IDENTIFYING THE GENETIC BASIS OF ATTENUATION IN MAREK’S DISEASE VIRUS VIA EXPERIMENTAL EVOLUTION

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ABSTRACT

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Marek’s disease virus (MDV), an oncogenic alphaherpesvirus of chickens, causes up to $2 billion in loses a year due to Marek’s disease (MD). Therefore control of this economically important disease is critical. The primary method to control MD is vaccination. Attenuated, or weakened, strains of MDV have been generated via repeated in vitro serial passage to generate avirulent MDV strains that have been used as successful MD vaccines. Despite introduction of several vaccines since the 1970’s, more virulent strains of MDV have evolved to break vaccinal protection. Therefore, development of new MD vaccines is necessary. To address this concern, we sought to better understand the molecular basis of attenuation in MDV to provide information that may assist in the rationale design of MD vaccines.

Three attenuated replicates of a virulent MDV were serially passed in vitro for over 100 passages. DNA and RNA from attenuated viruses were deep sequenced using Illumina next-generation sequencers to identify changes in DNA sequence or expression following attenuation. Top candidate mutations identified via sequencing were used to generate seven recombinant viruses using red-mediated recombineering for mutations within UL42, UL46, UL5, two involving LORF2 and two mutations within ICP4. These recombinant viruses were tested in vivo to determine the impact of these mutations on MD incidence, in vivo replication and horizontal transmission. Point mutations within UL42, UL46, LORF2-Promoter and ICP4 did not cause
observable phenotypic changes compared to the parental virus. A single point mutation within LORF2-Intron and a double mutant involving ICP4 both resulting in 100% MD in challenged birds but failed to transmit horizontally to uninfected contact birds. Finally, a point mutation within UL5 reduced MD incidence by over 90%, significantly reduced in vivo replication, and eliminated horizontal transmission.

Further characterization of this UL5 point mutation determined that it increased in vitro replication in growth curves, yet head-to-head competition of the Mut UL5 virus versus parental virus showed the parental virus outcompeted the mutant virus. Furthermore, serial passage of Mut UL5 in vivo did not result in increased in MD incidence, in vivo replication or result in reversion or compensatory mutations to UL5 after passage through birds. Trials testing vaccinal protection of the Mut UL5 virus showed the virus provided partial protection against challenge with virulent MDV, yet did not exceed protection achieved through use of traditional vaccines. Therefore, use of this point mutation in combination with other candidate mutations was tested. Addition of the UL5 mutation with ΔMeq, a candidate vaccine with high protection and replication but also induces bursal-thymic atrophy (BTA), resulted in a recombinant virus that replicated at low levels and did not cause BTA, yet reduced levels of vaccinal protection, indicating an intricate relationship between replication levels, BTA and vaccinal protection.

This study shows that a variety of genes are mutated during attenuation, and particularly mutations within DNA replication genes, such as UL5, appear to play an important role in attenuation. We also determined that experimental evolution is a process that not only can identify mutations involved in attenuation, but also offer protection as a vaccine to provide information for further development of MD vaccines.
ACKNOWLEDGEMENTS

I would like to thank everyone who made it possible not only to complete my project, but to make my time as a Ph.D student memorable and enjoyable. First off, I must thank my advisor Hans Cheng. As a scientist and advisor, I cannot think of anyone who is a better person to fill those roles. His knowledge, guidance, patience and enthusiasm provided the perfect and supportive work environment to not only be productive in regards labwork, but also intellectually develop as a scientist. I wish all graduate students could have the pleasure of working with advisors that were at least half the caliber of Hans, since they would be hard pressed to find ones that would come close to equaling his skills as a mentor.

As in my papers, I must acknowledge Laurie Molitor, Spencer Jackson and Lonnie Milam for their technical assistance in completion of my experiments. Without their help I might still be sitting out in the bird house bleeding chicks, or extracting DNA from countless samples with an obscene numbers of boxes of samples still sitting in the freezer waiting for me to work through.

Laurie showed me the ropes on how to conduct all the vital bird work, including how to challenge birds, work in necropsy and bleed birds like a pro, even those teeny tiny chicks less than a week old that at first seemed impossible to not immediately cause a hematoma and ruin all chances of obtaining any sample...all while somehow manage to have fun doing it too. Anyone who has ever touched a pipette while Lonnie Milam has worked at ADOL surely must thank him for his absolutely priceless knowledge and skills regarding nearly any assay you might even consider doing in a lab. From basic tissue culture, transfection, recombineering, growth curves, qPCR, DNA extraction, PCR, and nearly any other molecular biology technique his extensive
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ACUC</td>
<td>Animal Care and Use Committee</td>
</tr>
<tr>
<td>ADOL</td>
<td>Avian Disease and Oncology Lab</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>BTA</td>
<td>Bursal-thymic atrophy</td>
</tr>
<tr>
<td>CEF</td>
<td>Chicken embryo fibroblast</td>
</tr>
<tr>
<td>CVI988</td>
<td>A.K.A Rispens vaccine</td>
</tr>
<tr>
<td>DEF</td>
<td>Duck embryo fibroblast</td>
</tr>
<tr>
<td>DPI</td>
<td>Days post infection</td>
</tr>
<tr>
<td>DPV</td>
<td>Days post vaccination</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>gB</td>
<td>Glycoprotein B</td>
</tr>
<tr>
<td>HB</td>
<td>Horsfall-Bauer isolation unit</td>
</tr>
<tr>
<td>HSV-1</td>
<td>Herpes simplex virus 1 or Human herpesvirus 1</td>
</tr>
<tr>
<td>HVT</td>
<td>Turkey herpesvirus</td>
</tr>
<tr>
<td>ICP4</td>
<td>Immediate early transcriptional regulator gene RS1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IRL</td>
<td>Internal repeat long region of MDV genome</td>
</tr>
<tr>
<td>IRS</td>
<td>Internal repeat short region of MDV genome</td>
</tr>
<tr>
<td>m</td>
<td>Pathotype of MDV designated “mild”</td>
</tr>
<tr>
<td>MD</td>
<td>Marek’s disease</td>
</tr>
<tr>
<td>MDV</td>
<td>Marek’s disease virus</td>
</tr>
<tr>
<td>MEQ</td>
<td>“MDV EcoQ” the MDV oncogene</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocytes</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td>PI</td>
<td>Protective index</td>
</tr>
<tr>
<td>TRL</td>
<td>Terminal repeat long region of MDV genome</td>
</tr>
<tr>
<td>TRS</td>
<td>Terminal repeat short region of MDV genome</td>
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<tr>
<td>UL</td>
<td>Unique long region of MDV genome</td>
</tr>
<tr>
<td>US</td>
<td>Unique short region of MDV genome</td>
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<td>Pathotype of MDV designated “virulent”</td>
</tr>
<tr>
<td>vv</td>
<td>Pathotype of MDV designated “very virulent”</td>
</tr>
<tr>
<td>vv+</td>
<td>Pathotype of MDV designated “very virulent plus”</td>
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CHAPTER 1. INTRODUCTION

VIRUSES, ATTENUATION AND VACCINATION

Viral attenuation is a process describing the loss of virulence from a virulent, disease causing virus to an avirulent virus that is no longer able to cause disease. These attenuated or ‘weakened’ viruses often provide a useful and functional purpose as vaccines to protect against virulent, disease causing strains. Live viruses have been used to protect against disease causing strains since the 18th century when first employed by Edward Jenner’s use of the Cowpox virus as a vaccine providing protection against smallpox (1).

Prior to the 18th century, a variety of methods had been described as routes to preventing smallpox, such as a multitude of herbal remedies, dietary restrictions, temperature regulation and other treatments were documented, all with variable degrees of success (2). The most successful of these of methods were based on observations that survivors of smallpox were immune to future outbreaks. One method based on this observation that likely had been in use since the seventh century was known as variolation, in which material from the pustules of smallpox-affected individuals was used to inoculate naïve individuals with the intention to protect against smallpox (3). While this method did provide utility and protection against smallpox, it also conferred a high risk of disease transmission, not only for development of smallpox itself in 2-3% of individual variolated, but also transmission of other diseases, such as syphilis (2). To avoid dangerous complications from variolation, observations that milkmaids tending cows often developed cowpox marks, but did not contract smallpox or suffer any symptoms more severe than mild poxmarks from the cows. This provided an alternative to using smallpox directly in variolation. Instead, transmission of material from cowpox pustules led to protection against
smallpox without the risk of developing the deadly disease itself, as well as helping to name the process of vaccination thanks to the source material derived from the cow, or ‘vaca’ in Latin (4). This widespread application of a live virus as a vaccine for control of a deadly virus established the groundwork for proliferation of vaccine development, eventually culminating in the eradication of smallpox and the control of a multitude of diseases such as polio, measles, rabies, tuberculosis and many more diseases that are successfully prevented and controlled through the use of vaccination (1).

A variety of techniques are currently used to create vaccines. This includes heat or chemicals to produce inactivated vaccines from disease-causing viruses to generate a killed vaccine, or use of recombinant DNA to express only a specific region or epitope of the microbe to stimulate immunity against the pathogen, which are known as subunit vaccines. However, of the different vaccine types, live-attenuated vaccines are often considered the version that provides the greatest level of protection. Live-attenuated vaccines can provide a stronger and more robust immunity against pathogens and often can provide life-long immunity due to low levels of replication of live-attenuated viruses, leading to greater persistence of protection (5).

Live-attenuated vaccines have been generated by several methods, all of which are capable of producing an avirulent virus that is also protective against challenge with a virulent virus. One of the first methods implemented utilizes viral species that are naturally avirulent in certain species and able to protect against other closely related viruses that are pathogenic to that host, as illustrated by Jenner’s use of the closely related, apathogenic Cowpox virus against the deadly Smallpox in humans. A second method employed to generate attenuated viruses is based on prolonged exposure of the virulent virus to conditions detrimental or suboptimal for replication of the virulent virus, leading to a weakened virus poorly adapted for replication and
unable to cause disease when reintroduced into the host species as a vaccine. The first lab-
attenuated vaccine against rabies was created using this method in which neuronal samples from
affected rabbits were removed and dried for several days to weaken the rabies virus before
administering the neuronal tissue containing the virus as a vaccine (6). These initial rabies
vaccines provided an alternative following exposure to a rabid animal and were able to prevent a
lethal outcome that previously had no medical recourse. Vaccines based upon this technique
using infected tissues from species such as rabbits or sheep is still employed in some countries
such as Ethiopia and India today due to their inexpensive cost (7, 8), but further refinement and
development of rabies vaccines has resulted in even safer lines of rabies vaccines generated via
cultivation in cell lines, which the World Health Organization (WHO) recommends for
replacement of earlier tissue-derived vaccines (9).

In cell culture based methods for generation of attenuated viruses, virulent viruses are
grown and serially passed in tissue culture conditions, which are significantly different from the
_\textit{in vivo}_ host environment that the virulent viruses had evolved to replicate, transmit and cause
disease. It has long been known that passage of virulent viruses through non-target species or
sub-optimal conditions leads to a selection for avirulent viruses during this process (10). For
example, over the course of repeated passage in tissue culture, viruses can evolve and adapt to _\textit{in}
vitro_ conditions, leading to a loss of virulence when returned to the host species where they are
no longer are optimized for replication within _\textit{in vivo}_ conditions. One of the first, well-known
vaccines generated via this method of serial passage was the Sabin vaccine in which the polio
virus was cultivated in monkey kidney cells, resulting in attenuation of the polio virus for use as
a live attenuated vaccine (1). _\textit{In vitro}_ serial passage to attenuate virulent viruses is a process
used to generate many of today’s vaccines against diseases in addition to polio and rabies, such as measles, mumps, chicken pox, yellow fever, and influenza (5).

Widespread use of live attenuated vaccines is not just limited to controlling human diseases, but many diseases affecting livestock and animals as well. Marek’s disease (MD) is one such example of a major economic disease affecting livestock that is controlled by vaccination with live-attenuated viruses. MD is caused by an oncogenic alphaherpevirus of chickens known as Gallid herpesvirus 2, also commonly known as Marek’s disease virus (MDV). MD is a T-cell lymphoma resulting in paralysis, nerve lesions, tumors and eventually death of affected chickens. MD costs the poultry industry $1-2 billion annually as a result of losses due to MD, therefore control of MD is critical for modern poultry production (11). The primary method to control MD has been the use of vaccines, with the first anti-tumor vaccine in any species first developed for prevention of MD (12).

MAREK’S DISEASE AND VACCINATION

The first attenuated MDV developed in 1969, known as HPRS-16, was an attenuated virus generated via repeated serial passage of a virulent MDV strain through chicken kidney cells until after approximately 30 in vitro passages the resulting virus became apathogenic. This demonstrated that MDV could be attenuated through repeated passage in tissue culture, with the practical application of this technique for vaccine development. Shortly after demonstrating the attenuation of MDV via in vitro serial passage, a closely related herpesvirus of turkeys (HVT), known as Melaegrid herpesvirus 1, was determined to be apathogenic in chickens and also conferred vaccinal protection against challenge with virulent MDV. HVT was licensed and widely used as a vaccine against MDV throughout the 1970’s until more virulent strains of MDV classified as ‘virulent’ (v) arose that were able to break vaccinal protection of HVT (Figure 1-1).
This resulted in the introduction of Bivalent vaccines in the 1980’s containing a combination of two MDV vaccines, such as HVT plus an avirulent strain such as SB-1, which interacted synergistically to provide greater protection when combined than either virus provided alone. Bivalent vaccines provided the best protection against outbreaks of MD until the early 1990’s when even more virulent field strains classified as ‘very virulent’ (vv) evolved able to cause MD even in birds vaccinated with Bivalent vaccines. This resulted in the introduction of the current ‘gold-standard’ vaccine known as CVI988/Rispens. Rispens is currently the most protective vaccine commercially available able to protect against even strains classified as ‘vv.’ Despite the success of Rispens to control MD since the 1990’s, it appears that new strains classified as ‘very virulent plus’ (vv+) are now circulating within flocks, raising concerns that vaccines exceeding the protection currently provided by Rispens may soon be required (13).

Despite the success of MDV vaccines at reducing MD losses, vaccination itself may be driving the evolution of more virulent MD strains. In a vivid example of the Red-Queen hypothesis, MDV vaccines and virulent MDV field strains appear to be in an evolutionary arms race in which following introduction of a new, more protective vaccine, field strains of MDV respond in kind with new, more virulent strains able to overcome the new vaccine, leading to this cycle of continued vaccine development, introduction and viral evolution (14). While MD vaccines prevent the symptoms of MD such as tumors and nerve lesions, they are non-sterilizing vaccines, in which vaccinated birds are still able to be infected with virulent field strains of MDV (13). This results in both attenuated vaccine strains and virulent field strain of MDV co-infecting and replicating within the same bird, providing a selection force for the evolution of more virulent MDV strains able to break vaccinal protection.
FIGURE 1-1. Introduction of MD Vaccines and the Increased Virulence of MDV. Adapted Witter 1996
Due to the intrinsic limitations of non-sterilizing vaccines, it appears likely that successive lines of MD vaccines must be periodically developed and introduced to combat the continued evolution of more virulent MDV field strains. While vaccines derived from *in vitro* serial passage, such as HPRS-16 and Rispens, have been successfully created, there is concern that there may be a limit to the maximal vaccinal protection against MD as seen with current vaccine development. Protective indices of ten candidate vaccines were compared against Rispens, currently the most-protective licensed vaccine available. Two of the ten viruses had high levels of protection comparable to Rispens, indicating that new candidate vaccines can be developed to equal current protection levels (15). Unfortunately, none of the candidate had protection levels exceeding Rispens, indicating that development of a more protective vaccine to address the evolution of vv+ strains of MDV may be a difficult task.

**MAREK’S DISEASE VIRUS AND ATTENUATION**

In order to address this challenge, a better understanding of the genetic basis of attenuation may assist in the development of more protective candidate vaccines. As shown by Witter et al. (15), blind serial passage can reliably produce attenuated vaccines, of which some may provide appreciable levels of vaccinal protection, but with the current opportunities available with recombinant DNA technology and next-generation sequencing, it could be possible to design and engineer an attenuated virus able to provide even greater levels of protection against vv+ strains of MDV. In order to precisely design attenuated viruses, it is necessary to better understand the process of attenuation. Despite the utilization of attenuation of MDV since 1969 in generating candidate vaccines, little is known regarding what drives the process of attenuation at a genetic level.
Early observations of attenuated MDV strains showed a significant loss in the quantities of A-antigen, later identified as glycoprotein C (gC), which is encoded by UL44. Expression of gC was shown to be significantly reduced and lost after passage, correlating with a loss of virulence (16). Despite this correlation, expression of gC is variable, in which oncogenic viruses have been identified that do not produce gC (17) and attenuated viruses that produce high levels of gC expression (18). This indicates that loss of gC expression during serial passage is only a general trend and not the causative force for attenuation. This conclusion is further supported by sequencing of gC in attenuated and virulent strains that found no differences in the sequence or promoter of gC itself to account for changes in virulence of MDV strains, suggesting the role of other factors for alteration of gC expression besides direct mutation of gC (19).

Another proposed mechanism for the attenuation of MDV during in vitro serial passage involved the genomic region known as the 132 bp repeats. As the name suggests, this is a repetitive, 132 base pair long region normally present as two to three tandemly repeated copies (20, 21). During serial passage, a proliferation in the number of tandem copies of the 132 bp repeats occurs while viruses are becoming attenuated. Despite the correlation of this phenomenon with attenuation, recombinant viruses testing the impact of mutating the number of copies of the 132 bp repeats show expansion of these repeats is not sufficient for attenuation. Incorporation of 9 tandem copies of the 132 bp repeat region into a virulent MDV and following only two passages in vitro resulted in viruses with up to 20 copies of the 132 bp repeats, yet the resulting viruses were still virulent despite having the 132 bp repeat expansion pattern common in attenuated viruses (22). Furthermore, complete deletion of the 132 bp region in a virulent virus prior to repeated in vitro serial passage showed that despite lacking this region, the 132 bp
deletion virus was still able to become attenuated during prolonged passage in tissue culture, proving that the 132 bp repeats are not necessary for attenuation (23).

Understanding the basis of attenuation has been a topic of research for many years, yet despite the clear correlation of several traits common to attenuated viruses, such as the loss of gC expression and expansion of 132 bp repeats, it is clear a more systematic approach to determining candidate genes involved in attenuation is necessary. With the advent of next-generation sequencing (NGS) technology, it is now possible to sequence the complete genome of a multitude of viruses to compare variation among various virulent strains, attenuated viruses and vaccines. The complete genome of several MD vaccines, such as SB-1 and Rispens, have been sequenced and compared to oncogenic strains to find differences between vaccines and virulent MDV (24, 25). The serially passed and attenuated 648A strain of MDV has also been sequenced to identify differences in the attenuated virus relative to the parental 648A virus prior to passage to identify numerous mutations occurring during serial passage (26). Therefore, due to NGS technology, it is now possible to quickly and inexpensively sequence a multitude of viruses and identify mutations found in avirulent viruses compared to virulent progenitors. Despite the ability to catalogue these mutations, further steps are necessary to determine the effect of these mutations on virulence because, as seen with gC expression and the 132 bp repeats, correlation does not equate causation, in which mutations that appear correlated with attenuation may not be the causative factor for loss of virulence.

While NGS may provide a list of mutations for consideration, it is the role of recombineering for generation of recombinant viruses that can provide the necessary bridge between genotype and phenotype to determine the impact of mutations identified via NGS in biological context of the MDV genome as a whole. With the generation of BAC-cloned MDV
viruses containing the complete MDV genome incorporated into a BAC-backbone, a multitude of techniques are now available to allow the manipulation and mutation of MDV. Using Red-mediated recombineering, it is now possible to introduce defined point mutations, whole gene deletions and insertions to routinely be incorporated into BAC-cloned viruses to create recombinant viruses testing the impact of various mutations of interest and yield near-isogenic recombinant viruses containing only the desired alternations (27, 28).

A second benefit of BAC-cloned viruses is the ability to generate more homogenous viral populations. Cloning MDV genomes to create BAC-derived viruses allow genetically homogenous viral stocks comprised of one genotypically and phenotypically uniform virus of a designated virulence level. Compared to BAC-cloned viruses, which are highly uniform viral populations as all members are derived from the same transfected DNA molecule, MDV strains are often described as quasi-species composed of a variety of diverse viral genotypes (26, 29, 30). While these unique genotypes are all present within the same strain and each MDV strain is classified into categories based on their severity and virulence, not all members of the viral population are necessarily phenotypically, much less genotypically, identical and share the same virulence level (31), therefore, genetic homogeneity within strains have been shown to be an important component of pathogenicity in herpesviruses (32, 33). Traditionally, MDV strains have been classified as mild (m), virulent (v), very virulent (vv) or very virulent plus (vv+) based on their phenotypes and ability to be controlled by the successive lines of vaccines developed, yet it is not assured that any strain designated as one of the preceding virulence levels exclusively contains viral subpopulations that all share the same virulence level. For example, there is no limitation preventing a strain denoted overall as vv+ from also containing a mixture of subpopulations with unique genotypes and phenotypes that differ from the vv+ designation, such
as vv or v, or even avirulent viruses (31). During cloning of the Md11 strain of MDV, several unique genotypes were isolated as BAC clones, of which one had the entire terminal repeat short region of the genome replaced with part of a duck chromosome, supporting the viewpoint that MDV strains are often genotypically diverse collections of viruses only broadly described collectively as one strain (29). This genetically diverse quasi-species aspect of MDV complicates comparisons between parental and derived strains because, while there may be one consensus genotype sequenced for the strain, there are subpopulations within the progenitor virus which differ from the consensus sequence. This is a particular complication when trying to determine the source for genetic variation observed in serially passed attenuated strain which have been evolved from a virulent strain. During serial passage in vitro, an obvious phenotypic change occurs from the virulent parental virus to the resulting avirulent attenuated viruses, but there are two major forces that could account for that change; selection or de novo mutation. Due to the quasi-species nature of MDV strains, avirulent MDV genotypes already present at low frequencies within the parental virus could simply be selected for during repeated serial passage and drive an increase in frequency within the population, eventually resulting in replacing the majority of the virulent strain with avirulent sequences. As seen in previous studies, avirulent BAC-cloned viruses have been isolated from a very virulent strain, this phenomenon has already been observed and described (31).

