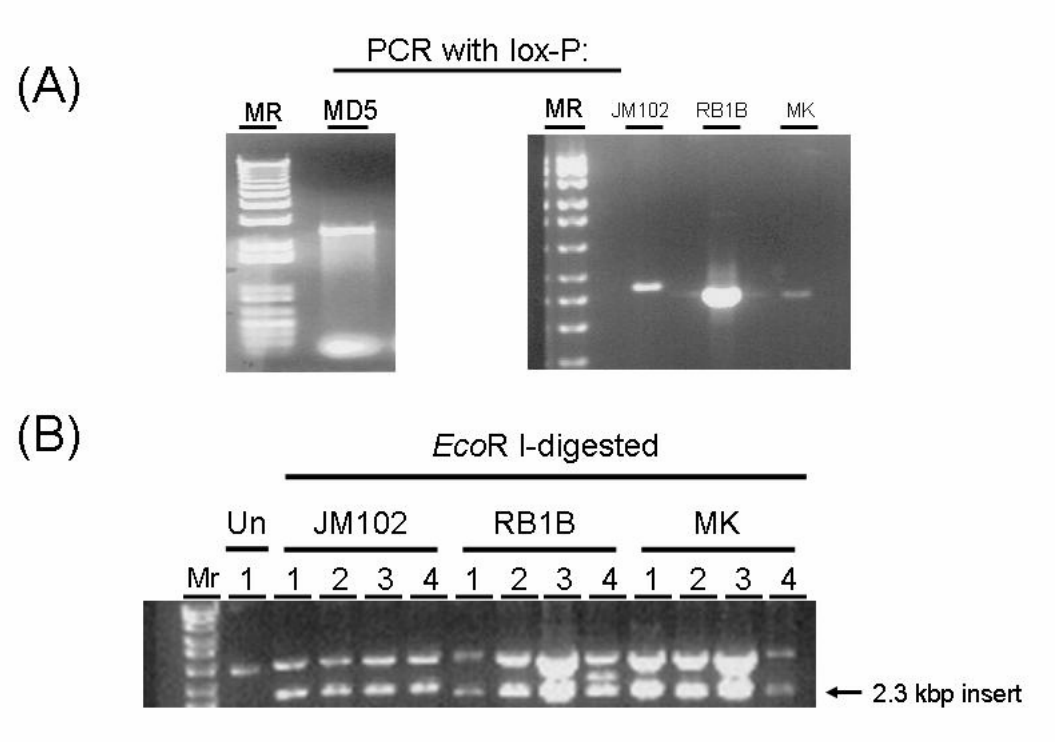


Figure 3.1 Generation of *lox*P-flanked *Eco*RI-Q fragments of MDV. Panel A shows the incorporation of *lox*P sites into the meq loci of the MD5 (left panel), JM102, RB1B, and MK (right panel). These products were then Topo cloned and digested with *Eco*RI. Panel B shows the results of this digestion. The band on the gel at 2.3 kbp indicates the presence of the *lox*P insert.

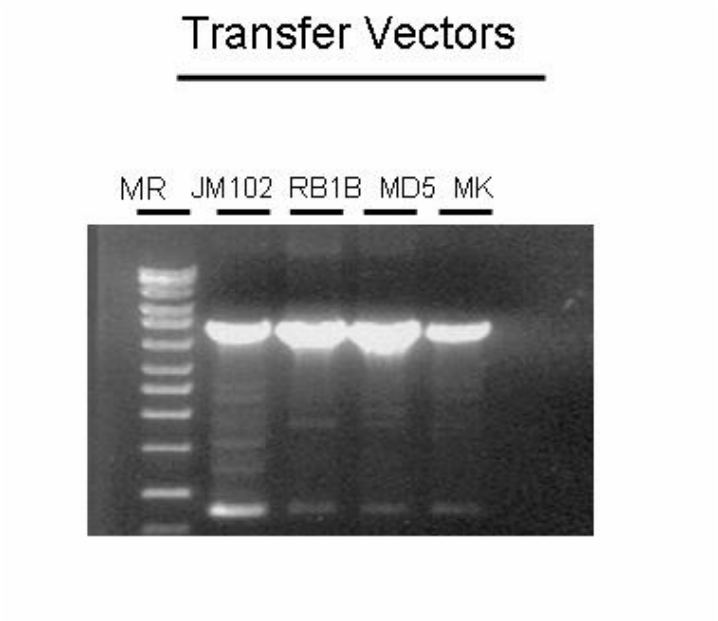


Figure 3.2. PCR Amplification of Transfer Vector Sequences. PCR amplification of 46 and 44 kbp are shown.

