SEQUENCE COMPARISON OF A BACTERIAL ARTIFICIAL CHROMOSOME (BAC)-BASED INFECTIOUS CLONE OF THE CVI988 (RISPENS) STRAIN OF MAREK'S DISEASE VIRUS (CVI988-699-2) TO A BACK-PASSAGED ISOLATE THAT HAS REVERTED TO VIRULENCE (CVI988-699-2 RV)

by

Juliana Rojas Amortegui

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Animal Science

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ABSTRACT

Marek's disease (MD) is pathology of chickens characterized by paralysis, immumosuppression, and the rapid induction of T-cell lymphomas. MD is caused by an oncogenic alphaherpesvirus, Marek's Disease Virus (MDV). Currently, MD is controlled through vaccination with non-oncogenic vaccine strains comprised of the herpesvirus of turkeys (HVT), or a combination of HVT and MDV-2 (strain SB-1) in a bivalent mixture. However, field strains of MDV have continued to evolve in virulence, necessitating the use of an attenuated MDV-1 strain, CVI988 (Rispens), initially developed in Europe. Commercial CVI988-based vaccines were established after 33 passages in cell culture, by which passage, the virulence of CVI988 had been attenuated. Presently, CVI988 is produced by four vaccine companies and each version of CVI988 confers various levels of protection.

In our lab, we have been able to generate a bacterial artificial chromosome (BAC)-based infectious clone of a low passage of CVI988 (~p25), that provides high levels of vaccine protection (33). This particular clone (CVI988-699-2) was reisolated from a visibly-protected broiler chicken (Bird tag #699) at seven weeks post-vaccination. CVI988-699-2 however, was found to retain some level of pathogenicity upon serial back passage in SPF leghorn chickens. Our initial goal was to attenuate CVI988-699-2 RV through the targeted deletion of the glycoprotein C (gC) gene with subsequent insertion of immunoregulatory genes (IFN-α/β, IL-1β). These were to be under the control of cellular type II keratin 5 (Ker-5) or viral (UL47) promoters for specific expression in the skin. After two years of attempting this mutagenesis, we

shifted the focus of this work to the mutations in CVI988-699-2 RV through examination of the whole genome sequence of this virus to the original CVI988-699-2.

To determine what changes had taken place in CVI988-699-2 during back passage, we inoculated chickens with a 10X dose (~25,000 PFU) of CVI988-699-2 and reisolated BAC-containing virus from tumors caused by this virus. We have termed this virus CVI988-699-2-RV for reverted-to-virulent. The sequence of the original CVI988-699-2 virus was performed by Dr. Stephen Spatz, USDA-SEPRL, using 454 pyrosequencing. The genome sequence of CVI988-699-2-RV was recently determined using the Pacific Biosciences (Pac-Bio) polymerase-based method, which yields up to 100 independent reads per nucleotide. In addition, Dr. Stephen Spatz determined the DNA sequence of CVI988-699-2 RV via 454 pyrosequencing with an average coverage of ~5,000 reads per nucleotide. Comparison of the sequences of the original CVI988-699-2, CVI988-699-2 RV (Pac-Bio) and CVI988-699-2 RV (454 pyrosequencing) revealed 51 and 5 genetic changes (nucleotide substitutions, insertions/deletions and sequence duplications), respectively. The basis of this project, therefore, was focused mutations that may induce reversion to virulence of CVI988-699-2. In addition, the construction of keratinocyte-specific expression cassettes may provide useful tools in the future generation of MD vaccines that could possibly block the transmission of challenge viruses from vaccinated chickens.

Chapter 1

INTRODUCTION

1.1 Marek's Disease

Marek's disease (MD) is a viral lymphoproliferative disease of chickens, caused by a cell-associated avian alphaherpesvirus, Marek's disease virus (MDV) (18, 3). MD was first reported by a Hungarian veterinarian, Dr. Jozsef Marek, in 1907 as fowl paralysis (18). During the 1920s, it was termed *neurolymphomatosis gallinarum* after infected chickens exhibited lymphoid tumors in addition to paralysis, although transmission of the agent was sporadic and onset of disease was greater than 16 weeks (41). With the development of high-density poultry production in the 1950s and 1960s, Marek's disease became the most serious problem in the poultry industry worldwide, because a new form of the disease, known as acute MD, appeared that caused lymphoproliferative infiltrations in visceral organs, peripheral nerves, and muscles in a shorter time frame (~12 weeks) (18, 45).

Chickens become infected with Marek's disease at an early age, with different clinical signs. The signs of MD are lymphoid tumors in the skin, nerves, eyes and internal organs. MD is associated with skin leukosis, poor performance, paralysis, and "gray eyes", a distortion of the shape of the iris and blindness due to lymphocyte infiltration. MD-induced tumors lodge in the visceral organs (heart, spleen, lungs, liver and gonads), and lytic infection is associated with thymic and bursal atrophy, splenomegaly, and stunting (10).