Alternatively, attenuation could be driven by de novo mutation occurring within virulent genotypes during serial passage as a result of errors during replication to generate new, avirulent genotypes within the virulent strain. Use of a virulent MDV-BAC cloned virus assists by ensuring a genotypically homogeneous viral population of only virulent viruses all sharing the same BAC-derived genome, therefore, complications arising from passing viral quasi-species is
eliminated, as well as guaranteeing that any genetic variation observed during passage must have resulted \textit{de novo} during replication of the \textit{in vitro} culture.

**IDENTIFYING THE GENETIC BASIS OF ATTENUATION IN MDV**

Due to the advantages offered by NGS technologies to inexpensively and quickly sequence the complete genome of several species, the availability of BAC-cloned MDV viruses allowing homogenous virulent viral stocks to be created, as well as recombineering technologies for generation of recombinant viruses to validate candidate mutations, a variety of techniques are now available to assist in exploring and elucidating the mechanisms and routes driving the attenuation of MDV during \textit{in vitro} serial passage. This will provide necessary information regarding attenuation and provide a starting point for the rational design of future vaccines. With a better understanding of the genetic mechanism driving attenuation it may be possible to precisely design and engineer an avirulent virus for use as a candidate vaccine without relying on blind serial passage \textit{in vitro} to generate a more protective vaccine, especially considering that current attempts to create more protective vaccines have failed to produce a vaccine significantly more protective than Rispens (15).

In order to address this question and elucidate the genetic basis of attenuation of MDV at the genetic level we had the following objectives:

**Objective 1:** Generate parallel, attenuated replicates of MDV, all initially derived from an identical, virulent MDV.

\textit{1a: Produce attenuated viruses through serial passage \textit{in vitro}.}

A virulent MDV BAC clone known as Md5B40BAC-c1 (B40BAC) was used as the initial homogeneous viral stock to infect chicken embryo fibroblast (CEF) cells. Three replicates
were grown in parallel and passed continuously for over 100 passages. Viral stocks of the three replicates were saved after every 10 passages for testing in bird trials to determine at which passage attenuation occurred.

1b: Determine at which passage each replicate became completely attenuated.

In vivo trials using the serially passed viral stocks to infect birds were conducted to determine when attenuation first occurred in each of the three parallel lines. Based on resulted generated in earlier studies it was predicted that the virulent MDV replicates would attenuated within approximately 30 to 100 passages (16, 24, 34, 35).

Objective 2: Identify sequence variation and expression differences in attenuated strains that appeared likely candidates responsible for creation of attenuated strains.

Sequencing both the DNA and mRNA from the lowest attenuated passage of the three MDV replicates identified differences in both the sequence and expression of genes in attenuated strains compared to the parental B40BAC. The frequency of these candidate mutations were then tracked over the course of serial passage using viral stocks saved every 10 passages. Mutations believed to be causative for attenuation were expected to be high frequency, nonsynonymous mutations that were found within genes commonly mutated within the replicate attenuated populations and whose frequencies increased during in vitro passage at similar timepoints that corresponded to a decrease in virulence in vivo.

Objective 3: Experimentally validate candidate mutations which appear to correspond with attenuation.

Mutations identified from the attenuated strains were used to replace the original virulent forms in the parental Md5B40BAC clone. The resulting recombinant viruses generated were
tested in birds to determine their ability to cause MD, or if incorporation of candidate mutations caused the engineered viruses to become avirulent. Recombinant viruses with an attenuated phenotype would confirm that mutations integrated in the recombinant viruses were responsible for changing the virus from a virulent to an attenuated form.

**Objective 4: Further characterization of mutations shown to alter virulence**

Mutations identified in Objective 3 shown to alter *in vivo* phenotypes of recombinant MDVs were further characterized to describe in greater detail their *in vitro* and *in vivo* characteristics. In particular, a point mutation within the UL5 helicase-primase subunit was identified which reduced virulence by nearly 90% and was selected for further characterization.

**4a: Comparison of *in vitro* replication of recombinant UL5 mutant virus to growth rates of the virulent parental virus.**

Mutations found at high frequencies in the attenuated viral populations were predicted to have had a selective advantage for *in vitro* growth, driving their increase in frequency during the course of serial passage. To determine if recombinant viruses resulting in reduced virulence *in vivo* also had an effect on *in vitro* replication one step growth curves were conducted comparing replication of the attenuated UL5 recombinant virus to the parental Md4B40BAC *in vitro.*

**4b: Serial passage of the attenuated UL5 recombinant virus through birds to identify restoration of virulence or increased *in vivo* replication due to adaptation during selection for improved *in vivo* replication.**

To determine how selective pressures within the bird may impact the point mutation within the recombinant virus, leading to either reversion or compensatory mutations occurring to
restore virulence and negate the effects of the point mutation, the UL5 recombinant virus was
serially passed through birds while selecting for individuals with the highest replication levels in
vivo for re-isolation and passage of the virus in vitro.

4c: Determine vaccinal potential of UL5 recombinant viruses

The UL5 recombinant virus was tested as candidate vaccines to determine if mutations identified
using the method outlined in Objective 1 was able to identify SNVs which not only reduce
virulence, but also provided vaccinal protection as proof of concept for this method in assisting
in the future rational design of vaccines. The UL5 point mutation was tested for vaccinal
protection as a single point mutation in a recombinant virus as well as a recombinant virus
containing both the UL5 point mutation and deletion of the Meq oncogene, which is another
mutation proposed for use as a candidate vaccine.
REFERENCES
REFERENCES


CHAPTER 2. CHARACTERIZING THE MOLECULAR BASIS OF ATTENUATION OF MAREKS DISEASE VIRUS VIA IN VITRO SERIAL PASSAGE IDENTIFIES DE NOVO MUTATION IN THE HELICASE-PRIMASE SUBUNIT GENE UL5 AND OTHER CANDIDATES ASSOCIATED WITH REDUCED VIRULENCE


ABSTRACT

Marek’s disease (MD) is a lymphoproliferative disease of chickens caused by the oncogenic Gallid herpesvirus 2, commonly known as Marek’s disease virus (MDV). MD vaccines, the primary control method, are often generated by repeated in vitro serial passage of this highly cell-associated virus to attenuate virulent MDV strains. To understand the genetic basis of attenuation, we used experimental evolution by serially passing three virulent MDV replicates generated from an infectious bacterial artificial chromosome (BAC) clone. All replicates became completely or highly attenuated indicating de novo mutation, and not selection amongst quasi-species existing in a strain, is the primary driving force for the reduction in virulence. Sequence analysis of the attenuated replicates revealed 41-95 single nucleotide variants (SNVs) at 2% or higher frequency in each population, and several candidate genes containing high frequency, nonsynonymous mutations. Five candidate mutations were incorporated into recombinant viruses to determine their in vivo effect. SNVs within UL42 (DNA polymerase auxiliary subunit) and UL46 (tegument) had no measurable influence, while two independent mutations in LORF2 (a gene of unknown function) improved survival time of birds but did not alter disease incidence. A fifth SNV located within UL5 (helicase-primase subunit) greatly reduced in vivo viral replication, increased survival time of birds, and resulted in
only 0-11% disease incidence. This study shows that multiple genes, often within pathways involving DNA replication and transcriptional regulation, are involved in de novo attenuation of MDV and provides targets for the rational design of future MD vaccines.

**IMPORTANCE**

Marek’s disease virus (MDV) is a very important pathogen in chickens that costs the worldwide poultry industry $1-2 billion annually. Marek’s disease (MD) vaccines, the primary control method, are often produced by passing virulent strains in cell culture until attenuated. To understand this process, we identified all the changes in the viral genome that occurred during repeated cell passage. We find that a single mutation in the UL5 gene, which encodes a viral protein necessary for DNA replication, reduces disease incidence by 90% or more. In addition, other candidate genes were identified. This information should lead to the development of more effective and rationally-designed MD vaccines leading to improved animal health and welfare, and lower costs to consumers.

**INTRODUCTION**

Marek’s disease virus (MDV, aka Gallid herpesvirus 2) is an oncogenic alphaherpesvirus that causes Marek’s disease (MD) in chickens, which is characterized by T-cell lymphomas, nerve lesions, and death in affected birds. MD costs the worldwide commercial poultry industry $1-2 billion in losses annually (1), therefore, control of MD is vital for economic viability. Vaccines have been successfully used to control MD since 1970 with the introduction of HVT, a related turkey herpesvirus (2). Since then, additional vaccines with better efficacy have been introduced to combat field strains that evolved to overcome existing vaccinal protection (3). This continuous need to improve MD vaccines is likely driven by the fact that MD vaccines are non-
sterilizing and, thus, do not prevent vaccinated birds from becoming infected with virulent field strains to collectively replicate and evolve within the same bird (4). Therefore, new vaccines must be periodically developed and introduced to combat the evolution of more virulent strains of MDV. Currently the most protective vaccine, known as Rispens or CVI988, is an attenuated MDV strain developed from in vitro serial passage of a virulent virus until the resulting isolate became avirulent (5).

The process of attenuation via in vitro serial passage has been used to successfully generate candidate vaccines against MD since 1969 (6). Despite this widespread and well-established history of use, the underlying mechanism(s) behind attenuation remains poorly understood. Expansion of 132 bp repeats within the inverted repeat regions flanking the unique long region of the MDV genome has been consistently observed during serial passage of MDV strains and, therefore, was once postulated to be a driving cause for attenuation. Despite the ubiquitous nature of this 132 repeat region expansion, this phenomenon is correlated with, but not causal for, attenuation because recombinant viruses completely lacking the 132 bp repeat region are still virulent and able to become attenuated via serial passage in vitro (7). Recently, next generation sequencing (NGS) has led to a massive expansion in sequencing power, allowing characterization at the molecular genetic level of the complete genomes for many viruses and strains, including serially passed MDV strains such as 648A (8) and several classical MD vaccines including Rispens and SB-1 (9, 10). This increase in sequencing power allows comparisons for identification of a range of polymorphisms among strains that might account for differences in virulence, yet connecting genetic variation with phenotypic differences still remains an additional and necessary step to understand attenuation at the genetic level.
To determine the genetic basis of attenuation, viral populations generated from the same infectious Md5 BAC clone of MDV were serially passed \textit{in vitro} to generate three attenuated replicates. For each replicate, the lowest attenuated passage was sequenced to \(\sim250-1,000\times\) depth of coverage via NGS. The resulting sequence information allowed us to accurately identify even low frequency mutations and track their occurrence within the viral populations as they evolved from the virulent parental virus and increased in frequency throughout the population. Furthermore, an additional benefit of using a BAC-derived MDV, besides ensuring initially uniform replicates before passage, is that candidate mutations identified in the serially passed attenuated replicates could be incorporated via Red-mediated recombination into the cloned viral genome allowing us to generate near-isogenic viruses. These recombinant viruses were then tested in bird trials in order to measure the phenotypic effects of candidate mutations. This approach led to the identification of several mutations that increased survival time of infected birds, and, most notably, a mutation within UL5, the helicase-primase subunit, that reduced MDV replication levels \textit{in vivo} and lessened virulence, resulting in a reduction in disease incidence by over 90\%.
MATERIALS AND METHODS

**Tissue Culture.** Chicken embryo fibroblasts (CEFs) and duck embryo fibroblasts (DEFs) were used to culture viruses. Cultures were maintained in 1:1 mixture of Leibovitz’s L-15 and McCoy’s 5A (LM) media supplemented with fetal bovine serum (FBS), 200 U/ml penicillin, 20 μg/ml streptomycin, and 2 μg/ml amphotericin B in a 37 °C, 5% CO₂ incubator. Cells were plated with 4% FBS LM media and maintained in 1% FBS LM media. For storage as viral stocks, infected cells were suspended in freezing media composed of 10% DMSO, 45% FBS, and 45% LM media and kept in liquid nitrogen.

**Viruses.** A virulent Md5 BAC-derived virus stock was generated by transfecting Md5B40BAC, an infectious pBeloBAC11 clone that contains the complete MDV Md5 genome (11), into CEF cells. Md5B40BAC-derived virus clone 1 (Md5B40BAC-c1) at passage 4 (p4) was used as the parental virus to infect three separate CEF plates with 200 plaque forming units (PFU) of virus. These three replicates were designated Rep 1 p5, Rep 2 p5, and Rep 3 p5. The original, uncloned Md5 strain p11 was also used to infect an additional plate of CEFs with 200 PFU of the virus and passed as a positive control. Plates containing a confluent monolayer of CEFs in 4% LM media were infected with the appropriate viral stock and after 24 hours, the media was changed and maintained in 1% media. When cytopathic effects developed on the entire monolayer, the viruses were passed by trypsinizing the monolayer and collecting infected cells in 1% LM media before co-culturing infected cells with fresh CEFs. Ten percent of the total cells harvested were transferred to a new plate containing a confluent monolayer of CEFs in 4% LM media to complete one passage. This process was conducted repeatedly on the three Md5B40BAC-c1 replicates and the Md5 strain for 100 passages. At every 10 passages (i.e., p15, p25, p35, etc., for
BAC-derived reps and p21, p31, p41 etc., for Md5 strain), viral stocks were saved for in vivo bird trials to determine virulence levels and identify the earliest passage at which attenuation occurred in each replicate.

**In Vivo Virulence Trials.** In order to determine virulence, viral stocks were used to challenge ADOL 15I5 x 71 maternal antibody negative chicks. A minimum of 17 day-old chicks were challenged intra-abdominally with 500 PFU of the designated virus and housed for eight weeks in Horsfall-Bauer (HB) units. Moribund birds, or those that survived up to eight weeks post challenge, were terminated and examined via necropsy for signs of MD, including tumors and nerve enlargement. All experiments were approved by the USDA, Avian Disease and Oncology Laboratory Animal Care and Use Committee (ACUC). The ACUC guidelines established and approved by the ADOL ACUC (April 2005) and the Guide for the Care and Use of Laboratory Animals by the Institute for Laboratory Animal Research (2011) were followed throughout the experiments.

**Virulence Trials of Mixed Virulent and Avirulent MDV.** Viral mixtures containing various known quantities of virulent Md5B40BAC-c1 p5 and avirulent ∆MeqBAC, a recombinant virus lacking both copies of the Meq (R-LORF7) oncogene (12), were used to infect birds. Mixtures contained 5, 10, 25, or 50 PFUs of the virulent Md5B40BAC-c1 p5 were mixed with ∆MeqBAC to yield a total of 500 PFU of virus to challenge each bird. An additional series of birds were infected with the same quantity of Md5B40BAC-c1 p5 as before (5, 10, 25, 50 PFUs) but without the addition of the ∆MeqBAC. Birds were challenged and disease incidence measured as described previously.
**Sequencing of Viral Stocks.** To identify changes in both the genome and gene expression of the attenuated replicates relative to the virulent progenitor virus, DNA and RNA of the lowest attenuated passage for each replicate, as well as the Md5 strain p11 and Md5B40BAC-c1 p5 parental viruses, were sequenced using the Illumina GAIIx platform (San Diego, CA). DNA from heavily infected plates was extracted and enriched for viral DNA using a micrococcal nuclease protocol (13) while RNA was extracted using the Stratagene Absolutely RNA Kit (Agilent Technologies; Santa Clara, CA). Library preparation and sequencing was conducted by the Michigan State University Research Technology and Support Facility (www.rtsf.msu.edu).

**Identification of Mutations.** After processing, filtering, and trimming reads based on quality scores, Illumina reads were mapped to the sequenced Md5B40BAC reference genome (HQ149526.1) using BWA (14). Single nucleotide variants (SNVs) present in attenuated replicates were called using VarScan (15) with a p<0.005. SNVs were identified from RNA sequence data following the same procedure described above, with analysis for differential expression utilizing BWA for mapping, followed by processing with Cufflinks (16).

**Tracking SNV Kinetics.** Primers were designed to amplify eight regions of the MDV genome containing 16 candidate SNVs identified via sequencing present at >20% in the attenuated replicates (Table 2-1). Template DNA was obtained from viral stocks of the serially passed replicates saved every 10 passages (from p15 to final attenuated passage of p65, p75 or p85 depending on replicate). SNV-containing regions were amplified using Phusion High-Fidelity DNA Polymerase (New England Biolabs; Ipswich, MA) and the resulting amplicons barcoded
and pooled for Illumina GAIIx sequencing. Sequenced reads were processed as previously described to determine SNV frequency at each passage level in the replicates.

**Pathway Analysis of Mutated Genes.** The Database for Annotation, Visualization and Integrated Discovery (DAVID) (17, 18) was used to identify gene pathways that were enriched for mutations in the attenuated viruses. A list of genes that contained mutations at 20% or greater frequency in any of the four serially passed replicates (Rep 1-3 and Md5 strain) was submitted to DAVID for analysis using the default parameters.

**Generation of Defined Recombinant Viruses.** Single point mutations in UL42, UL46, UL5, and two different mutations within LORF2 (aka MDV012) were incorporated individually into the parental BAC using two-step Red-mediated recombineering (19). Phusion High-Fidelity DNA Polymerase (New England Biolabs) and primer sets (Table 2-2) were used to mutate Md5B40BAC, yielding five independent recombinants: Mut UL42-D207G, Mut UL46-Q117R, Mut UL5-I682R and two involving LORF2, referred to as LORF2-Promoter and LORF2-Intron. Mutations in the BAC were verified in the bacterial stocks via Sanger sequencing before transfecting purified BAC DNA into DEF cells using the calcium phosphate method (20) to create viral stocks, which were again sequenced to confirm the desired mutation.
<table>
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<tr>
<th>Genomic Region</th>
<th>Primer Names</th>
<th>Primer Sequence</th>
<th>Genome Region Amplified</th>
<th>SNV Nucleotide Position: Amino Acid Change*</th>
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<td>17,545: intronic</td>
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<td></td>
<td>Primer J2-r</td>
<td>CTGTTGACTGTCGGAGTCT</td>
<td></td>
<td>150,169/174,285: T195A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>150,563/173,891: Q63H</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>150,567/173,887: G62V</td>
</tr>
</tbody>
</table>

*The nucleotide position in the Md5B40BAC-c1 reference genome HQ149525.1 followed by the amino acid change and their position in the protein. SNVs in repeat genes have both equivalent positions listed within IRL/TRL or IRS/TRS indicated.
<table>
<thead>
<tr>
<th>Recombinant Virus</th>
<th>Primer Name</th>
<th>Primer Sequence*</th>
</tr>
</thead>
</table>
| Mut UL42-D207G   | MDV055 D207G-f | TTGAAGCTGAAGAGGTTTTATGGCAGAAGCGTTTTGTTGATAAGTTTTGATAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGT
**In Vivo Characterization of Mutant Viruses.** Day-old, 15I5 x 71 maternal antibody negative chicks were infected with 500 PFU of the recombinant MDV. Eighteen infected chicks were housed in one HB isolator to measure MD incidence, while an additional 21 infected chicks were housed separately in another HB isolator with six uninfected contact chicks to test for horizontal transmission of recombinant viruses between birds. Additionally, one HB isolator containing uninfected negative control birds and another containing positive controls challenged with 500 PFU of the parental Md5B40BAC-c1 virus were included for each trial. For each recombinant virus, five birds were sacrificed at 7, 14, and 21 days post infection (dpi) to collect spleen samples for qPCR. All surviving birds after 10 weeks post-infection were euthanized and examined via necropsy. Recombinant viruses that showed altered disease incidence in preliminary trials were used to challenge two more isolators each with a minimum of 17 day-old, 15I5 x 71 maternal antibody negative chicks with 500 PFU of the virus, as previously described, in order to replicate measures of disease incidence.

**In Vivo Replication of MDV.** DNA extracted from spleens of infected birds was used to assay the *in vivo* replication levels of serially passed replicates and recombinant viruses relative to control virulent Md5B40BAC-c1 p5. Extracted DNA was used to quantify the relative copy number of MDV genomes present via qPCR using primers for chicken GAPDH and MDV gB with the Taqman Fast Universal PCR kit (Applied Biosciences; Foster City, CA) as described by Gimeno et al. (21).
RESULTS

Attenuation of Serially Passed Replicates

To assess the ability to identify *de novo* mutations in the MDV genome, three replicates derived from the virulent Md5B40BAC-c1 virus and one from the Md5 parental strain (p11), the strain originally used to clone the MDV genome, were serially passed *in vitro*. To determine the earliest passage at which viral attenuation occurred, birds were challenged with the passed MDV replicates and MD incidence measured. As expected, the genetically heterogeneous Md5 p11 strain became attenuated at p61, with no virulence observed after 50 serial passages (Fig. 2-1). In addition, all passed replicates became attenuated, with complete attenuation occurring at passage 65 and 75 for Reps 2 and 3, respectively, while Rep 1 retained low levels of virulence (6% MD) even at passage 85, indicating the rate of attenuation is a variable process even among initially identical replicates (Fig. 2-1).