3.2 Transfection Efficiency of MD5ΔMeq + Transfer Vectors

In our first attempt at transfection, we did not obtain adequate plaques to harvest for inoculation into chickens. We hypothesized that the MD5ΔMeq DNA used may be the source of the problem. Therefore, we tested various preparations of MD5ΔMeq-infected CEF DNA and determined which one was most conducive to plaque formation (Table 3.1). Based on these results, we used the MD5ΔMeq DNA from sample A, which gave the highest plaque count, for our next attempt at transfections.

We then performed (3) transfections for each strain using this MD5ΔMeq DNA. We were able to obtain visible plaques for these transfections, although the

plaque counts were still very low (Table 3.2). These transfections were scaled up from two 60mm plates to (1) T75 each. The (3) transfections for each strain were then combined prior to inoculation into chickens in order to generate a sufficient amount of virus.

Table 3.1 Plaque Counts for Three Samples of MD5Δmeq.

MD5Δmeq Plaque Counts	
Samples	Number of Plaques
1:100 MD5Δmeq A	895
1:100 MD5Δmeq B	885
1:100 MD5Δmeq C	739

Table 3.2 Transfection Plaque Counts.

Transfection Plaque Counts						
	Transfection #1		Transfection #2		Transfection #3	
Samples	Plate A	Plate B	Plate A	Plate B	Plate A	Plate B
MD5Δmeq	2	5	2	1	6	2
MD5Δmeq + JM102	2	4	1	5	2	3
MD5Δmeq + RB1B	1	7	6	7	1	1
MD5Δmeq + MK	5	26	8	12	2	0

3.3 Virus Reisolation from Spleen Samples

After each virus reisolation, Spleen Cell (SPC) samples were plated and subsequently stained for US1 protein expression by IFA. Plaques were then counted and the PFU per million cells was calculated (figure 3.3).

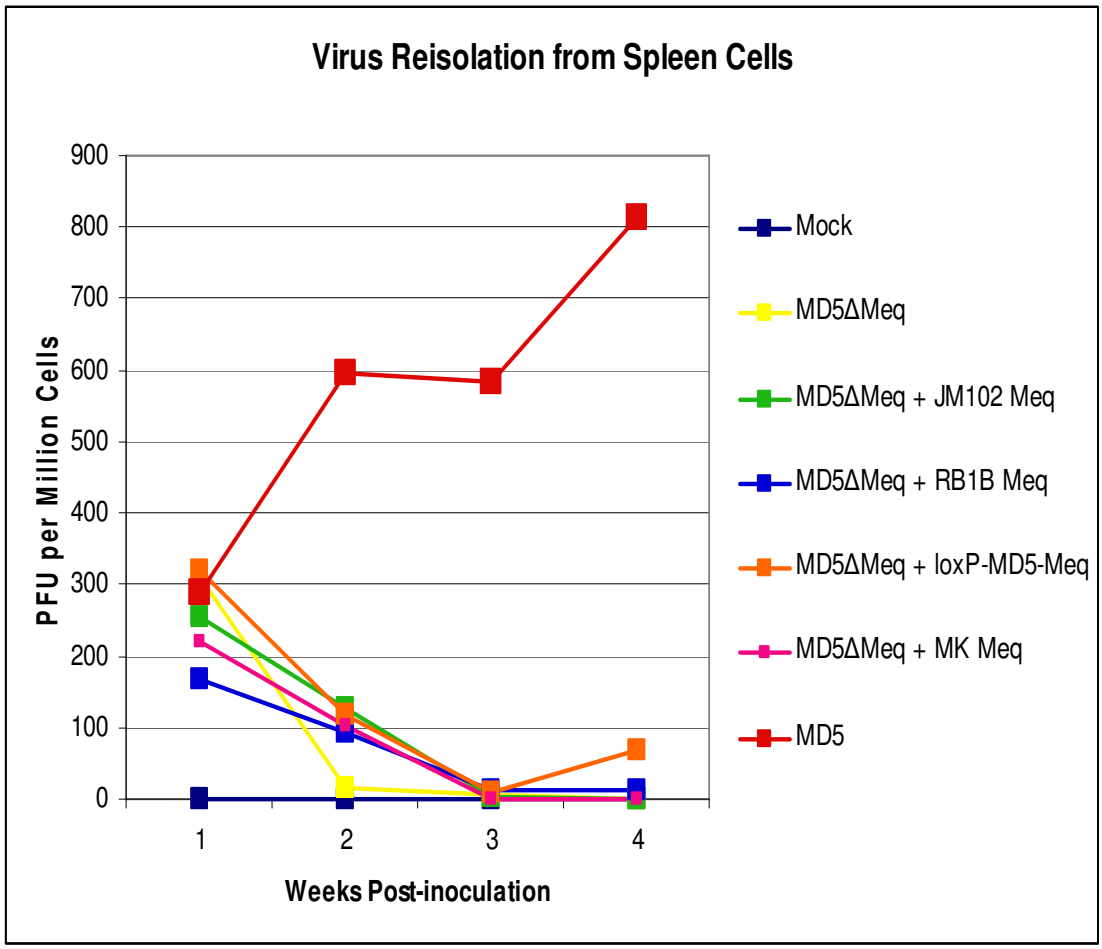
In the SPC, MD5 began to rise after week 1. Unfortunately, we were unable to obtain accurate counts for MD5 in week 3. The *lox*-P MD5 recombinant decreased after week 1 but rose slightly at week 4. All other recombinants (MD5ΔMeq-co-transfections) decreased throughout the experiment, as did the MD5ΔMeq control.