Losses due to MD have been controlled since the early 1970s via vaccination with non-oncogenic, related herpesviruses. Despite the success of vaccination, field strains of MDV have evolved to greater virulence. Marek's disease is considered to be the most expensive disease to control due to the cost of vaccination, the direct effects of the disease on chickens, and the indirect effects of immumosuppression which make chickens susceptible to bacterial, fungal, and other viral agents (5).

1.2 Marek's Disease Virus

Marek's disease virus (MDV) is an avian herpesvirus from the *Alphaherpesvirinae* sub-family, genus *Mardivirus* that causes nerve lesions and T cell lymphomas in chickens (39, 1). MDV strains have been divided into three serotypes that are antigenically-related viruses (MDV-1, MDV-2 and MDV-3) and differ in their virulence for chickens and ability to generate T cell lymphomas. The MDV-1 serotype includes all the oncogenic strains and their attenuated derivatives. The MDV-2 serotype is a non-oncogenic chicken herpesvirus; and the MDV-3 serotype is a non-oncogenic strain of herpesvirus originally isolated from turkeys (HVT) (19) and these are used as vaccine strains (54).

In general, all the serotypes of MDV have characteristics typical of the other alphaherpesviruses, in that their DNA sequence is similar 50%-80% (39), and the major regios of divergence are located in the repeats flanking their unique long regions. MDV-1 strains are further classified into four virulence groups, mild (m), virulent (v), very virulent (vv) and very virulent plus (vv+), based on their ability to cause disease, the severity of the disease, the rate at which disease is caused and the ability to overcome monovalent (HVT) or bivalent (HVT/SB1) vaccination (75).

1.3 The MDV Genome

The MDV genome is a double- stranded, linear DNA molecule of approximately 185 kbp in length (68). The MDV genome of MDV-1, MDV-2 and HVT serotypes, described previously, are organized like other alphaherpesviruses, such as herpes simplex virus 1 (HSV-1) (6). The genome size of MDV-1 is ~185 kbp long, MDV-2 is 165 kbp and HVT is 160 kbp (21). The genome of MDV consists of two unique regions, long (UL) and short (US), which are flanked by terminal and internal inverted repeat regions (TRL, IRL, TRS, and IRS) (6, 39, 58, 68). The TR and IR of the short and long regions are present in inverse orientation and are identical in sequence (40). The unique regions of the MDV genome encode herpesviral genes that are highly conserved. These genes encode products involved in replication (DNA polymerase, thymidine kinase, *etc.*), virus assembly (UL15, UL26, *etc.*), and virus structural antigens (capsid, tegument, and glycoproteins) (40).

1.4 MDV Pathogenesis

The pathogenesis of MDV has been divided into four phases (a) early cytolytic infection; (b) latent infection; (c) late cytolytic infection and immumosuppression; and (d) the transformation phase and development of lymphomas (9, 39, 70). The onset and duration of each phase depend on the phenotype of the strain, the level of challenge, the age, and genetic susceptibility of the chicken.

1.4.1 Early cytolytic infection

The first phase, early cytolytic infection occurs once the virus is inhaled into the respiratory tract (46) and virus is transferred to B-cells and macrophages (2). Following entry, MDV is transferred to the primary and secondary lymphoid organs

(bursa of Fabricius, thymus and spleen) (46,70) and replicates in these organs between 3 and 6 days post-infection. Both CD4+ and CD8+ T-cells are infected during this time, resulting in thymic atrophy as these cells undergo apoptosis (35). An early innate immune response to viral infection drives MDV into latency in primarily CD4+ T lymphocytes (9, 46).

1.4.2 Latency infection

Latency of MDV in CD4+ T-cells appears to be mediated by host-encoded factors and is evident at about two weeks post-infection (57, 70). Latency is defined as a lack of production of infectious virus, an alteration in MDV genome expression, with the presence of the viral genome.

An early initial immune response is essential for the induction of MDV latency. Interferon-γ (IFN-γ) and nitric oxide (NO) have been implicated in driving this process (17, 52). The importance of the early immune response to infection was demonstrated in studies by Buscaglia *et al*., in which suppression of immunity blocked the establishment of latency and increased the duration of the early cytolytic infection (7).

IFN-γ is very important during the MDV infection, since it can interfere with virus replication by activation of NK cells and macrophages and enhance production of other cytokines (IL-1β, iNOS, INF-α and/or TNF) (77). IFN-γ also has been shown to suppress virus replication in cell culture. High levels of NO also inhibit replication of the virus and downregulate T-cell proliferation (9, 52, 77).