MD Incidence in Defined Mixtures

Defined mixtures containing known quantities of virulent Md5B40BAC-c1 p5 and avirulent ΔMeqBAC were used to simulate intermediate time points of the serially passed replicates, which were mixed populations composed of both attenuated and virulent viruses at various frequencies. Additionally, equal PFUs of virulent Md5B40BAC-c1 p5 as used in the mixed populations, but without addition of ΔMeqBAC, were used as a comparison to determine if virulence was simply a matter of the raw quantity of virulent MDV used to infect a bird, or if infectivity would be influenced by the presence of additional avirulent ΔMeqBAC virions. Increasing the percentage of virulent virus within a total dose of 500 PFU increased the MD
induced by mixtures of Md5B40BAC-c1: ΔMeqBAC, as expected, but the same pattern did not hold for birds infected with identical levels of Md5B40BAC-c1 alone (Fig. 2-2). For example, birds infected with 5 PFU of the virulent Md5B40BAC-c1 along with 495 PFU of the avirulent ΔMeqBAC resulted in ~15% of the birds developing MD, yet birds challenged with 5 PFU of Md5B40BAC-c1 by itself resulted in over 90% MD (Fig. 2-2). Thus, our test for virulence is very sensitive, being virtually saturated at 5 PFU of Md5B40BAC-c1. While the quantity of additional avirulent virus reduces disease incidence following challenge with a mixed viral population, we still detect MD, even after infection with a population in which 1% of the PFU derive from virulent MDV. Therefore, mutations that lead to attenuation in our samples should exist in the population at substantial frequencies in order to explain the loss of virulence for the population as a whole.

Next-Generation Sequencing Analysis of Attenuated Replicates

Based on the results of tests above, the completely attenuated Rep 2 p65, Rep 3 p75, and Md5 strain p61, as well as the >90% attenuated Rep 1 at p75 and p85, were chosen for NGS to identify and quantify mutations in the attenuated viral genomes. The parental Md5B40BAC-c1 p5 virus used to generate the three replicate strains was also sequenced to determine any differences compared to the previously published Md5B40BAC reference sequence (GenBank accession no. HQ149525.1). This screening also allowed us to identify mutations already present in the progenitor virus prior to passage to eliminate pre-existing SNVs from consideration as causative mutations for attenuation. Analysis of the data identified six SNVs fixed at 100% in the sequenced Md5B40BAC-c1 p5 viral stocks that differed from the reference. Due to the presence of these variations in the progenitor stock and, consequently, all resulting BAC-derived
replicates, these SNVs were excluded from further analysis. While the number of SNVs identified in the sequenced viruses varied among replicates, 19-68% of called SNVs were present at frequencies of less than 2% in the viral populations (Table 2-3). To screen for the optimal candidate mutations involved in attenuation and eliminate false SNV calls due to sequencing error, mutations present at less than 2% were excluded from further analysis; the standard error of the frequency estimate was 0.9% and 0.4% for 250x and 1,000x depths of coverage, respectively. The total number remaining ranged from 41-95 SNVs, depending on the replicate (Table 2-4). These SNVs occurred within both coding and non-coding regions of the MDV genome, and of those within coding regions, over 60% were nonsynonymous mutations.

Among attenuated replicates, eight identical nucleotide changes were present at frequencies greater than 2% in at least two of the four attenuated viruses (data not shown). Five of the eight identical nucleotide changes were instances in which one viral replicate had the mutation at a high frequency while other replicates containing the same mutation were at low frequencies <10%. Only three mutations were present at moderate or high frequencies in two or more of the attenuated viral replicates. One mutation at moderate frequencies in two replicates was present at nucleotide 171,661, which is located downstream of SORF2a. This mutation occurred at frequencies of 22% and 27% in Rep 1 and Rep 3, respectively. Of the two high frequency mutations, one was identified within the three regions of the a-like sequence at positions 897, 140,705, and 183,749 occurring between 50-89% in the four attenuated replicates, while the final point mutation at nucleotide 100,014 in UL42 was found at 84% and 85% in Rep 1 and Md5 p61, respectively. Due to the logistical complexity of incorporating mutations within the three a-like regions present within herpesvirus genomes, that mutation was not considered for
creation as a recombinant virus, while the mutation within UL42 was deemed an excellent candidate.
FIGURE 2-1. Virulence of MDV replicates derived from a serially passed, BAC-cloned virus. Two bird trials (A and B) were conducted for Reps 1, 2, and 3 each at p55, p65, and p75, e.g., R1-A is Replicate 1, trial A at the indicated passages. In addition, the control strain Md5 was tested at p41, 51, and p61.
FIGURE 2-2. Disease incidence in chickens challenged with defined mixtures of virulent and avirulent MDV. Bird trials conducted in duplicate are indicated by “A” and “B,” e.g., 5:495-A was trial A where the inoculation consisted of 5 PFU B40-derived MDV and 495 rMd5ΔMeq.
<table>
<thead>
<tr>
<th>Mutation Frequency in Total Viral Population</th>
<th>Number of SNVs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Md5B40BAC-c1, p5</td>
</tr>
<tr>
<td>≤2%</td>
<td>33</td>
</tr>
<tr>
<td>3-10%</td>
<td>3</td>
</tr>
<tr>
<td>11-20%</td>
<td>2</td>
</tr>
<tr>
<td>21-30%</td>
<td>1</td>
</tr>
<tr>
<td>31-40%</td>
<td>2</td>
</tr>
<tr>
<td>41-50%</td>
<td>0</td>
</tr>
<tr>
<td>51-60%</td>
<td>0</td>
</tr>
<tr>
<td>61-70%</td>
<td>1</td>
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<td>71-80%</td>
<td>1</td>
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<tr>
<td>81-90%</td>
<td>0</td>
</tr>
<tr>
<td>91-100%</td>
<td>6</td>
</tr>
<tr>
<td>Total Number of Mutations Identified</td>
<td>49&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Six mutations in Md5B40BAC-c1 p5 were fixed 100% in our viral stocks relative compared to the reference Md5B40BAC sequence. Considering these mutations were present in the parental virus preparations, and therefore all serially passed replicates, these mutations were not considered in any further SNV analysis in the attenuated replicates.
<table>
<thead>
<tr>
<th></th>
<th>Rep 1, p75</th>
<th>Rep 1, p85</th>
<th>Rep 2, p65</th>
<th>Rep 3, p75</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of SNVs &gt;2%</td>
<td>95</td>
<td>85</td>
<td>41</td>
<td>67</td>
</tr>
<tr>
<td>Non-coding</td>
<td>67</td>
<td>57</td>
<td>22</td>
<td>43</td>
</tr>
<tr>
<td>Coding</td>
<td>28</td>
<td>28</td>
<td>19</td>
<td>24</td>
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<tr>
<td>Synonymous</td>
<td>7</td>
<td>10</td>
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<td>5</td>
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<tr>
<td>Nonsynonymous</td>
<td>21</td>
<td>18</td>
<td>12</td>
<td>19</td>
</tr>
<tr>
<td>Nonsynonymous mutations (%)</td>
<td>75</td>
<td>64</td>
<td>63</td>
<td>79</td>
</tr>
</tbody>
</table>
Besides SNV mutations, we looked for changes in gene copy number variation reflected by differences in the depth of sequenced coverage over the MDV genome of attenuated replicates versus virulent virus. Previous studies of the MDV 132 bp repeats has shown this region to greatly expand in copy number during serial passage, although this increase in copy number is not causative for the loss of virulence (7). In the virulent Md5B40BAC-c1 p5 virus before passage, the depth of coverage across the entire MDV genome was fairly consistent, with the only exception being a larger number of reads mapping to the inverted repeat region containing the a-like sequences and telomeric repeat regions-mTMR (Table 2-5). This expansion involving the repetitive a-like sequence and mTMR was also present in the three attenuated replicates. After serial passage, a 7-58x fold increase in depth of coverage corresponding to the 132 bp repeat regions was observed in the attenuated viruses, as would be expected (Table 2-5).

Candidate attenuation-causal genes were identified by screening for genes mutated more than once among the serially passed populations. Seventy-four percent of genes containing nonsynonymous mutations were mutated exclusively within only one of the three Md5B40BAC-c1 reps (Fig. 2-3). Five genes had nonsynonymous mutations in two of the three replicates: UL5 (helicase-primase helicase subunit), US8 (gE), US1 (ICP22), R-LORF4 (unknown function), and UL46 (VP11/VP12). There were only two genes with missense mutations in all three replicates: UL26 (VP24), which had an identical, but low frequency (<5%) mutation in all three replicates at nucleotide 59,317, and RS1 (ICP4). A third gene, LORF2, also contained unique mutations within all three BAC-derived replicates, although these mutations were not all nonsynonymous changes affecting protein sequence. Generally, mutated genes contained only 1-2 unique polymorphisms within a gene per replicate. Only ICP4 contained multiple, nonsynonymous mutations in all replicates (Table 2-6). In the completely attenuated Reps 2 and 3, both replicates
had multiple, high-frequency, nonsynonymous ICP4 SNVs such as G62V (Rep 2, 83.8%), T195A (Rep 2, 39.8%), Q63H (Rep 3, 100%), L256S (Rep 3, 65.9%), and S1630P (Rep 3, 85.7%). These mutations suggest ICP4 as a top candidate gene. The three Md5B40BAC-c1 replicates were not the only attenuated viruses containing high-frequency mutations within ICP4. The serially passed Md5 strain also possessed the SNV Y60C at 93%. All fully attenuated virus stocks except Rep 1 contained a high-frequency SNV in ICP4 within bases that encode amino acids 60-63, including the completely fixed mutation in Rep 3 encoding amino acid 62, suggesting that mutations within this region in particular may play an important role in attenuation.

Following sequencing of DNA to identify mutations in the attenuated replicates, SNVs were also called in the sequenced RNA isolated from infected plaques grown on CEF. While SNVs were first called in genomic DNA data, those mutations may not necessarily be expressed in mRNA, so the frequency of SNVs in DNA was compared to their frequency in RNA. Frequencies of mutations first identified in DNA were extremely consistent and comparable to their frequencies in RNA (Table 2-7). On the whole, SNV frequencies in RNA did not differ by more than 5% from their measured frequency called in DNA. Of the top candidate mutations identified, the sole exception to this trend was LORF2 in Rep 2 p65, which had the greatest difference in SNV frequency in RNA vs. DNA of 8.8%. This provides confirmation of the accuracy of the sequencing and variant calling, as well as indicating that there was no apparent preferential expression of wild-type or mutated alleles within a viral population.

RNA data were then analyzed to determine if there was differential gene expression between attenuated and virulent viruses. Comparing each attenuated replicate to the virulent parental virus identified 5-14 genes that had significantly differential expression (Table 2-8).
Analyzing genes that are differentially expressed among the attenuated replicates showed one gene, UL45 (envelopment protein), was differentially expressed in all attenuated replicates with at least a 1.5-2.9 fold increase in all attenuated viruses. In addition to UL45, R-LORF2 (vIL-8) was also differentially expressed in all replicates, although for Rep 1 there was only differential expression at p85. Despite the differential expression of these genes, there were no mutations within coding regions or nearby promoter regions of the genes themselves that occurred at frequencies exceeding 20%.

Analysis for gene pathways that were enriched for mutations among the attenuated replicates using DAVID only found one annotated cluster of functional genes that was enriched (DAVID Enrichment Score 0.56) for the attenuated replicates. This cluster contained genes involved in transcriptional regulation and regulation of RNA metabolic processes (GO:0006355 and GO:0051252), including the genes UL46, and ICP4 previously identified as candidate genes for attenuation.
<table>
<thead>
<tr>
<th>MDV Regions</th>
<th>MdS 40BACc1</th>
<th>Rep 1 p75</th>
<th>Rep 1 p85</th>
<th>Rep 2 p65</th>
<th>Rep 3 p75</th>
<th>MdS p11</th>
<th>MdS P61</th>
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<tbody>
<tr>
<td>Whole genome</td>
<td>246</td>
<td>1,011</td>
<td>909</td>
<td>357</td>
<td>600</td>
<td>406</td>
<td>213</td>
</tr>
<tr>
<td>132 bp in TRL</td>
<td>200</td>
<td>11,855</td>
<td>11,460</td>
<td>5,796</td>
<td>3,549</td>
<td>365</td>
<td>2,556</td>
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<tr>
<td>132 bp in IRL</td>
<td>208</td>
<td>11,892</td>
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<td>5,817</td>
<td>3,542</td>
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<td>a-like sequence</td>
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<td>3,667</td>
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<td>132 bp repeats</td>
<td>204</td>
<td>11,874</td>
<td>11,482</td>
<td>5,806</td>
<td>3,546</td>
<td>359</td>
<td>2,567</td>
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FIGURE 2-3. Genes with nonsynonymous mutations within attenuated MDV replicates. Serially passed MDV replicates were compared to identify commonly mutated genes shared among attenuated replicates.
<table>
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</tr>
</thead>
<tbody>
<tr>
<td>L47P</td>
<td>2.02</td>
<td>2.99</td>
<td>G62V</td>
<td>83.76</td>
<td>Q63H</td>
<td>100.00</td>
<td>Y60C</td>
<td>96.70</td>
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<tr>
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<td>3.70</td>
<td>T195A</td>
<td>39.76</td>
<td>L256S</td>
<td>65.94</td>
<td>Y271C</td>
<td>6.87</td>
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<td>8.14</td>
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<tr>
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<td>D1483V</td>
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<td></td>
<td></td>
<td></td>
<td>S1630P</td>
<td>85.70</td>
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</tbody>
</table>

<sup>a</sup> Indicates synonymous mutation
<table>
<thead>
<tr>
<th>Mutation Location</th>
<th>Mutation</th>
<th>DNA (%)</th>
<th>RNA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UL5, Rep 2-p65</td>
<td>I682R</td>
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<td>71.0</td>
</tr>
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<tr>
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<tr>
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\(^a\) Genes with an \(^*\) display more than a two-fold significant difference in expression between attenuated and virulent viruses.
Candidate Mutations and Characterization of Recombinant Viruses

Given that sequence analysis identified only a modest number of non-synonymous mutations in genes and the fact that the isolates were either completely or very highly attenuated, we further narrowed our focus to those mutations whose frequency exceeded 20% in the attenuated populations. We conducted targeted resequencing of 16 candidate SNVs identified in the attenuated replicates at consecutive 10 passage intervals from p15 until attenuation. This allowed us to estimate when these mutations first occurred and correlate increases in candidate SNV frequencies with decreasing virulence in vivo. Mutations that were at high frequencies in the final, attenuated population and whose increase in frequency occurred roughly around passages 55-65, when a drop in virulence of the serially passed populations was most predominant when tested in vivo (Fig. 2-1), were identified as top candidates, particularly if they were nonsynonymous mutations (Fig. 2-4). Of the candidate SNVs included in targeted resequencing, five mutations were picked as the top candidates to first test as recombinant viruses due to their location within either the unique long or short region of the MDV genome (Fig. 2-4). Based on mutation frequencies and a concentration of high frequency nonsynonymous mutations clustered around amino acids 60-63 in ICP4, ICP4 appears to be a top candidate for involvement in attenuation. However, ICP4 was not included among the five mutations initially tested using recombinant viruses due to its location within the repeat regions of the herpesvirus genome, as additional recombineering steps would have been required for mutating both copies of ICP4, instead of a single mutation necessary for genes located in unique regions. Despite this complication, characterizing ICP4 mutations via recombinant viruses is currently underway.

These five mutations within four genes were incorporated into Md5B40BAC to generate the following recombinant viruses: Mut UL42-D207G, Mut UL46-Q117R, Mut UL5-I682R, Mut
LORF2-Promoter, and Mut LORF2-Intron. The first three recombinant virus mutations altered amino acids within the dsDNA-binding protein/DNA polymerase processivity subunit, the VP11/VP12 tegument protein, and the helicase-primase subunit, respectively, while the two final mutations both are non-coding mutations that may affect expression of a fourth gene of unknown function, known as LORF2.

In vivo bird trials of the five recombinant viruses indicated that two of the mutations, UL42-D207G and UL46-Q117R, had no effect on survival or MD incidence (Fig. 2-5 and Table 2-9). Three recombinant viruses, Mut LORF2-Promoter, Mut LORF2-Intron, and Mut UL5-I682R all increased length of survival in birds challenged with the recombinant viruses relative to the virulent Md5B40BAC-c1, but upon termination and examination via necropsy both LORF2 mutants exhibited 100% MD incidence; though interestingly, Mut LORF2-Intron did not appear to transmit horizontally. Only Mut UL5-I682R not only influenced survival time, but also resulted in an almost complete reduction in virulence. Birds infected with Mut UL5-I682R survived to the end of the 10 week experiment and upon termination, only 11% of infected birds developed any symptoms of MD, in comparison to 100% of control Md5B40BAC-c1-infected birds (Table 2-9). Further independent trials measuring disease incidence of Mut UL5-I682R resulted in 0% (0/17 birds) and 6% (1/18 birds) of birds developing MD.

To determine if attenuated replicates and recombinant viruses retained the ability to replicate in vivo or if loss of oncogenicity resulted from loss of viral replication in vivo, splenic DNA from infected birds was extracted to measure MDV replication in birds using qPCR ratios comparing copy numbers of viral gB DNA versus copies of chicken GAPDH genes averaged over the three collection time points (Fig. 2-6). The average relative number of gB/GAPDH copies in Md5B40BAC-c1 ranged from 0.14-0.33, based on four separate bird trials. The three
serially passed Md5B40BAC-c1 replicates exhibited about a 10-fold reduction in viral replication, while the recombinant Mut UL5 I682R also had a ratio comparable to these attenuated viruses of 0.016 (Fig. 2-6). The two virulent recombinant viruses, Mut UL42-D207G and Mut UL46-Q117R, had ratios comparable to the virulent virus (Fig. 6). The LORF2 mutations, which resulted in greater survival for challenged birds yet still exhibited 100% MD incidence, exhibited values of 0.13-0.14, which were towards the lower range of values observed with Md5B40BAC-c1. Virulent viruses, such as Md5B40BAC-c1, Mut UL42-D207G, Mut UL46-Q117R, and both Mut LORF2 recombinants, all exhibited significantly higher levels of MDV than the three serially passed attenuated viruses or Mut UL5-I682R. While attenuated viruses had low levels of replication compared to virulent viruses, their levels of replication were higher than the background of uninfected controls, indicating they still replicated in vivo. Not only was a low level of MDV detectable via qPCR in attenuated viruses, but viable viruses were able to be reisolated from peripheral blood lymphocytes of infected, but MDV negative, birds challenged with the attenuated viruses when plated on DEF cells to produce plaques (data not shown), further supporting that these attenuated viruses replicated in vivo.

Based on the dramatic decrease in virulence due to the UL5 mutation, a revertant of Mut UL5-I682R was created called Revt UL5-R682I. This revertant virus not only replicated at high levels in vivo, but also restored levels of virulence comparable to that of the wild-type, Md5B40BAC-c1 virus (Fig. 2-6 and Table 2-9).
FIGURE 2-4. Kinetics of candidate SNP frequencies over serial passage. Targeted resequencing of candidate SNPs allowed for the frequency of each mutation to be determined from the earliest passage in which the mutation occurred until attenuation.
FIGURE 2-5. Survival of birds challenged with recombinant MDVs. Mortality due to MD over the course of the 10 weeks was determined for the five recombinant viruses and the virulent B40-derived MDV positive control.
<table>
<thead>
<tr>
<th>Virus</th>
<th>MD+ Challenged Birds</th>
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<tbody>
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</tr>
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<td>17/17</td>
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<td>Md5B40-c1</td>
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</tr>
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</table>
FIGURE 2-6. Comparison of *in vivo* replication for attenuated replicates and recombinant viruses. Due to housing and chick availability, recombinant viruses were tested incrementally, therefore positive (Md5B40-c1) and negative (uninfected) controls are matched to the respective recombinant viruses tested collectively by matched symbols (i.e. *, ^, #, etc.). Trials for Mut UL5 were conducted in duplicate and each trial indicated is by “A” or “B.”
DISCUSSION

This work shows attenuation of a genetically homogeneous MDV reproducibly occurs after repeated serial passage in vitro, indicating that de novo mutations arising during passage are sufficient to generate new, avirulent viral populations. A variety of mutations were identified within each attenuated MDV replicate relative to the parental virulent virus, of which several candidate mutations were found within genes of interest including ICP4 (transcriptional transactivator), UL42 (DNA polymerase subunit), UL46 (VP11/VP12 tegument protein), UL5 (helicase-primase subunit), and LORF2 (unknown function). In particular, no single mutation rose to high frequency in every attenuated replicate, nor was any single gene mutated at high frequencies in every replicate. Two genes, ICP4 and UL26, were mutated in all attenuated replicates but, at least in Rep 1, the frequencies of the respective mutations suggest that they are not solely responsible for attenuation. Furthermore, we experimentally identified individual mutations in UL5 and, to a lesser extent, in LORF2, that resulted in a phenotypic change that contributed to attenuation, while mutations within UL5 have been shown to result in a reduction in virulence in other herpesviruses, suggesting UL5 is a factor in virulence of herpesviruses (22, 23).