3.4 Virus Reisolation from PBMC

After each virus reisolation, PBMC samples were treated as described above for SPC cells, and the PFU per million cells were calculated (Figure 3.4). For PBMC, MD5 rose steadily from weeks 1-3 followed by a sharp decline in week 4. RB1B decreased after week 1, had a brief increase in plaques in week 3, and decreased again in week 4. All other recombinants decreased in replication throughout the experiment.

3.5 Virus Reisolation from Contact-exposed Chickens

On week 5, a virus reisolation was performed for the contact-exposed chickens. Spleen cell (SPC) and PBMC samples were collected for each group. These samples were treated as previously described for the MDV-inoculated chickens, and the PFU per million cells was calculated (Tables 3.3). For both the SPC and PBMC, MD5 was the only group with significant numbers of plaques. All recombinant groups had very low plaque counts, with SPC having slightly more plaques for all groups with the exception of *lox*P-MD5 which had a higher plaque count in the PBMC.



. Figure 3.3 Virus Reisolation from Spleen Cells. The line graphs above show virus replication as PFU per million cells from spleen cells collected during virus reisolations at 1, 2, 3 and 4 weeks post-inoculation.

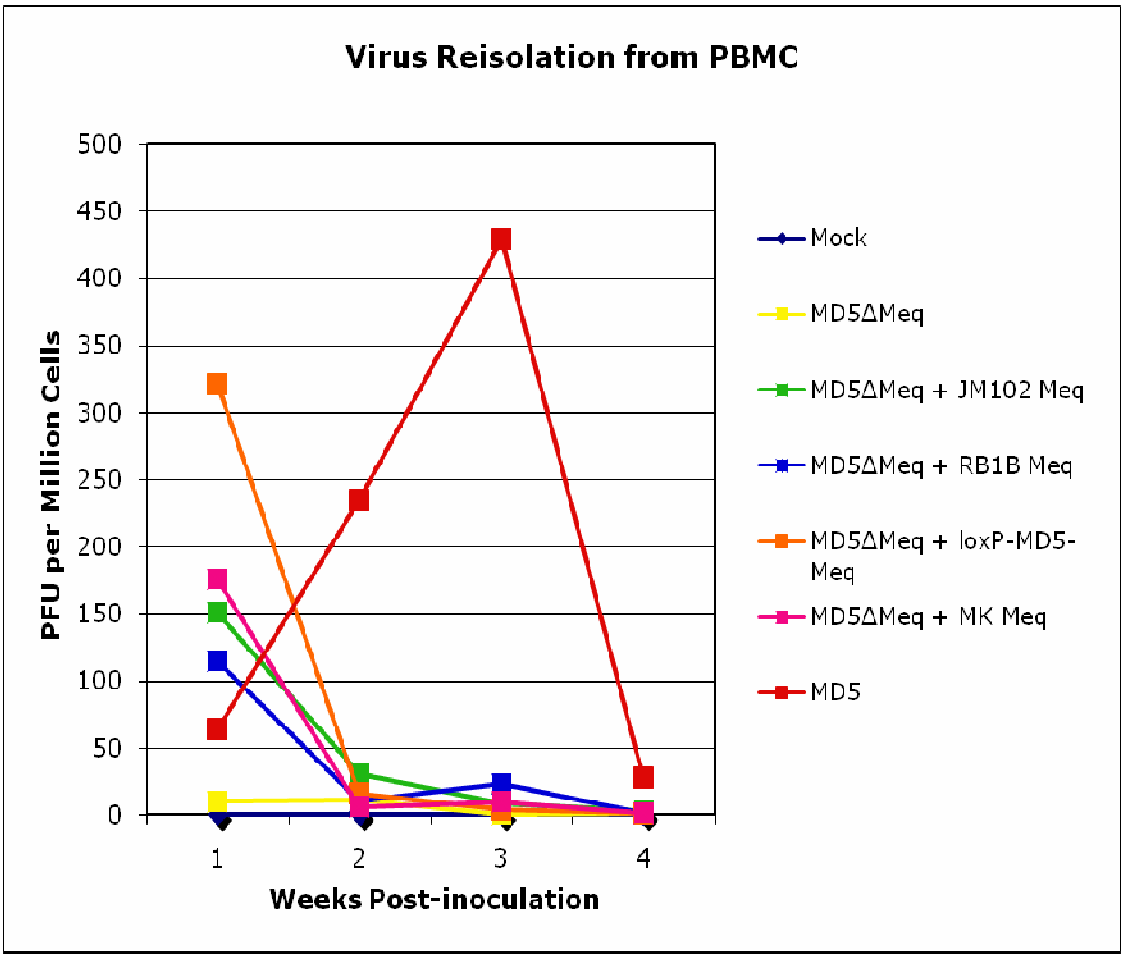


Figure 3.4 Virus Reisolation from PBMC. The line graphs above show virus replication as PFU per million cells from PBMC collected during virus reisolations at 1, 2, 3 and 4 weeks post-inoculation.

Table 3.3 Virus Reisolation for Contact-exposed Chickens. The table below shows virus replication in contact-exposed chickens as PFU per million cells from SPC and PBMC samples collected at 3 weeks post contact.