While attenuation of a virulent virus via serial passage in vitro is not a new or unusual phenomenon unique to MDV, the use of virus generated from a BAC-cloned MDV in this study allowed for a more detailed insight into the mechanism(s) of attenuation than previous studies (6, 8). When trying to understand the driving mechanism that causes a virulent viral population to become avirulent, the two most probable explanations are selection or mutation. Like many viruses, MDV strains, even those that are plaque purified, are often described as quasi-species composed of genetically diverse genotypes existing within a strain population (8, 11). Thus,
while the viral strain may be described as a virulent, there can be unique subpopulations that do not share the same degree of virulence compared to the whole population. Therefore, viral attenuation could be driven by selection for pre-existing variation of low-frequency, avirulent genotypes within a strain. A second explanation proposes *de novo* mutations that occur during viral replication over serial passage generate new avirulent genotypes to attenuate the virus. Therefore, by passing both a traditional virulent MDV strain and viruses from a BAC-derived clone, we could distinguish whether attenuation requires pre-existing variation within a strain, or if normal mutation rates occurring during serial passage are sufficient to generate avirulent viruses *de novo* from the genotypically homogenous virus. Not only did the Md5 strain become attenuated, as expected, but the three serially passed Md5B40BAC-c1 replicates attenuated as well. This result shows, that despite beginning from a single virulent genotype, *de novo* mutation is sufficient to generate avirulent viruses, and attenuation is not simply selection upon pre-existing variation within quasi-species. However, as the Md5 strain became attenuated at earlier passages, it is possible that selection of pre-existing variants in the population may also be a contributing mechanism.

To better understand attenuation, we then compared duration of time before attenuation between the serially passed replicates, and the first noticeable difference was that time until attenuation varied between replicates. While a reduction in virulence occurred in all replicates, attenuation did not occur simultaneously in all replicates despite being derived from identical BAC-derived virus stocks before passage. Rep 2 was completely attenuated at p65, Rep 3 at p75, and the virulence of Rep 1 was greatly reduced, but not completely attenuated, at p85. Despite these differences, all replicates showed dramatic decreases in virulence with a 50-80% decrease in MD incidence within a span of ~10 passages. This rapid change suggests a small number of
mutations of large affect are likely responsible for attenuation. In contrast, if attenuation was the result of an accumulation of many small, additive mutations that progressively accrued over time, we would have expected a slower, more gradual decline in virulence (24, 25).

Simply looking at the total number of mutations that occurred at >2% in the viral population, no replicate had greater than 100 mutations with the number of SNVs in the three replicates ranging from 41 to 95. While this is a relatively small number of mutations within a genome of ~184 Kb, the actual number of candidate mutations is reduced even further to yield a combined list of only 16 candidate SNVs from the three Md5B40BAC-c1 replicates when considering only mutations occurring at high frequencies exceeding 20% in the population, which is still a conservative cut-off value. As a result, we conclude that there are only a small handful of probable candidate mutations occurring at high frequency to cause attenuation of the population. Further filtering of these SNVs based on frequency kinetics in relation to decreasing virulence identified five candidate mutations and genes underlying attenuation.

Further comparisons among the three attenuated replicates to find commonly mutated genes identified ICP4 as a prime candidate gene. ICP4 contained several nonsynonymous candidate SNVs at frequencies greater than 60% in the completely attenuated MDV replicates, including one mutation completely fixed at 100% in a replicate. As ICP4 is an immediate early transcriptional regulator that controls expression of other immediate early, early, and late genes, it is reasonable that mutations altering normal ICP4 function could have a cascade affect, altering regulation of many downstream genes. RNA-seq analysis of attenuated viruses identified 5-14 genes that were differentially expressed but when compared to the list of SNVs identified there were no mutations within the genes themselves to explain their differential expression. Instead, mutations within upstream regulators, such as ICP4, could explain this observation in which
mutations within ICP4 could lead to larger, widespread effects within the population resulting from relatively few mutations occurring within ICP4.

The results of in vivo trials of recombinant viruses involving a single SNV within UL5 encoding the helicase-primase subunit showed that a single mutation can have a substantial impact on the phenotype of recombinant viruses. Unlike the other four recombinant viruses that retained 100% disease incidence in vivo, the UL5 I682R mutation reduced MD incidence by at least 89% or more, depending on the trial. This mutant virus also had much lower levels of viral replication in vivo compared to the virulent Md5B40BAC-c1. All serially passed Reps had comparable levels of MDV replication in vivo, as did the Mut UL5-I682R. As a result of this dramatic phenotypic change due to the mutation within UL5, we created a revertant virus to verify that the results of in vivo trials could be attributed to the desired single point mutation and not extraneous changes that may have occurred unbeknownst to us during recombineering and generation of viral stocks. Challenging birds with this virus clearly showed the reversion restored a wild-type phenotype for survival, levels of in vivo replication, and disease incidence, confirming our conclusion that the single point mutation within Mut UL5-I682R was responsible for the observed phenotypic changes. While this single mutation did not cause complete attenuation of the virus, it does show that even a single nucleotide change is capable of causing dramatic changes in virulence. This result clearly warrants further study to determine if introduction of additional candidate mutations, such as the next-highest frequency candidate mutation within UL46 (VP11/VP12 tegument protein) identified within the same replicate, is able to have an additive or epistatic influence to cause complete attenuation in a recombinant virus.
This dramatic decrease in disease incidence exhibited by the mutation within UL5-helicase-primase within this study indicates the importance of this gene as a factor in virulence. Sequence comparison of Gallid herpesvirus-1, an alphaherpesvirus also known as infectious laryngotracheitis virus (ILTV), identified a nonsynonymous mutation within UL5 that was unique to the vaccine strain and enabled the attenuated virus to be distinguished from four other virulent ILTV strains (26). Mutations within UL5 have been shown to cause a reduction in virulence in herpesviruses other than Gallid herpesviruses as well. Studies involving HSV-1 have identified several mutations within UL5 that have been determined to cause a reduction in virulence (27). Biochemical analysis of the UL5 Gly 815 mutant shows a decrease in binding affinity of single-stranded DNA and reduced turnover rate (28). This mutation in HSV-1 is unable to replicate in vivo alone, but when additional purified components of the heterotrimeric complex are provided, the mutant was able to perform normal helicase-primase functions. While the three components of the functional complex, UL5, UL8, and UL52, are all necessary for normal function in vivo, during in vitro replication UL8 is not. Growth curves measuring the in vitro replication for our Mut I682R relative to the Md5B40BAC-c1 are yet to be conducted, but it is clear the recombinant Mut UL5 replicates in vitro to form plaques and generates high viral titers. Despite this ability to replicate in vitro, its ability to replicate in vivo is severely reduced compared to the virulent wild-type, which has been observed in other UL5 mutants. Since serial passage of MDV in tissue culture drives selection for improved in vitro replication, it would be anticipated that the mutation within UL5 would not detrimentally affect in vitro replication or even, as a result of selection for improved in vitro growth, the observed Mut I682R could potentially improve in vitro growth. Whether in vitro replication is altered by the UL5 mutation compared to the wild-type BAC, growth curves quantifying replication of the Mut UL5-I682R
virus may offer an explanation for how this mutation rose to a high frequency in the attenuated populations.

While this high frequency SNV in UL5 resulting in greatly reduced virulence was only present in Rep 2, three additional mutations within UL5 were also found within Rep 1, although these SNVs were present at much lower frequencies than the mutation found within Rep 2. As seen in ICP4, it appears that identical mutations shared among replicates are rare, but there were several commonly mutated genes between attenuated replicates, such as with ICP4, UL5, and LORF2. Experimental evolution studies involving over 100 replicates of E. coli have shown high degrees of convergent evolution between replicates at the level of genes and pathways, while identical nucleotide mutations were rarely shared among the replicates (29). Based on these conclusions, we looked for commonly mutated pathways among attenuated replicates to determine if there were key pathways involved in attenuation. After screening all mutations for the most probable candidates we identified five top candidate genes, of which two of the five candidates were genes involved in DNA replication; a mutation within UL5 and a second one within UL42. Considering that mutations within genes involving DNA replication are probable targets for selection to cause adaptation of viruses during in vitro growth, wider inspection of all mutations within attenuated replicates of the seven genes required for DNA replication in herpesviruses (UL5, UL8, UL9, UL29, UL30, UL42 and UL52) revealed multiple mutations within genes involved in DNA replication, especially within Rep 1. Of these genes, we found eight mutations at frequencies >2% within all the seven genes required for DNA replication within Rep 1, p85. Clustering of mutations within DNA replication genes, such as the UL5 (helicase-primase) and UL42 (DNA polymerase subunit), as well as the significant phenotypic effect of the mutation tested within UL5, suggests that mutations in the genes associated with the
DNA replication pathway are targeted for selection during serial passage leading to attenuation of the virus. To further identify commonly mutated pathways, we used DAVID to determine pathways that were enriched for mutants in the attenuated replicates. Considering only high frequency, nonsynonymous mutations at >20% in the four attenuated viral populations, we found pathways containing genes involving transcriptional regulation were enriched for mutations. It is possible that pathways involving transcriptional regulation, at both the DNA-dependent and RNA metabolism levels, are mechanisms which, when disturbed, can lead to attenuation. By finding enrichment for pathways involving transcriptional regulation due to multiple mutations within genes involved in DNA replication, such as the Mut I682R in UL5, supports our hypothesis that serial passage in vitro selects for increased replication and adaptation for growth in tissue culture. Mutations altering normal interactions of these gene products would impact viral replication and lead to the changes in phenotype observed in attenuated viruses.

In conclusion, our results show that it is possible to attenuate viral BAC-derived clones as a result of de novo mutation, and that it is apparent there is variation among viruses in the manner of how they become attenuated. All serially passed replicates contained unique candidate mutations and, while there may be commonly mutated genes among attenuated viruses such as ICP4, it appears that there is no single route that all viruses must follow to become attenuated. Multiple candidate mutations have been identified and tested to determine their effect within genes of known and unknown function such as LORF2, DNA polymerase subunit, VP11/VP12 tegument protein and helicase-primase subunits. Singly, no one mutation was able to cause complete attenuation, yet one SNV within the helicase-primase subunit had a dramatic decrease in virulence indicating an important role for this gene in MDV attenuation. Additional work to further characterize candidate mutations identified in this study may identify a minimum number
of mutations required to create a fully avirulent virus through changing only a small handful, perhaps 2-3 key mutations. This could allow the precise engineering of avirulent viruses for production of candidate vaccines via mutation of critical genes involved in attenuation, without relying on blind serial passage and random chance to generate the next superior MD vaccine candidate.

ACKNOWLEDGEMENTS

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REFERENCES
REFERENCES


CHAPTER 3. MUTATIONS WITHIN ICP4 ACQUIRED DURING IN VITRO ATTENUATION DO NOT ALTER VIRULENCE OF RECOMBINANT MAREK’S DISEASE VIRUSES IN VIVO

ABSTRACT

Marek’s disease (MD) is a T-cell lymphoma of chickens caused by the oncogenic Marek’s disease virus (MDV). MD is primarily controlled by live-attenuated vaccines generated by repeated in vitro serial passage. Previous efforts to characterize attenuated MDVs identified numerous mutations, particularly a convergence of high-frequency mutations around amino acids 60-63 within ICP4 (RS1). Therefore, ICP4 was considered a candidate gene deserving further characterization. Recombinant MDVs were generated containing a single Q63H mutation or double Q63H+S1630P mutations. Despite the repetitive nature of mutations within ICP4 in attenuated viruses, neither recombinant virus decreased virulence, although one mutant reduced in vivo replication and failed to transmit horizontally. Our results indicate these mutations are insufficient to reduce disease incidence in infected birds and suggest variants in ICP4 do not directly alter virulence, but rather may enhance MDV replication rates in vitro, offering an explanation for the widespread occurrence of ICP4 mutations in a variety of attenuated herpesviruses.
INTRODUCTION

Gallid herpesvirus 2, also commonly known as Marek’s disease virus (MDV), is an oncogenic alphaherpesvirus of chickens. Afflicted birds display symptoms of Marek’s disease (MD) including depression, cachexia, and paralysis due to viral-induced T cell lymphomas that ultimately result in death. The primary mode of MD control has been via the use of vaccines, which has significantly reduced losses due to MD since the 1970’s (1). Despite the widespread use of vaccinal control of MD, virulent strains of MDV able to overcome vaccines have emerged, leading to the need for periodic introduction of new and more effective MD vaccines approximately every 10 years (2). Currently the most protective vaccine commercially available, CVI988/Rispens, has been in use in the United States since the 1990’s. Concern regarding the potential for MD outbreaks in Rispens-vaccinated flocks has highlighted the importance for vaccine development (3). One common method for vaccine production has been the use of live attenuated viruses generated via repeated in vitro serial passage. Several MD vaccines, such as HPRSS-16 and Rispens, were generated by in vitro serial passage and provide testament to the utility of this process in vaccine development (4, 5).

To better understand this process and identify candidate genes involved in attenuation, previously we serially passed and sequenced the complete genome of four attenuated MDV replicates (three Md5BAC-derived viruses and one Md5 strain) to identify candidate mutations and genes involved in attenuation (6). Several candidate mutations present within genes in the unique long (UL) or unique short (US) regions of the viral genome were characterized via recombinant viruses. Among the five point mutations tested, one recombinant virus revealed a single nucleotide mutation in UL5 (helicase-primase subunit) able to reduce disease incidence by 90% or more. Due to the additional complexity involved in mutating genes present within the
two long and short repeat regions (TRL/IRL and TRS/IRS) in MDV, these candidate mutations were not initially tested.

Therefore, the purpose of this study was the characterization of a top candidate gene, ICP4, identified within the repeat regions of the MDV genome. ICP4, encoded by RS1, is an immediate early transcriptional regulator in herpesviruses located within the TRS/IRS region of MDV and a gene commonly mutated in all of the attenuated replicates. All four passed viruses had mutations within ICP4, with three viruses containing high frequency (80-100%) nonsynonymous mutations within amino acids 60-63 in ICP4. Three other high frequency (40% or higher) nonsynonymous mutations in ICP4 were also observed, including one at amino acid position 1630 (85% frequency). Due to these numerous high frequency, parallel mutations within the attenuated replicates, ICP4 was considered a candidate gene for attenuation. Using Red-mediated recombineering, we generated two recombinant viruses to examine how these mutations in ICP4 impacted virulence of MDV.
MATERIALS AND METHODS

Recombinant Viruses and Tissue Culture. ICP4 mutations were incorporated into Md5B40BAC, the pBeloBAC11 clone containing the entire MDV (Md5 strain) genome, using Red-mediated recombineering (7–9). To generate recombinant viruses containing the desired single nucleotide variant (SNV) within both copies of ICP4, one copy of ICP4 was first deleted using the ΔICP4 primer set (Table 3-1) based on the process of manipulating genes within repeat regions described by Engel et al.(10). Point mutations were then incorporated within the remaining copy of ICP4 via additional rounds of recombineering using the primer sets Mut ICP4 Q63H and Mut ICP4 S1630P. The resulting mutants, designated as Mut ICP4-1 and Mut ICP4-2, both contained the mutation Q63H, while the latter also had the additional SNV S1630P. The Q63H mutation was fixed 100% in one of the serially passed attenuated replicates during our previous study (6), while the S1630P mutation was the second highest frequency ICP4 mutation within the same replicate present at 85%. All introduced mutations were confirmed via Sanger sequencing of mutated BACs before transfecting BAC DNA into duck embryo fibroblasts (DEF) via the calcium phosphate method (11). Additionally, mutations in the generated viral stocks were confirmed by Sanger sequencing. Viral stocks were then titrated on DEF cells to quantify the number of plaque forming units (PFU) in frozen viral stocks.
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</tr>
</tbody>
</table>

Nucleotides in uppercase indicate the region of primer homologous to MDV for integration into the MDV genome while nucleotides in lowercase are regions complementary to the kanamycin cassette. The single lowercase nucleotide in bold and underlined indicates the point mutation altered for generation of recombinant viruses.
**In Vivo Characterization of Mutant Viruses.** Maternal antibody negative, ADOL 15I₅ x 7₁ day old chicks were challenged intraabdominally with 500 PFU of each recombinant MDV or the original Md5B40BAC-c1 as a positive control, and housed in Horsfall-Bauer (HB) isolators for 12 weeks. Uninfected birds were housed as negative controls in a separate isolator. All surviving birds, or those that were moribund during the course of the experiment, were terminated and examined via necropsy to determine disease incidence. To determine horizontal transmission, uninfected contact birds were housed in the same HB unit with challenged birds. Five infected birds were removed for sampling at 7, 14 and 21 days post infection (dpi) to collect spleen tissues to quantify in vivo replication of viruses via qPCR comparing the relative quantity of the MDV gB gene to chicken GAPDH gene using a Taqman Fast Universal PCR kit (Applied Biosciences; Foster City, CA) and primers described by Gimeno et al. (12). All in vivo bird trials were approved by the USDA, Avian Disease and Oncology Laboratory Animal Care and Use Committee (ACUC). Based on ACUC guidelines established and approved by the ADOL ACUC (April 2005) and the Guide for the Care and Use of Laboratory Animals by the Institute for Laboratory Animal Research (2011), procedures for bird housing and care were followed throughout the duration of the experiment.

**Sequence Comparison of ICP4 among Alphaherpesviruses**

The complete amino acid sequence of the immediate early transcriptional regulator gene ICP4 from 20 different species of alphaherpesviruses was obtained from the NCBI database. All 20 sequences were aligned using ClustalW (13) and a maximum likelihood phylogenetic tree constructed in Mega6 (14), while pairwise comparisons for each of the 20 ICP4 sequences relative to herpes simplex virus 1 (HSV-1) ICP4 (GenBank accession no. AGZ01922.1) were
aligned via the UniProt protein resources database (15) to calculate the percentage of shared sequence identity among ICP4 sequences.

RESULTS

In Vivo Trials

Disease incidence resulting from challenge with either Mut ICP4-1 (single ICP4 Q63H SNV) or Mut ICP4-2 (double Q63H and S1630P SNVs) were 100% and 95%, respectively. Compared to the parental virus that showed 84% MD incidence, these mutations did not reduce virulence of the viruses in infected birds (Table 3-2). While most challenged birds developed MD, the disease incidence in contact birds varied significantly between the two recombinant ICP4 viruses. Contact birds housed with the Mut ICP4-1 virus all developed MD, yet no contact birds mixed with birds challenged with the Mut ICP4-2 recombinant virus developed MD (Table 3-2).

While both recombinant viruses resulted in over 90% disease incidence, there was delayed mortality for birds challenged with the double Mut ICP4-2 recombinant virus compared to Mut ICP4-1 and Md5B40BAC-c1. Birds challenged with the single Mut ICP4-1 recombinant virus experienced survival comparable to the original Md5B40BAC-c1 (Fig. 3-1). On the other hand, the double Mut ICP4-2 virus exhibited lower mortality from week 4 onwards (Fig. 3-1), with approximately 20% of birds surviving the full length of the 12 week experiment compared to birds challenged with either Mut ICP4-1 or Md5B40BAC-c1, which all exhibited severe morbidity and euthanasia of all birds by week 10.

Replication of MDV within the spleen of birds sacrificed at 7, 14 and 21 dpi as determined by qPCR showed a range among the three viruses (Fig. 3-2). Compared to Mut ICP4-1 and the wild-type Md5B40BAC virus, the double mutant Mut ICP4-2 recombinant virus
showed lower levels of \textit{in vivo} replication at 7 and 14 dpi, with significant differences in replication at day 14 (ANOVA p-value=0.0136), though this difference was not observed at 21 dpi. Further pairwise comparisons of \textit{in vivo} replication at day 14 showed significant differences in replication between Mut ICP4-1 and Md5B40BAC-c1, with differences between Mut ICP4-1 and Md5B40BAC-c1 falling short the threshold for significance (T-test p= 0.0101 and p=0.0557, respectively)
### TABLE 3-2. MD Incidence of Recombinant ICP4 Mutant Viruses

<table>
<thead>
<tr>
<th>Challenge Virus</th>
<th>MD Incidence in Challenged Birds</th>
<th>MD Incidence in Contact Birds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># MD+ Birds/ Total Birds</td>
<td>Percent MD</td>
</tr>
<tr>
<td>Mut ICP4-1</td>
<td>10/10*</td>
<td>100</td>
</tr>
<tr>
<td>Mut ICP4-2</td>
<td>18/19</td>
<td>95</td>
</tr>
<tr>
<td>Md5B40BAC-c1</td>
<td>16/19</td>
<td>84</td>
</tr>
</tbody>
</table>

*Due to chick mortality during the first two weeks of age in isolators challenged with Mut ICP4-1, fewer total birds were available compared to Mut ICP4-2 and Md5B40BAC-c1.
FIGURE 3-1. Survival curves of birds challenged with ICP4 recombinant MDV. Mortality of birds during the 12 week experiment until termination was compared for the two ICP4 mutant viruses relative to the parental Md5B40BAC virus.
FIGURE 3-2. Viral replication rates of birds challenged with ICP4 recombinant MDV. *In vivo* replication of recombinant ICP4 MDV versus parental Md5B40BAC virus.
Homology of Alphaherpesviruses ICP4 Protein Sequence

Comparing the prototypical alphaherpesvirus (HSV-1) to the 20 alphaherpesviruses sequences via pairwise alignment of HSV-1 to each respective species sequence showed a high degree of variation among ICP4 between species. Amino acid sequence identity for ICP4 of the 20 species surveyed relative to HSV-1 ranged from approximately 15-71%, with an average shared sequence identity of 36% of amino acids identical among the query species and HSV-1 (Table 3-3). Alignment of HSV-1 to Mardivirus sequences show an average sequence identity of 17% among the three Mardivirus species, versus an average shared sequence identity of 39% for HSV-1 alignments with non-Mardivirus species. Alignment of HSV-1 ICP4 and the virulent MDV ICP4 sequences revealed the lowest degree of shared sequence identify of approximately 15%.

Alignment of all 20 ICP4 sequences showed a high degree of variation among alphaherpesviruses surveyed, not only in coding sequence but also in total length. The average length of ICP4 in the 20 alphaherpesviruses surveyed was approximately 1,500 amino acids. However, the average length of ICP4 in Mardivirus species infecting chicken (Gallid herpesvirus 2, Gallid herpesvirus 3 and Meleagrid herpesvirus 1) revealed significantly larger (2,173 amino acids) ICP4 proteins, with Gallid herpesvirus 2 having the longest sequence of any herpesvirus species surveyed at 2,323 amino acids (Table 3-3). The majority of these nearly 700 additional amino acids are found at the N terminus of ICP4 in Mardiviruses. Clustal alignment of the 20 herpesvirus species show that for the first 500 amino acids of MDV ICP4, the only species with homologous amino acids to this region are Gallid herpesvirus 3 and Meleagrid herpesvirus 1, both of which also belong to the Maridivirus genus (Fig. 3-3). Furthermore, the cluster of high frequency mutations within ICP4 identified in the attenuated MDV replicates around amino acids
60-63 occurs within this additional region that is unique relative to other alphaherpesviruses. Even among Mardiviruses that possess a longer ICP4, the region around amino acids 52-69 is exclusively found only in the virulent Gallid herpesvirus 2 species, preventing functional predictions for this mutated region based on homology to other species (Fig. 3-3).

Alignment of ICP4 sequences showed the regions flanking the second mutation at amino acid 1630 are present in the majority of herpesvirus species, yet among those amino acids present, there is considerable variation in amino acids present at and around this position (Fig. 3-4). The MDV wild type serine is an amino acid seen in other herpesvirus species, such as Gallid herpesvirus 1 and both HSV-1 and HSV-2. The mutation of a serine to proline residue at position 1630 is predicted to be a tolerated change by programs such as SIFT (16), as well an evolutionarily acceptable amino acid considering that proline is commonly seen in several herpesvirus species, such as Equid herpesvirus 1, Suid herpesvirus 1, Psittacid herpesvirus 1, and the closely related Melaeagrid herpesvirus 1 (Fig. 3-4). Based on the alignment to HSV-1, the MDV S1630P ICP4 mutation corresponds to roughly the same serine residue at position 683 in HSV-1. This position is expected to be a globular region within the third defined region of ICP4 in HSV-1. This position is also characterized as occurring within a functional domain implicated for nuclear localization (17), yet the corresponding amino acid regions within MDV ICP4 do not appear to contain a known nuclear localization signal surrounding the mutated region in MDV ICP4 based on various prediction programs (NucPred, NLS Mapper and NLStradamus; data not shown).
<table>
<thead>
<tr>
<th>Alphaherpesvirus Species</th>
<th>Protein Length (a.a.)</th>
<th>Sequence Identity to HSV-1 ICP4 in Percent</th>
<th>Protein ID Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psittacid herpesvirus 1</td>
<td>2033</td>
<td>14.4</td>
<td>refNP_9444441.1</td>
</tr>
<tr>
<td>Gallid herpesvirus 2</td>
<td>2323</td>
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<td>gblAAT65019.1</td>
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<tr>
<td>Meleagrid herpesvirus 1</td>
<td>2164</td>
<td>18.0</td>
<td>refNP_0733661.1</td>
</tr>
<tr>
<td>Gallid herpesvirus 3</td>
<td>2033</td>
<td>18.6</td>
<td>gblAEI00305.1</td>
</tr>
<tr>
<td>Gallid herpesvirus 1</td>
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<td>22.2</td>
<td>gblAAA461111.1</td>
</tr>
<tr>
<td>Cercopithecine herpesvirus 9</td>
<td>1279</td>
<td>25.3</td>
<td>refNP_077485.1</td>
</tr>
<tr>
<td>Human herpesvirus 3</td>
<td>1310</td>
<td>27.0</td>
<td>gblAHB80301.1</td>
</tr>
<tr>
<td>Anatid herpesvirus 1</td>
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<td>gblADU04083.1</td>
</tr>
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<td>Canid herpesvirus 1</td>
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<td>dbjIBAA32781.1</td>
</tr>
<tr>
<td>Suid herpesvirus 1</td>
<td>1456</td>
<td>30.0</td>
<td>gblAEM64140.1</td>
</tr>
<tr>
<td>Equid herpesvirus 1</td>
<td>1486</td>
<td>32.6</td>
<td>dbjIBAD83403.1</td>
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<td>Felid herpesvirus 1</td>
<td>1398</td>
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<td>Bovine herpesvirus 1</td>
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<td>1168</td>
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<td>Macacine herpesvirus 1</td>
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<td>refNP_851933.1</td>
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<td>57.7</td>
<td>refYP_164516.1</td>
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<td>Human herpesvirus 2</td>
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<td>69.3</td>
<td>gblAEV91413.1</td>
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<tr>
<td>Chimpanzee alpha-1 herpesvirus</td>
<td>1268</td>
<td>71.1</td>
<td>gblAFV26963.1</td>
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<tr>
<td>UL36 Gallid herpesvirus 2 *</td>
<td>3319</td>
<td>7.0</td>
<td>gblABV31147.2l</td>
</tr>
</tbody>
</table>

*Indicates the UL36 sequence used as an artificial outgroup for construction of the ICP4 maximum likelihood phylogenetic tree.
FIGURE 3-3. Amino acid alignment of N-terminal end of ICP4. Individual sequences for the three mardiviruses are shown while all other 17 herpesvirus ICP4 sequences are combined and shown as a non-mardivirus consensus sequence. The region containing amino acids 52-69 unique to MDV in Gallid herpesvirus 2 is indicated by the boxed region, while amino acids 60, 62 and 63 are designated with an *.
FIGURE 3-4. Amino acid alignment of 20 ICP4 species and HSV-1. The S1630P mutated position in Gallid herpesvirus 2 is indicated by *. Identical amino acids at the same position are indicated in black boxes, while amino acids with comparable properties but different identities are indicated by shaded boxes.
Phylogenetic trees of all 20 alphaherpesvirus differed when considering either a combination of multiple genes or only ICP4. Our phylogenetic tree constructed based on the complete sequence of ICP4 from the 20 herpesvirus species had an overall topology and grouping comparable to trees previously constructed using a compilation of several genes (18, 19). However, despite the overall similarity for most genera between the two trees, there was a change in the relationship of the virulent Iltovirus, known as ILTV, and the Mardiviruses between trees based only on ICP4 or using multiple genes. Combining several different genes to construct one overall phylogenetic tree groups the closely related Gallid herpesviruses 1 and 2 together as sister taxa, with the avirulent Melaeagrid herpesvirus 1 branching from the Gallid herpesvirus node to collectively classify the Mardiviruses into one group known as α3 viruses, while the virulent Iltovirus, Gallid herpesvirus 1, that causes avian infectious laryngotracheitis in chickens, branches separately from the Mardivirus lineage and is classified singly into a group known as α4 (18, 19). However, when considering only the ICP4 amino acid sequence, there was a change in the relationship of the virulent Iltovirus and the Mardiviruses. The resulting ICP4 tree has the most closely related sister taxa classified as the virulent Gallid herpesvirus-2 and ILTV, which are shown to split from the avirulent Gallid herpesvirus-3 and Meleagrid herpesvirus 1 lineage (Fig. 3-5). This yields classification of the three Mardiviruses and single Iltovirus species along lines of virulence, unlike previous studies that classifies these species relationships based on genera lines, in which the split between groups is among Mardi and Iltoviruses instead of virulent and avirulent viruses. This relationship is dependent on unique ICP4 sequences, as trimming ICP4 sequences to only use regions known to be highly conserved among alphaherpesviruses, such as regions 2 and 4 (17, 20), results in a phylogenetic tree with
the expected grouping of the Mardivirus and Iltovirus genera as separate branches, restoring the
groupings of α3 and α4 (data not shown).
FIGURE 3-5. Maximum likelihood phylogenetic tree based on the complete amino acid sequence of ICP4 from 20 herpesvirus species. Artificially rooted with the outgroup of UL36 amino acid sequence from Gallid herpesvirus 2.
DISCUSSION

During previous *in vitro* serial passage of MDV to find candidate genes involved in attenuation, ICP4 was found to be mutated repeatedly in all attenuated MDV replicates sequenced (6). These attenuated viruses contained nonsynonymous mutations at amino acids 60, 62 and 63 at frequencies of 96%, 83% and 100%, respectively, in three of the four attenuated populations. Besides these clustered mutations, three more nonsynonymous mutations exceeding frequencies of 40% or more were also seen at other positions within ICP4 in the attenuated replicates. In addition to these serially passed Md5 replicates, mutations within ICP4 in attenuated MDV have been identified by other groups as well. During serial passage and sequencing of an attenuated 648A strain of MDV, three high frequency mutations within ICP4, two nonsynonymous and one silent mutation, were found within the attenuated strain (21). Even during complete genome sequence analysis of the current “gold-standard” MD vaccine known as Rispens, mutations were found within ICP4 (22). Studies including alphaherpesviruses besides MDV also show a similar trend of mutations within ICP4 in attenuated viruses. Complete genome sequencing of an attenuated and highly passed vaccine strain of the pseudorabies virus, Suid herpesvirus 1, identified 11 mutations within ICP4 that were unique to a vaccine strain compared to two sequenced virulent strains (23). Furthermore, in the Oka vaccine strain of the varicella-zoster virus (VZV), also known as human herpesvirus 3 (HHV-3), a disproportionate number of mutations (15 mutations, of which 8 were nonsynonymous) were found within ICP4 in the vaccine strain relative to the virulent parental virus (24).

Despite evidence from a variety of attenuated alphaherpesviruses suggesting mutations within ICP4 may be a causative factor driving attenuation of the virus, the two recombinant viruses tested containing high frequency candidate SNVs within ICP4 did not cause any
reduction in virulence of MDV, although the Mut ICP4-2 recombinant virus did fail to transmit horizontally to contact birds. Recombinant MDV viruses that are virulent and induced MD in challenged birds but failed to transmit horizontally to cause MD in contact birds have been previously reported for mutations in UL44 (gC) and UL13 (protein kinase) (7, 25), as well as with a recombinant virus with a point mutation within LORF2 (6). One possible explanation for the failure of Mut ICP4-2 to infect contact birds is due to lower levels of \textit{in vivo} replication at days 7 and 14, during the times traditionally classified as early cytolytic and latent phases of infection (26). The lowest virus load among the three time points was seen during the transition between the early cytolytic phase to latency (7 dpi) for Mut ICP4-2. This could affect transmission of the recombinant virus because as chickens mature they develop an age-related resistance towards MD. Therefore, young chicks are most susceptible to MDV infection, and due to a decrease or delay in shedding of virulent MDV by Mut ICP4-2, the potential for transmission and infection to older contact birds would decrease (7). Alternatively, these mutations within ICP4 may impair replication within the feather follicles, leading to an inability to spread through shed feather dander. Despite the indication that mutations within ICP4 appear to be correlated with attenuation in a multitude of alphaherpesviruses, recombinant viruses containing point mutations within ICP4 show that mutations within ICP4 alone are not sufficient for attenuation in MDV. This conclusion is supported by experiments using the Oka vaccine strain of VZV in which transactivation of downstream promoters regulated by ICP4 was compared between mutated versions of ICP4 from the vaccine strain versus the parental virus. Cohrs \textit{et al.} (27) established that regulation and transcription of downstream promoters between the mutated and wild-type ICP4 was comparable, leading to their conclusion that mutations within ICP4 alone are not sufficient to cause attenuation.
Mutations within the VZV Oka vaccine strain did not affect transactivation of viral promoters, but it is known that ICP4 contains different regions with specific functions in addition to transactivation, such as DNA binding, nuclear localization, and regulation of late genes (17). Therefore, we sought to computationally determine what function observed mutations within MDV ICP4 may impact, particularly those within amino acids 60-63, which contained mutations in three completely attenuated MDV replicates via sequence comparisons to other alphaherpesviruses. Alignment of the complete amino acid sequence of ICP4 revealed significant variation among alphaherpesviruses, which has previously been described. It is known that certain regions of ICP4, such as regions 2 and 4, are highly conserved among herpesviruses, while other regions are not particularly well conserved among species, such as regions 1, 3 and 5 (17, 20). Specifically, comparisons among genera show that Mardiviruses have significantly longer ICP4 of approximately 2,173 amino acids in length compared to the average length of 1,500 amino acids for non-Mardiviruses. Furthermore, in our attenuated MDV replicates containing several high frequency mutations around amino acids 60-63, these mutations occur within a region found uniquely in MDV yet absent in other closely related Mardiviruses. Considering that MDV is not only virulent, but is also an oncogenic virus, unlike all other alphaherpesviruses which do not cause tumors, it is difficult to predict what role these unique regions in MDV ICP4 provide during the life cycle of this oncogenic herpesvirus, particularly in light of the fact that closely related apathogenic Mardiviruses lack this commonly mutated region of ICP4.

The second mutation at amino acid 1630 occurs at a position present within the 20 herpesvirus species surveyed unlike the first mutation at amino acid 63. The mutation of a serine residue to a proline at position 1630 appears to be an evolutionarily accepted change, with
proline commonly found in several other species of herpesviruses. Despite this apparently tolerated amino acid change, it is only with addition of this second mutation at position 1630 in conjunction with the mutation at amino acid 63 that changes in horizontal transmission were observed. The role for this second mutation at position 1630 is difficult to predict due to the high degree of variation among herpesvirus species for amino acids within this designated region 3 of ICP4, which has been shown to be less highly conserved than regions 2 and 4 (17, 20), leading to a poorer understanding of functional roles for diverse regions such as region 3. Analysis of MDV’s ICP4 primary structure via software designed for modeling and prediction of protein structure, such as for MDV ICP4 sequences predict that the regions of MDV ICP4 containing the two mutations engineered are found within disordered, coiled regions of the protein but due to lack of MDV ICP4 structural predictions, little data is available for hypothesizing roles for the observed mutated regions.

Due to limitations of comparisons of ICP4 for specific amino acid positions among the herpesviruses surveyed, further comparison of the entire ICP4 sequence via phylogenetic analysis of the 20 alphaherpesvirus species based on the complete ICP4 amino acid sequence showed a discrepancy between the relationship of Mardiviruses and Iltoviruses of chicken compared to traditional trees based on compilation of data involving multiple genes. Previous studies have classified the three Mardiviruses (Melaeagrid herpesvirus 1, Gallid herpesvirus 2 and Gallid herpesvirus 3) collectedly in one group known as α3 viruses, while the Iltovirus, Gallid herpesvirus 1 (ILTV), was classified in a separate group designated α4, as would be expected (19). Instead, based on ICP4 sequences, these relationships are no longer in line with genera classification, but show closer homology between the virulent viruses of Gallid herpesvirus 2 and ILTV now grouped as sister taxa, despite belonging to different genera. Thus,
it appears there may be a greater commonality between evolutionary pressure affecting the sequence of ICP4 within virulent viruses compared to avirulent viruses for Mardi and Iltoviruses.

Collectively, these data suggests that mutations within ICP4 may play a supporting role in attenuation, cooperatively with the addition of other mutations that reduce virulence. Due to a selective advantage for viruses able to replicate faster during serial passage, mutations that increase in vitro replication would be expected in attenuated viruses despite the fact that those mutations themselves are not causative for the loss of virulence resulting from serial passage. It is possible that mutations within ICP4 seen in numerous attenuated viruses may not reduce virulence but would appear correlated with attenuation due to positive selective forces during in vitro passage. This would result in mutations that impact in vitro replication to occur concurrently with mutations that reduce virulence in vivo and are the causative mutation for attenuation and the loss of virulence. One such potential scenario involves a point mutation within the UL5 helicase-primase gene identified at 65% in an attenuated MDV viral population that likely could have occurred concurrently within the same viral genome containing the ICP4 mutation G62V present at 83% in the same population. This mutation in UL5 reduced virulence by nearly 90% (6), therefore, it may be postulated that mutations within ICP4, which increase in vitro replication and lead to a selective advantage in vitro (24), could result in attenuated viruses generated after repeated serial passage due to combination with additional mutations that alter virulence, such as the UL5 mutation.

Despite the large body of examples correlating mutations within ICP4 with attenuation in alphaherpesviruses, it remains unknown what function these mutations may impart to attenuated viruses. Without further characterization it would be difficult to further postulate what role these additional 700 amino acids confer in MDV relative to other alphaherpesviruses, or understand
the impact of the numerous mutations within ICP4 seen not only within attenuated MDV, but among other alphaherpesviruses as well.

Conclusions

Despite showing multiple nonsynonymous mutations at high frequency in attenuated MDVs, neither Q63H nor S1630P mutations were sufficient to reduce viral virulence and are not causative factors for the loss of virulence during in vitro serial passage. Therefore, recombinant MDVs with defined ICP4 mutations are not likely to be viable candidates as MD vaccines.

ACKNOWLEDGEMENTS

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REFERENCES


CHAPTER 4. A SINGLE NUCLEOTIDE MUTATION WITHIN THE UL5 HELICASE-PRIMASE SUBUNIT GENE NOT ONLY REDUCES VIRULENCE, BUT ALSO PROVIDES VACCINAL PROTECTION AGAINST MAREK'S DISEASE

ABSTRACT

Marek’s disease virus (MDV) is an oncogenic herpesvirus that afflicts chickens with the disease known as Marek’s disease (MD). This virus causes tumors, nerve lesions, immunosuppression, and death of affected birds. Vaccines are the primary method for control of MD but due to the periodic evolution of field strains, it is necessary to explore the development of new MD vaccines. MD vaccines are often attenuated MDV strains generated through serial passage in vitro. We previously used experimental evolution of MDV to provide a better understanding of the genetic basis of attenuation. During complete genome sequencing of evolved MDV populations, we identified a point mutation within the UL5 helicase-primase gene that reduced disease incidence by 89-100%. To determine if experimental evolution also identifies mutations that provide protective qualities as potential vaccine candidates, we tested the UL5 recombinant virus as a vaccine and compared its protection to commercial HVT and bivalent (HVT + SB-1) vaccines. Both commercial vaccines resulted in higher protection against MD than the UL5 recombinant virus, although the UL5 virus did provide protection against developing MD in 46-70% of birds challenged. This indicates that a mutation within the UL5 helicase-primase gene not only reduces virulence, but also confers protection against challenge with virulent MDV, providing support that not only can experimental evolution identify candidate mutations involved in attenuation, but also identify potential candidates for use in vaccine development.
INTRODUCTION

Marek’s disease virus (MDV; aka Gallid herpesvirus 2) is an oncogenic alphaherpesvirus that cause Marek’s disease (MD) in chickens. Annually, MD causes nearly $1-2 billion losses worldwide, therefore, control and prevention of this disease is important for the poultry industry (1). Vaccines are the primary method for controlling MD, which were first employed in 1969 with the use of the live attenuated strain HPRS-16 (2). A related turkey herpesvirus, known as HVT which is nononcogenic in chickens, was introduced shortly afterwards and has been widely used to protect against MD since 1971 (3). Despite the introduction of early MD vaccines, field strains of MDV evolved to greater virulence that were no longer controlled by vaccines such as HVT (4). This led to the introduction of bivalent vaccines based on HVT with the addition of a second vaccine, such as the nononcogenic SB-1 strain, in which the two strains interacted synergistically to protect better than single vaccine preparations alone (5). While this combination provided protection for several years, more virulent strains once again emerged requiring vaccines with greater protection, leading to the U.S. introduction in the 1990s of the Rispens, or CVI998 strain, currently considered the gold standard of MD vaccines (6). Despite, or likely as a result of, the availability and use of these protective vaccines, virulent strains are now appearing able to cause outbreaks even within flocks vaccinated with Rispens (4).

This evolution of more virulent strains requiring the successional introduction of more efficacious vaccines illustrates the importance of continuous vaccine development. Several vaccines, including both HPRS-16 and Rispens, are live attenuated viruses resulting from serial passage in vitro (2, 7). Considering the history of in vitro serial passage for generation of attenuated vaccines, this process could further yield new vaccine candidates. Despite the utility of this method for vaccine development, the underlying genetic mechanism behind the process of
attenuation remains unclear. With the advent of next-generation sequencing, many MDV strains have now been sequenced, including vaccines such as SB-1 and Rispens, as well as attenuated viruses such as a highly passed strain of 648A (8–10). Sequencing complete MDV genomes provides valuable insight into genetic differences between virulent and nononcogenic viruses, yet still requires validation of candidate polymorphisms identified to determine their impact on virulence.