		SPC	PBMC
Isolator	Virus	PFU per million cells	PFU per million cells
1	Mock	0.0	0.0
2	MD5ΔMeq	2.0	1.5
3	MD5ΔMeq + JM102	7.0	6.0
4	MD5ΔMeq + RB1B	3.0	1.0
5	MD5ΔMeq + loxp-MD5	0.5	1.5
6	MD5ΔMeq + MK	4.0	2.0
7	MD5	1019	429.5

3.6 Tumor Incidence and Mortality

Birds were monitored daily during the experiment and any that died were removed and recorded. From these data, survival curves were generated for both inoculates and contact-exposed birds showing the percentage of birds surviving from each group at each day of the experiment (Figure 3.5).

In the MDV-inoculated birds, all recombinants with the exception of RB1B had 0% mortality after 14 days post-inoculation (any mortality prior to 14 days is considered to be due to non-MDV-specific causes). The mock and MD5Δmeq also

experienced no mortality. The MD5 group showed significant mortality, with the most significant decline beginning after week 4 (day 28) of the experiment (Figure 3.5).

For the contact-exposed birds, all groups experienced a 0% mortality rate with the exception of MD5. This group had two separate declines, one beginning around day 16 and the other around day 30 of the experiment (Figure 3.6).

During the final necropsy, tumors were observed in several of the groups (Table 3.4). The RB1B co-transfected group had the highest tumor incidence in the co-transfected inoculated groups; however, no tumors were seen in the contact-exposed birds. The *Lox*P-MD5 and MK co-transfection inoculated groups had comparable tumor incidence in inoculated and contact birds. The positive control group, MD5, had high tumor incidence in both inoculated and contact-exposed birds.