Previously, we serially passed three replicate strains of a virulent, BAC-cloned MDV virus and sequenced the resulting attenuated viruses. We identified candidate mutations believed to play a role in attenuation and created recombinant viruses testing 5 unique mutations via Red-mediated recombineering. One nonsynonymous point mutation within UL5, which encodes the helicase-primase subunit, significantly reduced both in vivo replication and virulence when used to challenge birds. With the availability of BAC-derived MDVs and ability to create recombinant viruses by Red-mediated recombineering, there is now the opportunity to design and engineer candidate vaccines incorporating mutations into the MDV genome to quickly and precisely create avirulent viruses to yield a more protective attenuated virus. Therefore, we wanted to determine if this single nucleotide mutation within UL5 conferred any protection against challenge with virulent MDV and could be considered for future development of vaccine candidates, or if the UL5 mutation decreased virulence with no bearing on protection against MD.
MATERIALS AND METHODS

Viruses and Cell Culture. Viral stocks were propagated in secondary duck embryo fibroblast cells in a 1:1 mixture of Leibovitz’s L-15 and McCoy’s 5A (LM) media supplemented with 1-4% fetal bovine serum (FBS), penicillin (10,000 U per 100 ml), 200 μg/ml streptomycin, and 0.2 μg/ml amphotericin B in a 37 °C, 5% CO₂ incubator. The recombinant virus, Mut UL5-I682R, was generated from the Md5B40BAC using Red-mediated recombineering, as previously described (11). Md5B40BAC-c1 (12) and JM/102W challenge viruses, as well as the bivalent vaccine, were derived from ADOL stocks, while the HVT vaccine originated from a commercial source (13).

In Vivo Challenge. A minimum of seventeen maternal antibody negative, 15I₅ x 7₁ chicks were vaccinated intraabdominally upon day-of-hatch with 2,000 plaque forming units (PFU) of HVT, bivalent HVT+SB-1 (1,000 PFU of each virus to yield a collective dose of 2,000 PFU in total), or the recombinant Mut UL5-I682R. Vaccinated birds were then infected five days later with 500 PFU of the virulent challenge virus, either JM/102W or Md5B40BAC-c1, and housed in Horsfield-Bauer isolators for 10 weeks. Isolators of unvaccinated birds were also challenged with virulent viruses as unvaccinated controls. To determine how increasing the quantity of PFUs administered to match commercial doses affected disease incidence due solely to Mut UL5-I682R, an additional set of birds challenged with 2,000 PFU Mut UL5-I682R only was included. Moribund birds, and all birds that survived past 10 weeks, were terminated and examined via necropsy for signs of MD to determine disease incidence. All in vivo bird trials were approved by the USDA, Avian Disease and Oncology Laboratory Animal Care and Use Committee (ACUC). Based on ACUC guidelines established and approved by the ADOL ACUC
(April 2005) and the Guide for the Care and Use of Laboratory Animals by the Institute for Laboratory Animal Research (2011), procedures for bird housing and care were followed throughout the duration of the experiment.

**Vaccinal Protection of Viruses.** To compare the protection of Mut UL5-I682R versus commercial HVT or bivalent vaccines against challenge with virulent MDV, the protective index (PI) of vaccines was calculated based on MD incidence. The percent MD incidence in birds challenged only with virulent MDV strains was compared against levels of MD incidence resulting from vaccinated birds infected with the same virulent virus, and the protective index calculated as follows;

\[
\text{PI} = \frac{\% \text{ MD incidence in unvaccinated} - \% \text{ MD incidence in vaccinated}}{\% \text{ MD incidence in unvaccinated}}.
\]

Fischer’s exact test was used to determine if there were statistically significant differences in levels of protection between Mut UL5-I682R and commercial vaccines when challenged with the same virulent MDV strain.
RESULTS

Unvaccinated birds challenged with virulent virus, either JM/102W or Md5B40BAC-c1, developed MD and resulted in 89-100% mortality of infected birds before the end of the experiment (Fig. 4-1 and 4-2). On the contrary, between 89-100% of birds vaccinated with either commercial vaccine, either HVT or bivalent, survived the full length of the experiment and upon termination had disease incidences <11% (Fig. 4-1 and 4-2, Table 4-1). Vaccination with the recombinant virus Mut UL5-I682R resulted in approximately 80% of birds surviving the full length of the experiment regardless of which challenge virus, either JM/102W or Md5B40BAC-c1, was used to infect birds (Fig. 4-1 and 4-2). Examination upon termination revealed birds vaccinated with Mut UL5-I682R resulted in 30% and 48% MD following challenge with virulent JM/102W or Md5B40BAC-c1 viruses, respectively (Table 4-1). Of the birds administered 2,000 PFU of recombinant UL5-I682R virus only, 90% survived the full 10 week experiment and upon termination 23% of birds were MD positive, indicating increasing the quantity of the Mut UL5-I682R virus delivered by four-fold also increased MD incidence relative to earlier trials, in which 500 PFU of Mut Ul5-I682R caused 0 to 11% MD.
FIGURE 4-1. Survival of birds challenged with JM/102W virus. A minimum of 17, 1515 x 71 maternal antibody negative chicks were vaccinated with either HVT or Mut UL5-I682R virus at day of hatch, while unvaccinated birds were used as controls, before challenge with 500 PFU of JM/102W virus at 5 days of age and mortality shown over the course of the experiment until termination.
FIGURE 4-2. Survival of birds challenged with Md5B40BAC-c1 virus. A minimum of 17, 1515 x 71 maternal antibody negative chicks were vaccinated with either bivalent (HVT+SB-1) vaccine or Mut UL5-I682R of virus at day of hatch, while unvaccinated birds were used as controls, before challenge with 500 PFU of Md5B40BAC-c1 virus at 5 days of age and mortality shown over the course of the experiment until termination.
### TABLE 4-1. MD Incidence and Vaccinal Protection

<table>
<thead>
<tr>
<th>Virus</th>
<th>MD+ Birds/Total Birds</th>
<th>% MD</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM/102W</td>
<td>18/18\textsuperscript{a}</td>
<td>100</td>
<td>N/A</td>
</tr>
<tr>
<td>UL5 + JM/102W</td>
<td>6/20\textsuperscript{b}</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>HVT + JM/102W</td>
<td>0/20\textsuperscript{c}</td>
<td>0.0</td>
<td>100</td>
</tr>
<tr>
<td>Md5B40BAC-c1</td>
<td>16/18\textsuperscript{A}\textsuperscript{*}</td>
<td>89</td>
<td>N/A</td>
</tr>
<tr>
<td>UL5 + Md5B40BAC-c1</td>
<td>10/21\textsuperscript{B}</td>
<td>48</td>
<td>46</td>
</tr>
<tr>
<td>Bivalent + Md5B40BAC-c1</td>
<td>2/18\textsuperscript{C}</td>
<td>11</td>
<td>87</td>
</tr>
<tr>
<td>UL5 (2,000 PFU)</td>
<td>4/17</td>
<td>23</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Fisher’s exact test was used to compare MD within unvaccinated, Mut UL5 vaccinated or commercial (HVT or Bivalent) vaccinated groups and statistically significant groups indicated by lowercase letters for groups challenged with JM/102W and uppercase letters for groups challenged with Md5B40BAC-c1 (P<0.05).

\*Two birds that died before the end of the experiment were nonspecific for signs of MD, either nerve lesions or tumors. Due to the early age of mortality before manifestation of classical MD signs these birds were classified as nonspecific deaths and not MD positive, yet their death were likely the result of MD induced transient paralysis.
**PI of Viruses.** To compare protective efficacy of the vaccines, the PI of the three vaccinating strains were calculated as described previously. Without vaccination, the JM/102W virus caused 100% MD incidence in the unvaccinated birds. Vaccination with HVT before challenge with JM/102W protected all birds against MD, resulting in a PI of 100 and MD incidence of 0%, while the recombinant Mut UL5-I682R resulted in 30% of birds developing MD when challenged by JM/102W (PI=70). Comparing protection of Mut UL5-I682R versus the bivalent vaccine when challenged with Md5B40BAC-c1, a more virulent vv strain of MDV, showed a similar trend. Without vaccination, the virulent challenge virus alone caused high MD incidence of 89%. After vaccination with the bivalent vaccine, challenge by Md5B40BAC-c1 resulted in only 11% MD (PI=87) compared to vaccination with Mut UL5-I682R that had higher levels of MD, 48% (PI=46). Birds administered 2,000 PFU of only the Mut UL5-I682R virus developed MD at higher levels than previous trials involving only 500 PFU of the virus (11). The higher dose of Mut UL5-I682R used in all the vaccine trials caused 23% MD without addition of a following challenge virus. Statistical analysis of disease incidence comparing unvaccinated versus vaccination with Mut UL5-I682R or either commercial vaccines (HVT or bivalent vaccines) using Fisher’s exact test showed significant differences among all three groups (P<0.05). Comparing unvaccinated birds challenged with either virulent virus (JM/102W or Md5B40BAC-c1) versus birds vaccinated with Mut UL5-I682R prior to challenge showed significant differences between vaccinated and unvaccinated birds. Furthermore, comparing MD in birds vaccinated with Mut UL5-I682R and birds vaccinated with a commercial vaccine there also was a statistically significant difference between protection conferred by Mut UL5-I682R compared to HVT or bivalent vaccines.
In all trials, the commercial vaccine strains (HVT or bivalent) provided higher protection, although not always complete protection, compared to Mut UL5-I682R against challenge by virulent virus. While the Mut Ul5-I682R recombinant virus did not protect as well as commercial vaccines, it did improve the survival of birds and protected birds against virulent MDV strains, although not to as high degrees as standard vaccine strains.

**DISCUSSION**

Due to the increasing evolution of more virulent strains of MDV able to overcome vaccinal protection of even the current, most efficacious vaccines, new candidate vaccines must be developed to address the disease threat of these new strains. Previously, we used experimental evolution of three MDV replicates to identify candidate mutations involved in attenuation during serial passage *in vitro*. Recombinant viruses testing top candidate mutations revealed the recombinant virus, Mut UL5-I682R, dramatically altered *in vitro* phenotype of the virus by causing a reduction in disease incidence by nearly 90% or more depending on trial. We tested the Mut UL5-I682R recombinant virus in vaccine trials to determine any protective qualities due to this SNP, as well as to provide a proof-of-concept that experimental evolution is a viable route for identification of candidate SNPs and genes for potential use as vaccine candidates.

Comparing protection of the recombinant Mut UL5-I682R versus commercial vaccines indicated that the commercial vaccines all provided higher degrees of protection against challenge by virulent viruses. Despite the superior protection conferred by commercial vaccine strains, Mut UL5-I682R still was able to provide a fair level of protection and improved survival of birds when challenged with either virulent (v) or very virulent (vv) MDV strains. Considering that the Mut UL5-I682R recombinant virus results in 0-11% MD in birds infected with low, 500 PFU doses, and up to 40% MD when 2,000 PFUs are administered in vaccine-level doses,
indicates that the recombinant mutant containing a single SNP clearly is not a vaccine candidate currently. Considering that this recombinant virus significantly reduces disease incidence and *in vivo* replication (11), while also providing some level of protection against virulent MDV strains, instead suggests this mutation could potentially be used collectively with other mutations to generate a completely apathogenic virus, which in turn could be considered for use as a candidate vaccine.

Additional candidate mutations previously identified within attenuated viruses could be considered for incorporation into the UL5 recombinant virus for potential vaccine research and development, although a recombinant virus involving mutations within both within UL5 and the oncogene Meq may be a superior alternative for initial consideration. The Meq gene has previously been identified as the oncogene of MDV and a virus in which the Meq gene has been deleted, known as rMd5ΔMeq, resulted in an virus which no longer causes MD (14, 15). Deletion of the Meq gene in rMd5ΔMeq has been shown to not only result in an avirulent virus that does not caused MD, but also serves as candidate vaccine that provides higher protection than even Rispens, the current most efficacious vaccine (16). Despite the improved protective qualities of rMd5ΔMeq, this virus still induces bursal-thymus atrophy (BTA) in maternal antibody negative birds and, as a result, has not been approved as a commercial vaccine (17). Attempts to eliminate bursal-thymus atrophy by serial passage of rMd5ΔMeq *in vivo* resulted in a virus that no longer caused BTA in maternal antibody negative birds, but also lost the superior protective efficacy of the parental rMd5ΔMeq relative to Rispens when used in commercial birds (18). The authors noted that with the loss of BTA after 40 passages *in vitro*, they also no longer were able to detect viral DNA, indicating that the attenuated rMd5ΔMeq p40 virus was unable to replicate *in vivo*. They concluded that BTA is likely the result of vigorous replication of the low
passage rMd5ΔMeq within the lymphoid organs, and that \textit{in vitro} passage eliminating \textit{in vivo} replication of the high passage p40 rMd5ΔMeq virus led to loss of BTA as well. Based on the hypothesis that high levels of viral replication within lymphoid organs cause BTA and loss of \textit{in vivo} viral replication eliminates BTA, suggests one possible solution would be creation of a Meq deletion virus able to replicate in \textit{vivo}, albeit at low levels as to not induce BTA. The candidate mutation we have identified within UL5 is one candidate SNP to propose introducing into a Meq deletion virus. By itself, the UL5 mutation causes greatly reduced levels of \textit{in vivo} replication and, as shown in this study, confers protection against challenge with virulent MDV.

Incorporating both mutations involving deletion of Meq and addition of the UL5 point mutation into a single recombinant virus would be predicted to generate a virus lacking oncogenicity and that replicates at low levels \textit{in vivo}, although the resulting impact on BTA and PI compared to Rispens and rMd5ΔMeq remain unknown.

This experiment shows that a previously identified mutation within UL5 not only greatly reduces virulence, but also imparts protection against challenge with virulent MDV, although the PI of the Mut UL5-I682R recombinant virus is lower than current vaccines. This single mutation alone is not appropriate for use as a vaccine, yet it is possible that the use of this SNP, in conjunction with other identified mutations such as deletion of Meq, may be considered for development of new vaccines addressing the evolution of emerging vv+ strains of MDV. This experimental also shows that experimental evolution of MDV is a process not only able to identify candidate mutations involved in attenuation and but identifies mutations that provide protection against challenge by virulent MDV and that can be considered for use in vaccine development.
ACKNOWLEDGEMENTS

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REFERENCES
REFERENCES


CHAPTER 5. ADDITION OF A UL5 HELICASE-PRIMASE SUBUNIT POINT MUTATION ELIMINATES BURSAL-THYMIC ATROPHY OF MAREK’S DISEASE VIRUS ΔMEQ RECOMBINANT VIRUS, BUT REDUCES VACCINIAL PROTECTION

ABSTRACT

Marek’s disease virus (MDV) is an oncogenic alphaherpesvirus and the causative agent of Marek’s disease (MD), a T-cell lymphoma of chickens. Despite widespread usage of vaccines since the 1970’s to control MD, more virulent strains of MDV have emerged that break vaccinal protection, necessitating the development of new and more protective MD vaccines. The rMd5ΔMeq (ΔMeq) virus, a recombinant Md5 strain MDV lacking the viral oncogene Meq, is one candidate MD vaccine with great potential, but unfortunately it also causes bursal-thymic atrophy (BTA) in maternal antibody negative chickens, prohibiting approval as a vaccine. Previously, we identified a point mutation within UL5 that reduced in vivo replication in attenuated viruses. We proposed that introduction of the UL5 point mutation into the ΔMeq virus would reduce in vivo replication and eliminate BTA yet retain high protective abilities. In birds, the ΔMeq+UL5 recombinant MDV had reduced replication compared to the original ΔMeq virus, while weights of lymphoid organs indicated that the ΔMeq+UL5 did not induce BTA, supporting the hypothesis that reduction of in vivo replication would also abolish BTA. Vaccine trials of the ΔMeq+UL5 virus compared to other ΔMeq based viruses and commercial vaccines show that, while the ΔMeq+UL5 does provide vaccinal protection, this vaccinal protection was also reduced compared to the original ΔMeq virus. Therefore, it appears that a very delicate balance is required between levels of replication able to induce high vaccinal protection, yet not as high as to induce BTA.
INTRODUCTION

Marek’s disease (MD) is an economically significant disease of chickens that costs the worldwide poultry industry $1-2 billion a year (1). The causative agent of MD is the oncogenic herpesvirus known as Gallid herpesvirus 2, commonly referred to as Marek’s disease virus (MDV). Characteristic signs of MD include depression, transient paralysis, nerve lesions, tumors, and death of affected birds. The primary method used to successfully control MD since the 1970’s is vaccination. The first vaccine against MD was the attenuated strain of MDV known as HPRS-16, which was shortly followed by the introduction of another vaccine utilizing the closely related turkey herpesvirus known as HVT (2, 3).

While vaccines have been used to successfully prevent MD and reduce losses in the poultry industry for over 40 years ago, more virulent strains have evolved requiring the introduction of a successive line of new vaccines (4). Disease breaks within HVT-vaccinated flocks resulted in the need to introduce bivalent vaccines combining two vaccines that interacted synergistically, such as HVT and another attenuated virus such as SB-1, to yield a vaccine that was more protective than either vaccine alone (5). Bivalent vaccines controlled losses due to MD until the evolution of more virulent field strains led to the introduction of the CVI988-Rispens (Rispens) vaccine in the U.S. (6, 7). Presently, the Rispens vaccine is considered the gold-standard vaccine against MD and provides the highest level of protection commercially available.

Despite the level of success in controlling MD, there is concern that more virulent strains of MDV are evolving able to overcome even Rispens vaccinal protection (8). Currently a
recombinant cosmid-based Md5 strain MDV in which the Meq oncogene has been deleted, known as rMd5ΔMeq, has shown great potential in several trials. Unfortunately, rMd5ΔMeq causes bursal-thymus atrophy (BTA) in maternal antibody negative birds and, as a result, has not been licensed for commercial use as a vaccine. Attempts to eliminate BTA of rMd5ΔMeq via repeated serial passage in vitro to attenuate the virus resulted in viruses that did not cause BTA in antibody negative birds (9), but failed to replicate and lost the superior protective qualities of the initial rMd5ΔMeq virus in commercial antibody positive birds (10). It is postulated that BTA seen in the otherwise avirulent rMd5ΔMeq virus is the result of vigorous in vivo replication of the virus within the bursa and thymus, leading to atrophy of those lymphoid organs (10, 11).

During previous efforts to identify the driving factors for MDV attenuation at a genetic level, we identified a point mutation within UL5, the helicase primase subunit, that resulted in significant reduction of in vivo replication of the virus, while significantly reducing MD incidence to 0-11% as well as providing protection against challenge with virulent MDV (12). Considering the hypothesis that loss of BTA seen in the high-passage rMd5ΔMeq resulted from considerable reduction in replication after passage, we predicted that addition of the UL5 point would reduce replication of ΔMeq to low levels seen in the UL5 point mutant, while simultaneously eliminating BTA characteristic of low passage ΔMeq, but that it would still confer vaccinal protection.

A second Meq deletion mutant generated using a BAC construct, known as Md5B40BACΔMeq, has previously been created and shown to be as protective as rMd5ΔMeq, and equaling or even surpassing protection of Rispens (13). Therefore, use of the BAC cloned MDV allowed us to use Red-mediated recombineering to create a double mutant of Md5B40BACΔMeq+UL5 (12). We compared in vivo replication and ratios of lymphoid organ
weight for low passage ΔMeq, high passage ΔMeq (p41), and the double mutant, 
Md5B40BACΔMeq+UL5, against uninfected birds. Furthermore, we tested the three preceding 
ΔMeq-based viruses as well as two commercially approved vaccines, CVI988 and bivalent 
(HVT+ SB-1), for vaccinal protection against challenge with 648A, a very virulent plus (vv+) 
strain of MDV.
MATERIALS AND METHODS

Viruses and Tissue Culture. Md5B40ΔMeq BAC generated and characterized by Silva et al. (13) was used for incorporation of the UL5 point mutation using Red-mediated recombineering protocols and mutational primers as previously described (12). The resulting Md5B40BACΔMeq+UL5 (ΔMeq+UL5) recombinant mutant contained deletion of both copies of Meq and the nonsynonymous point mutation of amino acid I682R within UL5. Viral stocks of Md5B40ΔMeq BAC (ΔMeq BAC) at p5 and ΔMeq+UL5 p4 were used for birds trials, while rMd5ΔMeq p39 (9, 10) was plated and passed through chicken embryo fibroblast (CEF) for one additional passage to amplify viral stocks before use of the resulting rMd5ΔMeq p40 (ΔMeq p40) for bird trials. The very virulent plus (vv+) MDV 648A (p7) (6) strain was used as the challenge virus. The two MD vaccines compared were (1) bivalent vaccine consisting of HVT (p10) and SB-1 (p14) strains and (2) Rispens (p43); of which all viruses used originated from ADOL stocks. Cells and viruses were cultured in a 1:1 mixture of Leibovitz’s L-15 and 100 McCoy’s 5A (LM) media containing 1-4% of fetal bovine serum (FBS) with antibiotics as previously described (12).

In Vivo Replication and Bursal Thymic Atrophy. ADOL 15I5 x 71 maternal antibody negative chicks were vaccinated with 2,000 PFU of ΔMeq BAC p5, ΔMeq p40, or ΔMeq+UL5 p4 at day of hatch and housed in Horsfall-Bauer (HB) unit isolators. In addition to vaccinated birds, an isolator of unvaccinated birds was maintained for negative controls. Birds were bled at days 6, 13, and 20 days post vaccination (dpv) and peripheral blood lymphocytes (PBL) isolated using Histopaque-1077 density gradient separation (Sigma-Aldrich). DNA extracted from PBLs was used for qPCR analysis to compare in vivo replication among vaccines. The Taqman Fast
Universal PCR kit (Applied Biosciences; Foster City, CA) and primers for amplification of chicken GAPDH and MDV gB were used to calculate the relative ratio of the number of MDV genomes versus chicken genome numbers as previously described (12, 15).

To monitor BTA, five birds from each of the four lots (ΔMeq BAC p5, ΔMeq p40, ΔMeq+UL5 p4, or unvaccinated) were sacrificed at 15 dpv, the time when atrophy is typically most severe (11), and their total body weight (g) and weights of bursa and thymus (mg) measured. The ratio of lymphoid organs over total body weight was calculated to compare against unvaccinated controls to assay atrophy of lymphoid organs in vaccinated birds. Ratios of lymphoid organs over total body weight were analyzed using ANOVA to determine differences between vaccinated lots and unvaccinated controls.

**Vaccinal Protection.** To assay vaccinal protection of Meq-deleted viruses, we compared the three Meq-deleted viruses previously described against two MD vaccines: Rispens/CVI988 and bivalent (HVT+SB-1). Maternal antibody negative, ADOL line 15I5 x 71 chicks were vaccinated with 2,000 PFU of ΔMeq BAC p5, ΔMeq p40, ΔMeq+UL5 p4, bivalent (HVT+SB-1) or Rispens at day of hatch and housed in HB units. After 5 dpv, vaccinated birds were challenged with 500 pfu vv+ 648A strain of MDV. As positive controls, one lot of unvaccinated birds were challenged with 500 PFU of 648A at 5 days of age. Unvaccinated and uninfected negative control birds were also housed and maintained with the experimental lots in HB units for 8 weeks. Birds that died before 8 weeks were examined via necropsy for cause of death, while after 8 weeks all surviving birds were terminated and examined via necropsy for signs of MD. To determine vaccinal protection, we calculated protective index (PI) of vaccines as follows; PI= ((% MD incidence in unvaccinated) – (% MD incidence in vaccinated))/ (% MD incidence in vaccinated).
unvaccinated)). To compare protection of vaccines, analysis using Fisher’s exact test was performed to identify statistically significant differences in MD among vaccinated groups.

RESULTS

In Vivo Replication. Unvaccinated birds and birds vaccinated with either ΔMeq+UL5 p4 or ΔMeq p40 all had very low background levels of MDV detected at all three time points (Fig. 5-1). ΔMeq and virulent 648A challenged birds had high levels of at 6 dpv and 13 days post challenge, respectively. No data is available at 20 days post challenge for 648A infected birds due to the severity of this vv+ virus causing mortality of nearly all birds within 20 days after challenge with 648A. There were statistically significant differences in replication at 6 dpv (p<0.01), in which the low passage ΔMeq BAC p5 had very high levels of replication at 6 dpv that dropped dramatically at 13 and 20 dpv to levels comparable to unvaccinated birds and the two other Meq-based viruses.

BTA of Meq-Based Viruses. Ratios comparing the weights of lymphoid organs, either individual bursa and thymus weights or total lymphoid organs, to the total body weight of birds at 15 dpv showed a significant difference in the weights of lymphoid organs in birds vaccinated with low passage ΔMeq BAC p5 (Fig. 5-2) compared to unvaccinated birds (p<0.001 in all trial comparisons). Individual bursa and thymus weights, or total lymphoid organ to body weight ratios of birds vaccinated with either ΔMeq p40 or ΔMeq+UL5 p4 were not significantly different from unvaccinated (ANOVA p>0.15 in all trial comparisons).

Protective Efficacy of Vaccines. Both trials showed severe mortality in unvaccinated birds challenged with 648A (Fig. 5-3). Due to infection with this vv+ strain of MDV, over 90% of
unvaccinated birds challenged with 648A were deceased within 2-3 weeks post challenge. In trial one, all birds vaccinated with ΔMeq p40, bivalent, or Rispens prior to challenge survived the full 8 week experiment, while ΔMeq BAC p5 had one death out of fifteen birds (7% mortality) and four out of 14 birds died when vaccinated with ΔMeq+UL5 p4 (29% mortality). In trial two similar trends were also observed in which 80-90% of birds vaccinated with either ΔMeq+UL5 p4, ΔMeq BAC p5, ΔMeq p40, or Rispens survived the full 8 week experiment compared to unvaccinated birds challenged with 648A, which were all deceased by 3 weeks post challenge (Figure 5-3b).

Examination via necropsy to determine MD incidence revealed very high protection following vaccination with ΔMeq BAC p5, ΔMeq p40, and Rispens. Of these highly protective vaccines, ΔMeq BAC p5 had the lowest protective index of 93, while ΔMeq p40 and Rispens had no MD positive birds, resulting in PIs of 100 for both viruses in Trial 1 (Table 5-1). The ΔMeq+UL5 p4 and bivalent viruses had higher degrees of MD incidence in birds vaccinated with these viruses, resulting in PIs of 57-43 or 87-73 for each respective virus, showing they did not confer as strong of protection as the previous three viruses, but they still provided significant protection against challenge with vv+ MDV. Results of trial two also supported these conclusions in which ΔMeq BAC p5, ΔMeq p40, and Rispens all provided high protection against MD (PIs of 94, 94 and 88, respectively), while lower levels of protection were provided by vaccination with ΔMeq+UL5 p4 (PI 44).
FIGURE 5-1. *In vivo* replication of MDV within PBLs in vaccinated, unvaccinated or 648A challenged birds at 6, 13 and 20 days post vaccination. Unvaccinated birds challenged with 648A experienced severe mortality around 2 weeks post infection, resulting in no data for the final timepoint indicated with an “x”. Panel A designated trial one and panel B indicates trial 2.
FIGURE 5-2. Weight of lymphoid organs in vaccinated and unvaccinated birds relative to total body weight in Trial 1 (Panel A) and Trial 2 (B).
FIGURE 5-3. Survival of vaccinated birds following challenge with vv+ 648A MDV. Panel A shows data from the first trial, with panel B depicting data from second replicate trial. Data for birds vaccinated with bivalent vaccines was unavailable for trial two due to flood causing complete loss of the isolator housing that lot.
<table>
<thead>
<tr>
<th>Trial</th>
<th># MD+ Birds/Total Birds</th>
<th>Percent MD</th>
<th>Protective Index (PI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unvaccinated</td>
<td>A 16/16</td>
<td>100%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>B 17/17</td>
<td>100%</td>
<td>0</td>
</tr>
<tr>
<td>ΔMeq+UL5 p4</td>
<td>A 6 or 8 /14</td>
<td>42-57%</td>
<td>57-43</td>
</tr>
<tr>
<td></td>
<td>B 10/18</td>
<td>56%</td>
<td>44</td>
</tr>
<tr>
<td>ΔMeq BAC p5</td>
<td>A 1/15</td>
<td>7%</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>B 1/18</td>
<td>6%</td>
<td>94</td>
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<tr>
<td>ΔMeq p40</td>
<td>A 0/14</td>
<td>0%</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>B 1/18</td>
<td>6%</td>
<td>94</td>
</tr>
<tr>
<td>Bivalent (HVT+SB-1)</td>
<td>2 or 4 /15</td>
<td>13-27%</td>
<td>87-73</td>
</tr>
<tr>
<td></td>
<td>B n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Rispens</td>
<td>A 0/15</td>
<td>0%</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>B 2/17</td>
<td>12%</td>
<td>88</td>
</tr>
</tbody>
</table>

n.d. Indicates no data available due to a flood of isolator for bivalent vaccination in Replicate 2
DISCUSSION

While low passage ΔMeq is a highly effective vaccine able to protect against vv+ MDV strains, it also induces BTA in maternal antibody negative chickens, prohibiting commercial licensing of the virus as a vaccine (11). In addition to BTA, another trait characteristic of low passage ΔMeq is the vigorous in vivo replication of ΔMeq during early cytolytic replication. This characteristic was previously described, and is apparent in this study as well (11, 16). Due to loss of vigorous in vivo replication following serial passage of ΔMeq, it was postulated this high replication within lymphoid organs was the cause of BTA. We hypothesized that addition of a point mutation within UL5 shown to cause greatly reduced in vivo replication would abrogate BTA in low passage ΔMeq, resulting in an avirulent virus that does not induce tumors and replicates at very low levels as to not cause BTA. Comparing replication via qPCR of PBLs and the weight of lymphoid organs of the three ΔMeq based viruses compared to unvaccinated birds supported the hypothesis that reducing in vivo replication in the ΔMeq+UL5 p4 virus also resulted in a low passage ΔMeq-based virus that failed to cause BTA. In addition to the anticipated phenotypic changes in regards to in vivo replication and lymphoid organ weight, addition of the UL5 point mutation also affected vaccinal protection of the ΔMeq+UL5 p4 compared to the original ΔMeq BAC p5 virus. This illustrates the fine interplay between even a single point mutation intended to reduce in vivo replication and broader implications on vaccinal protection, suggesting a delicate balance between vaccinal protection, in vivo replication and BTA.

Previous studies have explored the interaction between MD vaccines and replication. Studies comparing vaccinal protection of serially passed and fully attenuated MDV strains relative to earlier passages still containing residual levels of virulence, designated as partially
attenuated viruses, showed that partially attenuated viruses had higher viral loads in PBLs and conferred significantly higher levels of protection than the fully attenuated passages of the viruses (17). Further studies exploring the interaction between protection and replication of candidate vaccines compared a set of several vaccine candidates categorized as high protective (HP) or low protective (LP) vaccines in order to identify characteristics that appeared common among high or low protective vaccines (18). One trait the authors noted that distinguished high from low protective vaccines was in vivo replication. Looking at viral load of HP viruses revealed higher viral loads in all tissues quantified, with significantly higher levels in lymphoid organs such as the bursa, thymus and spleen.

These studies collectively suggest a correlation of greater vaccinal protection with high in vivo replication. Unfortunately it is not uncommon to find that highly protective, high replicating MD vaccine candidates also induce BTA. One such example is the virus known as RM1, which was generated by cocultivation of MDV and reticuloendotheliosis virus (REV), leading to retroviral insertions into the MDV genome. RM1 was determined to be attenuated and replicated efficiently in vivo, yet failed to cause MD and was typified by severe BTA (19). Further characterization revealed that not only was the attenuated RM1 virus very protective as a vaccine, but also exhibited high tissue tropism for early dissemination and replication to the thymus (20). Among several vaccine viruses examined, RM1 showed the earliest and highest replication within lymphoid organs, specifically the thymus, compared to vaccine viruses that did not cause BTA.

Additional studies looking at in vivo replication within the thymus comparing both RM1 and low passage ∆Meq against in vivo replication of viruses that do not induce BTA show both RM1 and low pass ∆Meq have significantly higher levels of replication within the thymus (11).
This supports the hypothesis that increased replication, specifically within lymphoid organs such as the bursa and thymus, leads to atrophy of these sensitive organs. A confounding factor for vaccine development is that despite the negative effects causing BTA due to high levels of \textit{in vivo} replication, increased replication appears to be a factor impacting higher degrees of vaccinal protection conferred by viruses. Therefore, \textit{in vivo} replication and the protection qualities of candidate vaccines appear to be highly interconnected, although not the only factor believed to play a role in protection, leading to complications when trying to eliminate BTA by altering excessive replication while still producing a protective vaccine.

Modulation of \textit{in vivo} replication has been a method proposed for generation of attenuated vaccines by increasing replication fidelity of the viral polymerase in a polio mouse model. High-fidelity polio vaccines exhibiting altered tissue tropism resulted in attenuated viruses with diminished transmission and shed of virus (21). These high-fidelity replication variant viruses were also able to generate excellent immune responses and induce antibody levels comparable to, or even exceeding, current Sabin polio vaccines. Considering the trend that moderate-to-high levels of replication tends to result in more protective MD vaccines than their low replicating counterparts, it may be advantageous to try to direct replication and limit tissue tropism for these vaccine candidates. Considering that viruses such as RM1 and low passage ΔMeq replicate at high levels in lymphoid organs, and those tissues suffer atrophy as a result of this vigorous replication, it may be beneficial to restrict the tissue tropism of prolific replication to avoid BTA. By diverting the highest replication, this may allow low passage ΔMeq viruses to maintain their high levels of replication in tissues other than the bursa and thymus and prevent BTA, while also provoking a strong immune response due to vigorous early cytolytic replication. Considering that MDV is a highly cell-associated virus whose lifecycle involves preferential
infection and replication within B and T cells, it may be difficult to directly apply this approach with MDV, but it is clear that alternative approaches besides simply reducing in vivo replication should be considered for development of protective candidate vaccines.

Ongoing development of more protective MD vaccines clearly illustrates the difficulty in generating vaccines that are not only more protective, but also do not cause undesirable secondary reactions, as seen with low pass ΔMeq or RM1 viruses which induce BTA. Attempts to generate new vaccine candidates have yielded many viruses which confer vaccinal protection equaling that currently provided by Rispens, yet it has been difficult to surpass the protection of Rispens (22). Two candidate vaccines, RM1 and low passage ΔMeq, both met or exceeded vaccinal protection commonly seen due to Rispens vaccination, but both viruses also induce BTA in maternal antibody negative birds, prohibiting their approval as commercial vaccines (18). Attempts to eliminate BTA in low passage ΔMeq in previous studies by extensive serial passage resulted in a virus with reduced in vivo replication and which did not cause BTA, but also reduced protection compared to the original low passage ΔMeq (10). This study aiming to eliminate BTA in low passage ΔMeq by reducing in vivo replication via addition of the UL5 point mutation showed similar results as previous trials, in which BTA was lost at the expense of vaccinal protection.

Clearly there is a fine balancing act between generating a highly protective vaccine that elicits a strong immune response with mild residual pathogenicity, compared to completely viruses that are completely avirulent yet may not induce as vigorous immune response as the virus’ partially virulent counterpart. As shown in this study and supported by previous work, it appears easier to eliminate undesirable BTA characteristics of a virus such as ΔMeq, but a far more complicated task to do so without also simultaneously reducing vaccinal protection.
Perhaps new routes must be considered to address this problem of BTA in highly protective viruses, such as trying to restrict the tissue tropism of replication, instead of simply reducing replication to eliminate BTA, which has been shown to be an unsatisfactory method to address BTA without also undermining high vaccinal protection.

ACKNOWLEDGEMENTS

We would like to thank Laurie Molitor, Spencer Jackson and Lonnie Milam for excellent technical support.
REFERENCES


CHAPTER 6. CHARACTERIZATION OF MAREK’S DISEASE VIRUS POINT MUTATION WITHIN UL5 HELICASE-PRIMASE SUBUNIT IDENTIFIED WITHIN ATTENUATED VIRUS SHOWN TO REDUCE VIRULENCE AND \textit{IN VIVO} REPLICATION

ABSTRACT

A nonsynonymous point mutation identified within UL5, which encodes the helicase-primase protein, subunit in a serially passed, attenuated MDV was shown to reduce \textit{in vivo} replication and MD incidence when incorporated into a recombinant virus, known as Mut UL5. This mutation was further characterized to determine if addition of a second top candidate mutation would result in complete loss of virulence. Growth curves quantifying \textit{in vitro} replication were conducted comparing the mutant UL5 virus to parental wild-type virus, and the UL5 mutant was serially passed \textit{in vivo} while selecting for increased \textit{in vivo} replication and assaying for reversion of the point mutation during replication within birds. Addition of a second point mutation did not significantly affect \textit{in vivo} replication or MD incidence compared to the single UL5 virus, and serial passage of the single UL5 virus through birds did not yield any revertant viruses or result in increased replication of disease incidence. Growth curves showed significantly higher numbers of Mut UL5 plaques compared to the wildtype virus, yet head-to-head competitions of the two viruses resulted in the parental virus outcompeting the Mut UL5, suggesting traditional growth curves may not provide complete pictures of the true biological ramifications of \textit{in vitro} replication for viral populations and quasi-species collectively replicating within a shared culture.
INTRODUCTION

Marek’s disease virus (MDV) is an oncogenic alphaherpesvirus that causes Marek’s disease (MD) in chickens, which is characterized by depression, paralysis, nerve lesions and t-cell lymphomas that ultimately culminates in the death of affected chickens (1). Control of MD has primarily been through the use of vaccines, of which many highly effective MD vaccines are attenuated viruses created through in vitro serial passage (2). Due to the continued evolution of more virulent field strains of MDV, the development of more protective MD vaccines has been a continued topic of research since the first introduction of MD vaccines in the 1970’s (3–7). In efforts to better understand the process of attenuation and provide information that may assist in future vaccine development, we previously serially passed three BAC cloned MDV replicates and conducted complete genome sequencing of the attenuated replicates via Illumina sequencing (8). We identified several candidate mutations and created recombinant viruses testing the phenotypic impact of those mutations and identified a point mutation within the UL5 helicase-primase subunit that reduced disease incidence by >90% while significantly reducing in vivo replication. Due to its dramatic impact, we wished to further characterize this point mutation. Considering that the UL5 recombinant virus still resulted in low levels of MD, we wanted to determine if adding a second, high-frequency mutation identified in the attenuated replicate would interact in a synergistic manner to result in complete loss of virulence and a fully attenuated recombinant virus. Furthermore, we sought to determine if the point mutation affected in vitro replication, which may provide a selective advantage during serial passage and explain its rise in frequency within the attenuated viral population. Finally, we also wanted to determine if, despite the low in vivo replication of the Mut UL5 virus, it was possible to select for improved in vivo replication, and, if so, whether increased in vivo replication would result in increased
virulence through reversion or compensatory mutations that may arise counteracting the original UL5 point mutation.

MATERIALS AND METHODS

Tissue Culture: Cell and Virus Propagation

Chicken embryo fibroblast (CEF) and duck embryo fibroblasts (DEF) were used to propagate viruses. All cells were cultured in Leibovitz’s L-15 and McCoy’s 5A (LM) media (1:1 ratio) and supplemented with fetal bovine serum (1-4% FBS) and penicillin, streptomycin, and amphotericin B (10,000 U per 100 ml, 0.2mg/ml streptomycin, and 0.02µg/ml respectively). Recombinant viruses were generated via Red-mediated recombineering using techniques previously described (8–10) and mutational primers indicated in Table 6-1.

UL5+UL46 Double Mutant In Vivo Replication and MD Incidence

We generated and tested a double recombinant virus of Md5B40BAC UL5-I682R+UL46-Q117R (Mut UL5+UL46) to see if addition of the UL46 point mutation had a synergistic or additive effect on virulence compared to the single UL5 mutation. The double Mut UL5+UL46 virus was created by addition of the UL46-Q117R mutation into the UL5 mutant BAC using primers listed in Table 6-1 and Red-mediated recombineering as described earlier. The single Mut UL5-I682R recombinant virus and double Mut UL5+UL46 recombinant viruses were used to challenge groups of 18 maternal antibody negative ADOL line 151×71 chicks, injected intraabdominally with 500 PFU of virus at day of hatch. Five uninfected contact birds were also housed with infected birds to determine horizontal transmission of viruses. Birds were sacrificed for tissue collection at days 7, 14 and 21 post infection and spleen extracted DNA used
for qPCR to determine viral load. Birds were housed for (10) weeks and examined via necropsy upon termination to identify MD positive birds and determine disease incidence.
<table>
<thead>
<tr>
<th>Primer Function</th>
<th>Primer Name</th>
<th>Primer Sequence 5’&gt;3’</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Recombineering Mutational Primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mut UL46-Q117R</td>
<td>MDV059 Q117R-f</td>
<td>ACACCTGCGGTGGTAAAAGAACTACACAGACTCGATATCGGCTATGTCTGTAAGCGGCAACAGGTAATCGATT</td>
</tr>
<tr>
<td></td>
<td>MDV059 Q117R-r</td>
<td>ATCAACACAAAGGTATACGCACGCTTACAGACATAGCAGGATACGAGTAATACGAGTAATTACACCAATAACCC</td>
</tr>
<tr>
<td>Mut UL5-I682R</td>
<td>MDV017 I682R-f</td>
<td>GATAGTTATGTCGATAATGTGAGTGGTCTGAGAGGAGTGAGAATATCTCACATAACAAACATGCCGAGTAGGTAACCAATCTGGAACACTCCTGCTGTTGTTTTATGATATTCTACCATCCTGCAGACTCGGCGTGGTCCTGACCAAAACCACAAACCACAAAAC</td>
</tr>
<tr>
<td></td>
<td>MDV017 I682R-r</td>
<td>AGCAAGGGGAAAGATTTCTATTCCTGACATTTGTTTATTGATATTCTACCATCCTGCAGACTCGGCGTGGTCCTGACCAAAACCACAAACCACAAAAC</td>
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<tr>
<td><strong>Pyrosequencing Primers</strong></td>
<td></td>
<td></td>
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<td>UL5 I682R f-biotinylated</td>
<td></td>
<td>atgtgatctgagagagatgaga</td>
</tr>
<tr>
<td>UL5 I682R r</td>
<td></td>
<td>ttccccctctgctgtgtg</td>
</tr>
<tr>
<td>UL5 I682R sequencing</td>
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<td>cgcatgtgtttatggaat</td>
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<tr>
<td><strong>TaqMan qPCR Primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gB .5</td>
<td></td>
<td>cggtagctttctagttcag</td>
</tr>
<tr>
<td>gB .3</td>
<td></td>
<td>ccagttggtcaacgctga</td>
</tr>
<tr>
<td>gB TM Probe 1</td>
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<td>Fam-catttttcgaagctagtgcagga-tamra</td>
</tr>
<tr>
<td>GAPDH 2.5</td>
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<td>acaggaggtgctgcccagaa</td>
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<tr>
<td>GAPDH 2.3</td>
<td></td>
<td>actttccccacactgctagtga</td>
</tr>
<tr>
<td>GAPDH TM Probe 2</td>
<td></td>
<td>Vic-tcatccacgttccact-tamra</td>
</tr>
</tbody>
</table>
Viral Growth Curves

Growth curves comparing replication of the wild-type Md5B40BAC-c1 versus Mut UL5-I682R were conducted as follows. For each respective virus, 21 p60 plates containing a confluent monolayer of CEF cells were infected with a calculated quantity of approximately 50 PFU of the appropriate virus. Triplicate plates at 1, 2, 3, 4, and 5 days post infection for each virus were trypsinized with .025% trypsin solution to be individually frozen in vials for later titration of virus at each timepoint. The total number of plaques were counted for three plates at 6 days post infection to calculate the precise quantity of PFU actually delivered during inoculation of CEF plates. After collecting timepoints, the frozen vials were titered. Each vial was titered in triplicate in a series of dilutions ranging from ¼ to 1/1000 of the original infected p60 plates. Plaques were counted at 6 days post infection to calculate the number of plaques per p60 plate frozen at each timepoint.