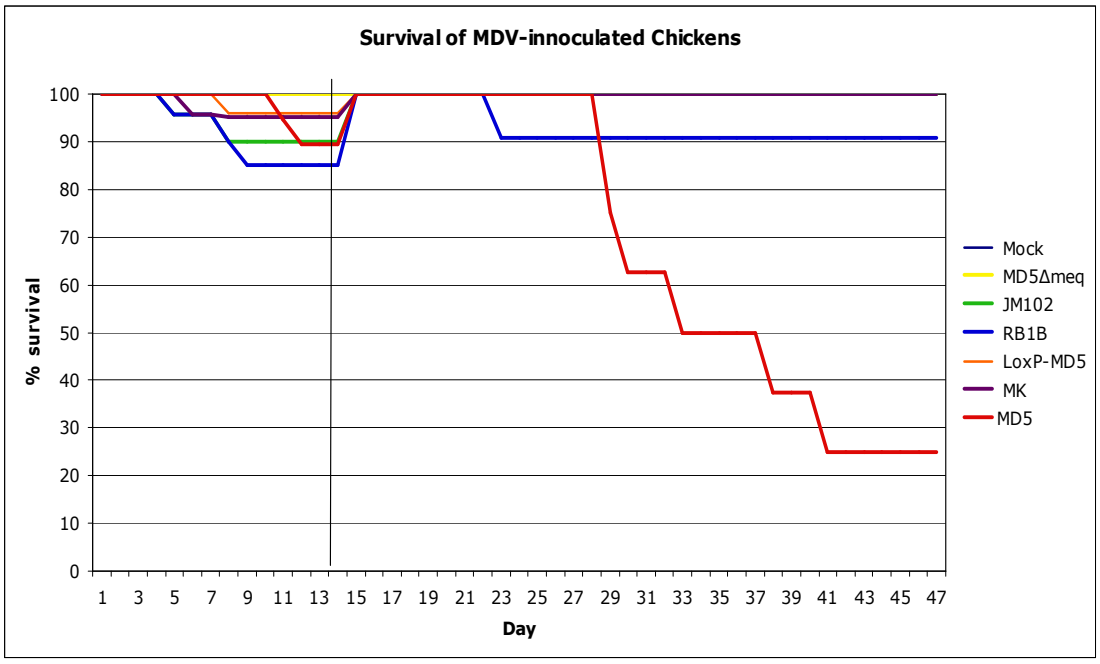


Figure 3.5 Survival Curve of MDV-inoculated Chickens. The above line graph shows the percentage of surviving chickens in each group at each day of the experiment. Any mortality that occurs in the first 14 days post inoculation are considered non-specific and are not attributed to the presence of the virus. The vertical line labeled 14 DPI (days post inoculation) indicates where the graph has been reset to 100%. Any subsequent mortality is assumed to be MDV-related.

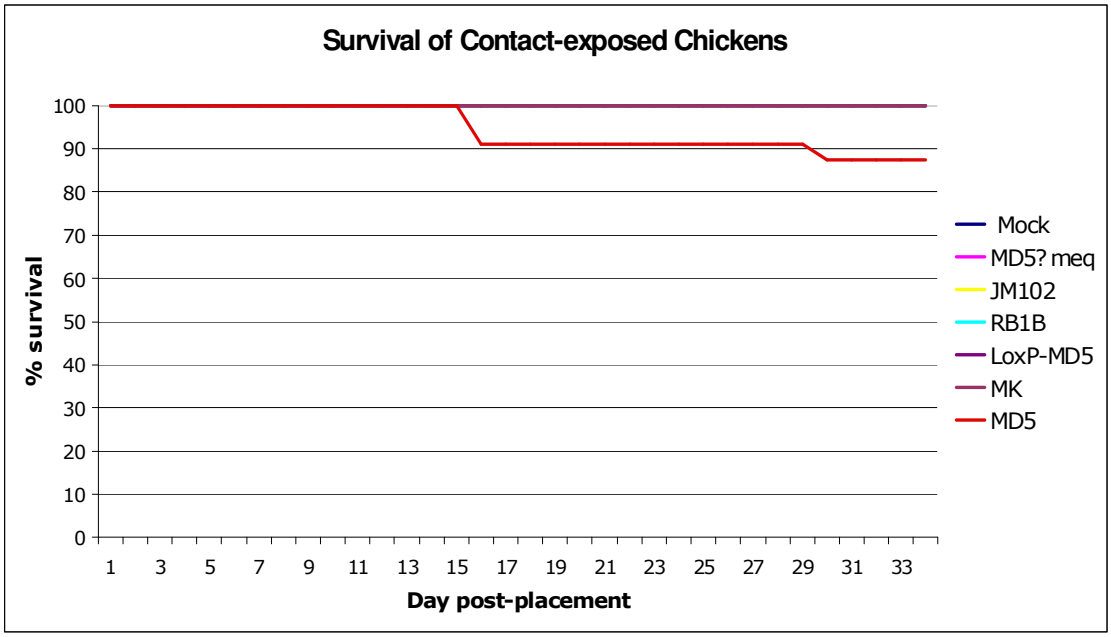


Figure 3.6 Survival Curve of Contact-exposed Chickens. This graph shows the percent survival of the contact-exposed chickens for each group throughout the experiment. Day 1 on this graph indicates the second week of the experiment when the contact birds were introduced into the groups.

Table 3.4 Tumor Incidence in MDV-inoculated and Contact-exposed Chickens. During the necropsy, any visible tumors were taken for analysis. This table shows the number of tumors isolated from each group for the MDV-inoculated and Contact-exposed chickens.

	Innoculates			**Contacts**		
	# tumors	tot. # birds	% tumors	# tumors	tot. # birds	% tumors
Mock	0	15	0	1	10	10
Md5^meq	0	18	0	0	11	0
JM	0	23	0	0	11	0
RB1B	5	23	21.7	0	11	0
lox-P	2	27	7.4	1	11	9.09
MK	2	23	8.7	1	11	9.09
Md5	9	22	40.9	9	11	81.8

3.7 Screen for Recombinant MDVs by PCR

At each week of virus reisolation, we purified DNA from SPC and PBMC samples for PCR amplification of the *meq* gene, which would indicate the generation of a recombinant in the co-transfected groups (Figure 3.7). Our analysis of SPC and PBMC samples from each virus reisolation did not indicate the presence of recombinant virus. Several samples were positive in the PBMC from week 2 (Figure 3.8 B, white asterisks). We have concluded that these were due to contamination of the samples, as the JM102 sample showed a *meq* band smaller than would be expected. Analysis of the SPC and PBMC samples taken from the virus reisolation of the contact-exposed birds also did not reveal any recombinant virus (Figure 3.8).

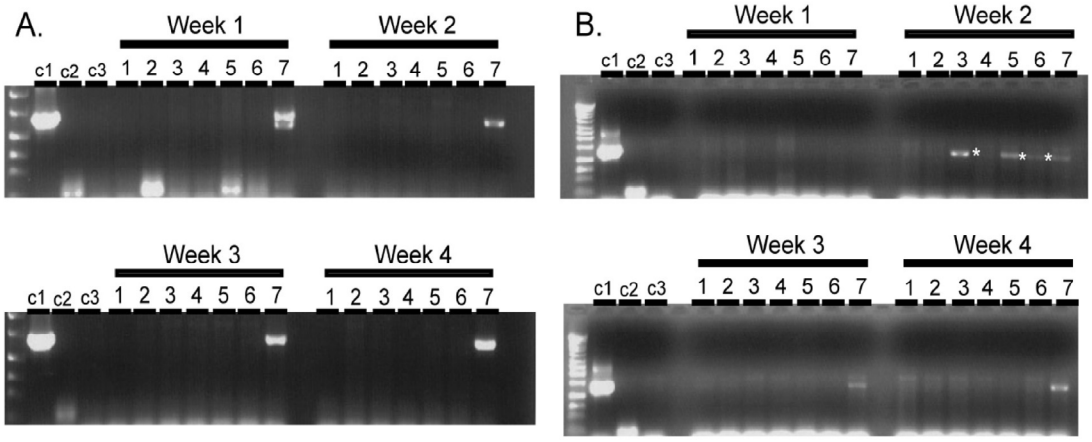


Figure 3.7 PCR Screening for Recombinant MDVs. Panel A shows pictures of an agarose gel of *meq* PCR amplicons. The templates used were DNA purified spleen cells taken for each group at each virus reisolation. The numbers above each lane correspond to the group from which the sample was taken (as previously listed in the Materials and Methods). These samples were amplified with primers Meq 5'F and Eco-Q R. PCR was performed using Accuzyme, Pfu polymerase. MD5 (C1), MD5ΔMeq (C2), and uninfected CEF cells (C3) were run as controls. The PCR products were run on a .6% agarose gel and were analyzed for bands indicating the presence of recombinant virus. Panel B shows the results of PCR amplification of DNA purified peripheral blood mononuclear cells from each virus reisolation. The PCR amplification, controls, and gel analysis of PBMC are identical to those described for the spleen cells.