Viral Competition

2,000 PFU of each Md5B40BAC-c1 and Mut UL5-I682R were mixed (1:1) to infect three p100 plates containing a CEF monolayer and 10% of harvested cells were passed onto a new, uninfected CEF monolayer once mature plaques appeared, on average 4-5 days post infection. At each passage, the remaining harvested cells to quantify the ratio of wild-type to mutant allele using pyrosequencing to track changes in allele frequencies. DNA was extracted from aliquots of harvested cells during trypsinization for passage using QIAamp DNA Blood Extraction Kit (Qiagen Cat# 51106) for PCR amplification and prepared using the Pyromark Gold Q96 pyrosequencing kit (Qiagen Cat# 972804) with primers described in Table 6-1 and run on a PSQ 96MA pyrosequencing machine.
Serial Passage of Mut UL5-I682R *In Vivo*

17 maternal antibody negative ADOL line 1515x71 chicks were challenged at one week of age with 500 PFU of either the parental Md5B40BAC-c1 or recombinant Mut UL5-I682R and designated as *in vivo* p0. One week post infection, all birds were bled and PBLs isolated, DNA extracted and Taqman qPCR conducted to identify birds with the highest levels of MDV for each of the viral lots. Approximately 3-5 of the birds with the highest levels of MDV were then re-bled at two weeks post infection and PBLs from each individual bird isolated and plated onto a p100 containing a CEF monolayer to reisolate MDV. Infected CEF plates were passed to amplify viral quantities by 2-3 passages until large quantities of plaques were produced, then harvested and frozen in vials to be titered. Extracted DNA from the amplified viral stocks was assayed using pyrosequencing to verify whether the mutant UL5 SNP was still present, or if it had reverted to the wild-type allele in the highest replicating viruses.

Titered viral stocks from the top 1-2 birds with the highest load of MDV for each viral lot were used to infect another set of isolators with 500 PFU of the derived Md5B40BAC-c1 and Mut UL5-I682R viruses described as “*in vivo* passage 1”. This process of infecting, bleeding, screening, selecting and amplifying viruses from birds with the highest levels of MDV was repeated for a total of three passages through birds to select for viruses with the highest levels of *in vivo* replication. During the final challenges of the *in vivo* p3 viruses, two additional groups of seventeen maternal antibody negative 1515x71 chicks were challenged with the original viruses prior to passage *in vivo* to directly compare replication and disease incidence prior and post passage. These birds were housed and examined via necropsy for MD upon termination 8 weeks post infection to compare disease incidence of the *in vivo* p0 and *in vivo* p3 viruses.
RESULTS

UL5+UL46 Double Mutant *In Vivo* Replication and MD Incidence

Recombinant viruses containing a single UL5 point mutation or a double mutant recombinant virus containing both the UL5 and UL46 point mutation were both shown to replicate at very low levels *in vivo* (Figure 1). Viral load of MDV from spleen tissue of infected birds revealed no significant differences in replication between the single or double recombinant mutants (ANOVA p=0.4559)

Birds challenged with recombinant UL5 viruses all experienced low levels of MD with disease incidences of 17% or less (Table 6-2). Although the double mutant had slightly higher levels of MD compared to the single mutant, the difference in the number of MD positive birds between the single and double mutant (1 and 3, respectively) was not statistically significant (Fischer’s exact test p=0.6026).

**Mut UL5-I682R In Vitro Replication; Viral Growth Curves and Competitions**

Growth curves comparing the parental Md5B40BAC-c1 and Mut UL5-I682R show the two viruses were initially plated with an average of 54 or 52 PFU, respectively, yet the Mut UL5-I682R virus produced higher number of plaques for all days 1-5 compared to the parental Md5B40BAC-c1 (Fig 6-2). There were statistically significant differences in the number of plaques produced between the two viruses at 1, 2, 3, and 5 days (Unpaired t-test p= 0.0042, 0.0004, 0.0017, and <0.0001, respectively). Differences in plaque numbers at day 4 were not statistically significant at p= 0.0537.

Head-to-head competition of Md5B40BAC-c1 vs Mut UL5-I682R showed that despite starting mixed cultures with approximately 50% of each virus, there was a steady progression
towards greater quantities of the wild-type Md5B40BAC-c1 allele with each passage in all three replicates of the mixed UL5:B40 cultures (Figure. 6-3). After 20 passages the frequency of the wild type allele composed approximately 83-90% of the viral DNA, indicating a greater yield of the wild-type viral DNA in mixed cultures than Mut Ul5-I682R.

**Serial Passage of Mut UL5-I682R In Vivo**

Screening viral stocks of the highest replicating viruses generated from in vivo serial passage via pyrosequencing to determine presence of either the mutant or wild-type allele within UL5 indicated that there was no reversion of the Mutant UL5 allele to the parental form in any of the viral stocks isolated from birds with the highest viral load (n=7).

Comparing disease incidence resulting from infection with pre and post in vivo passage viruses of both Md5B40BAC-c1 and Mut UL5-I682R did not result in an increase in virulence for either virus as a result of passage and selection for high viral load (Table 6-3). The virulent parental Md5B40BAC-c1 typically has 90-100% virulence and birds trials comparing the Md5B40BAC prior and post in vivo passage resulted in all developing MD, therefore this virus already induces the highest levels of MD, making increases in virulence of Md5B40BAC impossible to detect. There also were no significant increases in virulence of Mut UL5-I682R following passage in vivo. Several bird trials of Mut UL5-I682R show that MD incidence routinely falls within 0-11% MD. In vivo passage p3 of Mut UL5-I682R resulted in <10% MD incidence, a level of MD which falls within the range of MD typically seen from the pre-passage Mut UL5-I682R. Comparing viral load via qPCR of PBL and spleen samples from the two viruses pre and post passage shows that in both tissues, the Mut UL5 viruses replicated at very low levels which were statistically indistinguishable (T-test p>0.2989). For the higher replicating MD5B40BAC viruses, there were also no significant differences in viral load in PBLs before and
after passage (T-test p=0.400). Within spleen tissues there were higher average levels of MDV after *in vivo* passage, although this degree of increase was nonsignificant (T-test p=0.1024) (Fig 6-4).
FIGURE 6-1. *In Vivo* Replication of UL5 Single and Double Recombinant Viruses. Splenic DNA was extracted for qPCR analysis to quantify viral loads of the single UL5 mutant virus or double Mut UL5+UL46 recombinant viruses relative to uninfected birds (Neg) or wildtype Md5B40 challenged birds. Results from two separate trials of Mut UL5 are indicated by “a” and “b”.
TABLE 6-2. MD Incidence of Single and Double UL5 Recombinant Viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Number of MD+ Birds/Total Birds</th>
<th>Percent MD Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mut UL5</td>
<td>1/18</td>
<td>6%</td>
</tr>
<tr>
<td>Mut UL5+UL46</td>
<td>3/18</td>
<td>17%</td>
</tr>
<tr>
<td>Md5B40BAC-c1</td>
<td>10/10*</td>
<td>100%</td>
</tr>
</tbody>
</table>

* Number of birds present within Md5B40BAC-c1 challenged birds less than other lots due to early chick mortalities
FIGURE 6-2. Growth curves of Wild-type MdB40BAC-c1 and Mut UL5. 50 PFU of parental Md5B40BAC or Mut UL5 viruses were used to infect p60 plates containing a CEF. Triplicate plates of each virus were then trypsinized to harvested and freeze plates at 0, 1, 2, 3, 4 and 5 days post infection. Dilutions of these harvested plates were used to titer and calculate the number of plaques for all timepoints for the two viruses to compare plaque yield of Mut UL5 versus Md5B40BAC viruses.
FIGURE 6-3. Head-to-Head competition of wild-type Md5B40BAC-c1 vs Mut UL5. 2,000 PFU of either Mut UL5 (UL5) or Md5B40BAC (B40) viruses were used to infect p100 plates containing a CEF monolayer. Three plates were then infected with 2,000 PFU of both viruses and designated plates A, B, or C (B40:UL5 A, B, C). These plates were serially passed and at each passage stocks saved for DNA extraction for pyrosequencing analysis to quantify the proportion of DNA containing the wild-type allele or the mutant allele following serial passage.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Number of MD+ Birds/Total Birds</th>
<th>Percent MD Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-Passage (p0)</td>
<td>Post-Passage (p3)</td>
</tr>
<tr>
<td>Mut UL5</td>
<td>0/17</td>
<td>1*/17</td>
</tr>
<tr>
<td>Md5B40BAC-c1</td>
<td>17/17</td>
<td>17/17</td>
</tr>
</tbody>
</table>

* Indicates bird suggestive of MD+ but not entirely conclusive for MD based upon necropsy
FIGURE 6-4. *In Vivo* Replication of Md5B40BAC-c1 and Mut UL5 within PBL and spleen (s) tissue Pre and Post serial passage *in vivo*. Birds challenged with the Mut UL5 (UL5) or Md4B40BAC (B40) viruses were bled for collection of PBLs or splenic DNA and extracted for qPCR to quantify viral load of the respective viruses prior to *in vivo* passage (p0) or after three passages through birds while selecting for birds with the highest viral loads (p3) to determine MDV levels in viruses following selection for greater *in vivo* replication.
DISCUSSION

Initially, the UL5 point mutation resulted in a >90% reduction in MD incidence, yet addition of a second, high-frequency UL46 candidate SNP identified within the same attenuated replicate did not have an additive or synergistic effect on either MD incidence or in vivo replication. While the single UL46 point recombinant virus was comparable to the virulent parental virus for the common traits assayed in MDV infection, such as MD incidence, replication, survival duration or horizontal transmission (8), the double UL5+UL46 recombinant also showed that addition of the UL46 mutation did not confer any observable differences in those key in vivo phenotypes compared to the single UL5 recombinant. The nonsynonymous UL46 point mutation occurred at a high frequency (99%) within the attenuated replicate and was expected to confer some phenotypic effect due to the hypothesis that this mutation may provide a selective advantage in order to explain the near fixation of this mutation within the attenuated population.

In vitro characterization of replication has been performed by comparing the UL5 recombinant virus growth in vitro to the parental Md5B40BAC-c1. Growth curves showed the Mut Ul5-I682R virus produced significantly plaques than the wild-type virus. However, in head-to-head competitions the wild-type virus outcompeted the UL5 virus, eventually resulting in the wild-type allele composing 80-90% of the population following serial passage of the viral populations. One factor that potentially explains this discrepancy is that counting plaque numbers in growth curves does not take into account the total number of infectious cells produced within each plaque or plaque size. Comparing plaques, the number of infected cells, as well as the quantity of virus produced within each infected cell can vary, allowing for viruses to produce drastically different quantities of infected cells from one plaque to another. Therefore the UL5
mutant virus may have a greater number of overall plaques, yet the number of infectious cells, and in turn virus, produced by each plaque may be lower than the yield for each plaque of the Md5B40BAC-c1 virus, allowing for the outcompetition of the wild-type virus when approximately equal quantities of plaques are initially mixed.

Further work would be necessary to better understand the mechanism between replication of these two viruses to have a better understanding of if the observed differences in growth curve vs competition assays was the result of a biological difference in the viruses’ response to replication within a denser, genetically diverse population due to a difference in copies of MDV genomes produced or cells infected within a plaque, overall plaque size, soluble products released within conditioned media or other factors.

Nevertheless, these results show that under traditional in vitro growth curve assays, the Mut UL5 recombinant virus showed higher replication by producing more plaques than the wild-type virus. Yet under more biologically relevant conditions reflecting the quasi-species nature of MD viruses, in which mixed populations of the Mut UL5 and wild-type Md5B40BAC viruses were serially passed and allowed to replicate in head-to-head competition, the result of the head-to-head competition was unexpected based upon the result of the growth curve. This suggests that while in vitro growth curves may provide important information regarding replication dynamics of viruses, this assay may fail to encompass all aspects which may be pertinent for replication and competition within the biologically relevant context of populations.

Besides characterizing in vitro replication of the Mut UL5 virus, we also attempted to increase replication via selection and repeated serial passage in vivo, particularly with an emphasis on determining the likelihood for the mutation virus to revert or undergo a secondary compensatory mutation to restore wild-type levels of replication and virulence. Despite serially
passing and selecting viruses with the highest viral loads in birds, there was no significant increase of *in vivo* replication for either virus. Prior work involving *in vivo* serial passage of Md5B40BAC, the same progenitor virus used in this study, showed that the virus initially caused MD in 18% of the MHC-B13 Line 0 of maternal antibody positive chickens prior to passage, where after 5 passages virulence of passaged viruses increased to 94% (19). This previous work shows selection for increased *in vivo* replication and MD incidence can be observed significantly within a very small number of passages. However, our use of the Md5B40BAC virus within the highly susceptible line of maternal antibody negative 15$\times$71 birds leads to maximum MD incidence, making it difficult to select for significant increases and adaptation in an equally short time frame. Conversely, while the Md5B40BAC virus caused 100% MD incidence in birds both before and after serial passage, the Mut UL5 virus was at the opposite spectrum, resulting in <6% MD incidence both before and after passage. Despite selecting for the birds with the highest levels of MDV, even the highest loads of Mut UL5 virus were significantly lower than those of the virulent Md5B40BAC virus. Due to a lack of increases in either *in vivo* replication or MD incidence after selection and serial passage, it seemed likely that compensatory mutations occurred to counteract the Mut UL5 point mutation. Pyrosequencing of serially passaged viral stocks confirmed that the UL5 point mutation did not revert to the wild-type Md5B40BAC allele.

In summary, the UL5 point mutant was shown to result in significantly higher levels of *in vitro* replication via traditional growth curves, yet when grown in head-to-head competition with the parental Md5B40BAC virus, it did not outcompete the parental virus. This suggests traditional growth curves may fail to account for key biological aspects of replication within populations. Considering the impact of this single point mutation on both *in vitro* and *in vivo*
replication, experiments to determine stability of this mutation under selection during *in vivo* passage determined that mutations to counteract the detrimental reduction of *in vivo* replication do not readily occur even when purposefully selecting for increased replication, suggesting that despite concern over reversions of point mutations in vaccine candidates, point mutations may be more stable than initially anticipated.

**ACKNOWLEDGEMENTS**

We would like to thank Laurie Molitor, Lonnie Milam and Spencer Jackson for exemplary technical support, as well the USDA National Institute of Food and Agriculture for partial financial support via the National Research Initiative Competitive Grant number 2010-65119-20505.
REFERENCES
REFERENCES


FURTHER WORK

HORIZONTAL TRANSMISSION OF RECOMBINANT VIRUSES

Based on the result of birds trials involving two recombinant viruses (LORF2-Intron and ICP4-2, Chapters 2 and 3 respectively) that remained virulent when used to challenge birds, yet failed to transmit horizontally to uninfected contact birds, further trials of these viruses in greater depth to better understand the mechanism behind this loss of horizontal transmission would be warranted. Further studies focusing on horizontal transmission would involve greater numbers of contact birds, and trials evaluating the impact that the age of shedding birds post challenge has on transmission. Preliminary trials, of which the results were described in Chapters 2 and 3, involved a small number of approximately 5 contact birds housed in isolators with an average of 17 infected contacts birds. Challenged birds were infected with the recombinant viruses upon day of hatch and immediately housed with uninfected contact birds of the same age from the same hatch.

Future trials would contain a greater number of contact birds. It would be preferable to increase the number of contact birds to a minimum of 10 birds per isolator to be housed with at least 10 infected contact birds. Two different approaches could be followed in order to compare the impact the age of shedder birds has on MD incidence in contact birds. As previously conducted in the prior trials, challenged shedder birds would be infected and housed with contact birds from the same hatch immediately upon day of hatch. Alternatively, a second route would involve challenge and housing of shedder birds from separate hatches approximately 1-2 weeks prior to hatch and housing of the uninfected contact birds. This would allow shedder birds to establish viral replication and to shed MDV into isolators prior to addition of contact
birds, resulting in immediate exposure to infected dander, compared to earlier scenarios in which there was a delay in possible exposure in contact birds due to time required for infection to become established.

For further trials, sampling of dander would be required to quantify MDV shed from feathers. This could be accomplished through collection of whole isolator dander collected through an air filter, as well as through collection of dander obtained through vacuuming of individual birds. Invasive sampling of challenged birds sacrificed for collection of feather follicles would also allow immunohistochemistry straining to identify MDV replication within the feather follicles prior to release as feather dander. Quantification of MDV within dander and feather follicle samples will help determine if the recombinant viruses are completely deficient in horizontal transmission and no MDV is present, or if they are shed in dander and if so, at what levels compared to wild-type Md5B40BAC virus.

Besides collection and quantification of MDV in feather follicles and dander, viral load could be quantified through isolation of PBLs from both contact and challenged birds, as well as within the spleen collected from sacrificed, moribund, or terminated birds during examination via necropsy to determine MD.

**IN VITRO REPLICATION OF UL46 RECOMBINANT VIRUSES**

The Mut UL46 recombinant virus was a candidate mutation found at a high frequency of 99% within Rep 46, but this mutation had no *in vivo* phenotypic affect. Therefore we wish to determine if this mutation conferred a selective advantage *in vitro* during replication to offer an explanation regarding the spread of this mutation with an attenuated rep during serial passage. Recombinant viruses containing the single point mutations of Mut UL5 and Mut UL46, as well
as a recombinant virus containing both mutations have already been constructed and tested *in vivo*. Growth curves and *in vitro* competition comparing the single Mut UL5 to the wild-type Md5B40BAC-c1 have also already been conducted and described in Chapter 6. We propose addition of growth curves for the single Mut UL46 and Double Mut UL5+UL46 to compare the impact of the UL46 point mutation on replication compared to the single MutUL5 and the Md5B40BAC-c1. We also propose a head-to-head competition of viruses versus the parental Md5B40BAC-c1. Previously the single Mut UL5 point mutation was shown to produce more plaques in one-step growth curves compared to the parental Md5B40BAC-c1, yet in head-to-head competitions the parental Md5B40BAC-c1 outcompeted the Mut UL5 in mixed cultures, suggesting within a mixed population it is more complicated to predict outcome than based simply upon higher yield as determined in single viral cultures. Therefore *in vitro* characterization of recombinant viruses containing the UL46 mutation will help determine if addition of the UL46 point mutation increased in *vitro* replication compared to the parental virus, providing an explanation for the presence of this high frequency mutation within the attenuated replicate.

**FURTHER CHARACTERIZATION OF *IN VITRO* REPLICATION OF MUT UL5**

As described in Chapter 6, the Mut UL5 recombinant virus was shown to produce significantly higher numbers of plaques compared to the parental Md5B40BAC virus, yet when the two viruses were grown in head-to-head competitions at roughly initially equal quantities, the Md5B40BAC virus expanded to dominate nearly 90% of the population over the course of *in vitro* serial passage. To better understand this phenomenon, comparisons of plaque size and average numbers of infected cells produced per plaque would be compared between the Mut UL5 virus and Md5B40BAC virus to determine if the Mut UL5 virus yields smaller plaques with
fewer copies of MDV per plaque compared to the Md5B40BAC. Additionally, to explore the possibility that the two viruses may produce or cause infected CEF cultures to produce, soluble and diffusible products impacting growth of neighboring viruses, it would be proposed to explore the impact of conditioned media on growth of the two viruses. Conditioned media from plates infected with either Mut UL5 or Md5B40BAC viruses would be concurrently added to plates being infected with a defined PFU of viruses and assayed via qPCR to determine MDV quantities in infected cultures and plaque size measured via fluorescent microscopy with ImageJ software, as would also be conducted and compared to plates infected with a known quantity of MDV PFUs but cultured normally as described above.