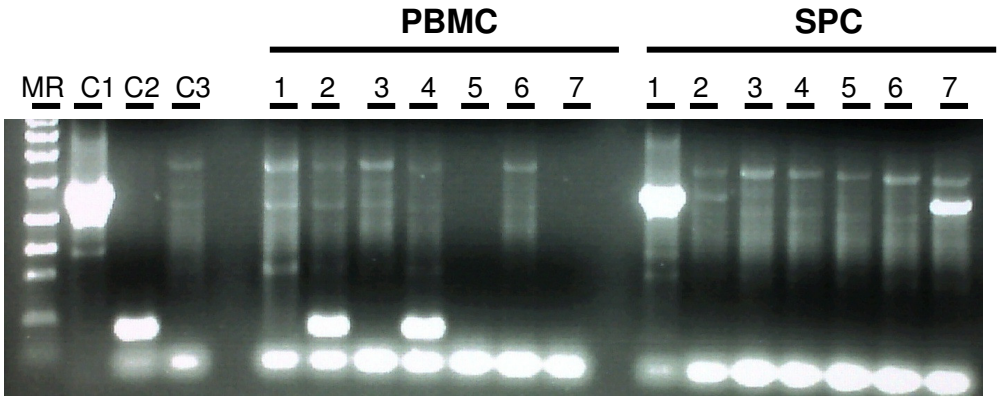


Figure 3.8 PCR Screening for Recombinant MDVs in Contact-exposed Chickens. A picture of an agarose gel of meq PCR amplicons is shown above. The templates used were DNA purified spleen cells and peripheral blood mononuclear cells collected during this virus reisolation. C1, C2, and C3 denote the controls used for this PCR. C1 was an intact MD5 virus, C2 was MD5Δmeq, and C3 is uninfected CEF.

Chapter 4

DISCUSSION

4.1 Comparison of MD5ΔMeq to MD5

MD5 causes acute lymphoproliferative disease that results in T-cell lymphomas in the visceral organs and peripheral nerves (9). MD5Δmeq on the other hand, replicates well in cell culture, but is apathogenic in chickens (9). Due to the loss of the *meq* gene, MD5Δmeq is non-oncogenic *in vivo*; however, it has been found to replicate in lymphoid organs and in feather follicular epithelium (FFE) (9). Therefore, the *meq* gene cannot be selected for in cell culture, but can be selected *in vivo* based on the ability of the virus to cause tumors.

As can be seen in the results of the co-transfections of the MD5Δmeq virus with the *meq* genes from various strains of MDV, plaques did form, but in very low quantities. Since the transfection efficiency was low, further analysis of the co-transfection procedure should be done in order to obtain more plaques for infection into chickens. Since tumors were present in several groups at the end of the experiment, it suggests that recombinant viruses were in fact generated, but were not present in a high enough concentration for isolation in the lab. Increasing the transfection efficiency of the virus DNA may therefore lead to an increase in recombinant virus, which may result in tumors being formed earlier in the study and subsequent isolation of recombinant virus.

4.2 Overall Meaning of Results

Throughout this experiment, there was no evidence that recombinant viruses had been generated. PCR analysis of SPC and PBMC samples taken each week did not indicate the presence of recombinants. In addition, the co-transfection inoculated groups had only one specific death (in an MD5Δmeq + RB1B inoculated bird). Unfortunately, samples were not taken from this bird. The MD5 group on the other hand had significant mortality in both the inoculated and contact-exposed birds. The virus reisolation data showed consistent declines in plaque concentrations for all groups except MD5, again indicating that no recombinant virus had been generated. Alternatively, there may have been recombinants generated, but the MD5ΔMeq may have provided strong vaccine protection from these viruses.

There was a slight increase in virus reisolation titers for LoxP-MD5 in the SPC samples and RB1B in the PBMC samples at weeks 4 and 3 respectively; however, this may have been due to variations between birds, but also may have been due to the generation of a low number of recombinant viruses.

At the end of this experiment, several tumors were observed in some of the groups, suggesting that recombinant virus may have been generated for the co-transfection groups. In addition, RB1B, LoxP-MD5 and MK co-transfection inoculated groups contained birds that had tumors in their contact-exposed birds, indicating transmission of the virus may have occurred. However, when these samples were examined via PCR, no evidence of recombinant virus was found unlike the prior trial in which one bird, 1137, was found to have a recombinant RB1B Meq in MD5.

Although we were unable to isolate any recombinant virus in the lab, the presence of tumors indicates that recombinant virus was present in our samples. These samples could be re-introduced into live chickens in an attempt to further amplify the

virus to a level that can be isolated in the lab. As stated previously, MD5Δmeq replicates well in cells culture but is apathogenic in chickens. Therefore, these samples must replicate *in vivo* to allow for the selection of viruses that have reincorporated the *meq* gene.

4.3 Conclusions

One explanation of these results is that the MD5Δmeq virus acts as a vaccine, and depresses the replication of any oncogenic recombinants at early times post-infection. A previous study done by Lee et al. (2008) found that MD5Δmeq actually provided better protection than CV1988/Rispens, the best vaccine currently available, when challenged by a very virulent plus strain of the virus (9). These results were subsequently confirmed in our laboratory (E. McDowell, M.S. Thesis). The initial study showed that the chickens vaccinated with the recombinant virus MD5Δmeq showed no MDV specific lesions or mortality (9). Significantly, only 39-41% of the chickens vaccinated with CV1988/Rispens did show signs of the disease (9). The MD5Δmeq vaccine was also found to significantly reduce the replication of the challenge virus (9).

Based on these results, it seems possible that any virus in our *inoculum* that did not recombine the *meq* gene may have acted as a vaccine, protecting the chickens from becoming infected with the recombinant virus, and preventing the reisolation of that virus from tumors. Although we did not isolate any recombinant virus during the experiment, there were several groups with tumors at the end of the experiment. This may be due to a decrease in the amount of MD5Δmeq present in the chicken. As the replication of MD5Δmeq virus declines, the recombinants may begin to induce tumors. However, tumor formation would be at a much lower rate than the

MD5 strain, hindering the ability to reisolate any recombinant virus from those tumors. Since MD5Δmeq replicated to near wild type levels within the first week, but then dropped off significantly afterwards, when MD5 increased in replication, the immune system must be "patterned" during this early replication of MD5Δmeq to block tumor development and progression.

4.4 Possible Alternative Strategies

The putative recombinant viruses we generated in our experiment were constructed through recombination in cell culture. Although this process works, as demonstrated with the 1137 virus, it is very time consuming and inefficient. In the future, this experiment may be done more efficiently and successfully using Bacterial Artificial Chromosomes (BACs). BAC clones were originally only made using an attenuated MDV; however, RB1B, a very virulent form of the virus, is now available as a BAC clone, and could be used to generate recombinant virus (16).

Using this method, the entire viral genome of MDV would be cloned as a bacterial artificial chromosome in *E. coli* (5). Mutations could then be incorporated into the MDV genome via homologous recombination of a marker gene, and then reinsertion of the gene of interest (5). This would allow mutations to be introduced into MDV quickly and efficiently.

This method of generating recombinant MDV is a faster and more efficient process than overlapping cosmid clones. There are some limitations to using overlapping cosmid clones that would not be an issue using BAC clones. Overlapping cosmid clones require cultured eukaryotic cells to replicate the virus. Specifically, you are restricted to secondary CEF cells (5). In addition, in order to generate recombinant virus, you must co-transfect eukaryotic cells, which is a time consuming process and

again requires a large supply of cells to be generated and maintained (5). BAC clones will allow MDV to be maintained without the use of eukaryotic cells (5). Using this method, generation of mutants and recombination of the virus would be a relatively quick and easy process, and this experiment could be re-done using less time and resources.

4.5 Biological Relevance of Work

Although we were unable to generate recombinant viruses in this experiment, the results that were obtained provide further insight into the function of *meq* in the pathogenesis of MDV. In the virus reisolation data for the inoculated birds, the MD5Δmeq group had comparable plaque concentrations to the MD5 group in the first week of the experiment. However, as the plaque concentrations for MD5 continued to rise in the subsequent weeks, the MD5Δmeq group experienced a dramatic decrease in plaque concentrations after week one of the experiment. This suggests that the *meq* gene is not involved in early stages of MDV infection. Specifically, *meq* is important in the onset and control of the latent stage, and in tumor formation, explaining why viruses lacking the *meq* gene are unable to maintain infectivity beyond the initial lytic phase.

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