At least two forms of latency appear to be established during MDV infection (71), in which latently infected cells can either rapidly or slowly reactivate virus, depending on the stage. Work by Brown *et al*., 2012 (4), provided some insight into

these stages of latency, as the MDV genome becomes regulated by changes in histone methylation in latently-infected and transformed cells. During latency, and in transformed cells, MDV lytic promoters are associated with repression-based histone methylation (H3K9 and H3K25), while latency-associated promoters are associated with activation-based histone methylation (H3K4). Similarly, more tightly repressive CpG methylation was found at lytic promoters in tumors, while transformation-associated genes were notably hypomethylated.

Latency is also associated with the production of spliced gene products of the Meq oncoprotein (see below) (44) with spliced forms of Meq having a higher affinity for CtBP-1, a scaffold protein for chromatin remodeling enzymes such as histone methyltransferases, acetylases/deacetylases, and DNA methyltransferases.

1.4.3 Secondary cytolytic infection

Following the establishment of latency, MDV can reactivate and resume lytic infection starting 14 to 21 days post infection (dpi) (9). During this secondary cytolytic infection, MDV disseminates into other organs, such as the adrenal glands, kidneys, Schwann cells, and epithelial tissue, including the feather follicle epithelium (FFE) (8). The FFE is the only location where fully productive virus replication occurs, shedding cell-free particles in the dander (46). MDV can be detected as early as 10 to 12 dpi in the infected FFE cells, and virus is shed from this skin intermittently for the life of the chicken, even from vaccinated and protected chickens (see Horizontal Transmission, below) (3).

1.4.4 Transformation

By 3 to 4 weeks post-infection, lymphomas develop and become lodged in the heart, liver, kidney, breast muscle, gonads, intestines, and spleen. MDV-induced lymphomas are comprised are complex mixtures of lymphoid and myeloid cells; however the transformed component are primarily CD4+T lymphocytes. (24). The transformed CD4+ T-cell has been characterized as being polarized as a regulatory T-cell through the proteomic examination of primary lymphomas (55). The MDV-transformed CD4+ T-cell expresses high levels of CD30, MHC-II and various other activation and polarization-associated antigens and cytokines.

Of the MDV gene products linked to transformation, the most essential appears to be Meq (for Marek's EcoRI-Q-encoded protein), a basic leucine zipper (bZIP) protein encoded in the TRL/IRL of the MDV genome and highly expressed in lymphomas and cell lines established from lymphomas. Meq has many of the hallmarks of viral oncoproteins. Meq, via its leucine zipper (ZIP) domain, can either form homodimers with itself or heterodimers with cellular bZIP proteins, such as c-Jun, JunB, ATF-2, etc. Meq also can bind to AP-1 like sites, thereby altering the expression of both cellular and viral genes during cell proliferation. Meq also interacts with p53, CDK2 and RB to shortened G_1 phase in the cell cycle. Meq is related with up-regulation of anti-apoptosis genes such as Bcl2, and down-regulation of pro-apoptotic genes such as Bax, Fas, FasL, and DAP5 (24). Meq also binds the C-terminal binding protein (CtBP), a protein that serves as a scaffold for chromatin remodeling enzymes (HDACs, HATs, HMTs). The interaction of Meq and CtBP is essential for MDV-mediated transformation (24).

Other transformation-associated gene products of MDV, such as, vTR, RLORF4, UL36, etc., have an important role in the pathogenicity of MDV. The virus-

encoding telomerase RNA (vTR) contributes to transformation of T-cell lymphomas and in supporting tumor dissemination. Telomerase is a ribonucleoproteic complex that protects chromosomal DNA from shortening during replication. The telomerase enzyme contains two essential, and several auxiliary proteins. One essential component is the telomerase reverse transcriptase (TERT), and the second a telomerase RNA (TR), which serves as a template for TERT. During cell division, telomerase adds repeat sequences (TTAGGG)n to the end of each chromosome. The secondary structure of telomerase RNA consists in four conserved domains: pseudoknot, CR4-CR5, Box H/ACA, and CR7 domains that function as template sequence, enhance telomerase activity, and provide TR stability respectively (14, 66). MDV encodes a viral homologue called virus-encoded telomerase RNA (vTR), which exhibits 88% sequence identity with the chicken TR (*chTR*) (14, 24, 66). Moreover, analysis of vTR functionality showed that vTR has a more efficient at interacting with the telomerase reverse transcriptase than the chicken TR (*chTR*) due to mutations affecting its structure (66). Telomerase activity is up-regulated in lymphoid cells undergoing activation and gastrointestinal epithelium in adults, but is also observed in oncogenically-transformed cells (24).

The open reading frame RLORF4 is encoded within the IR_L and TR_L regions of the MDV genome downstream of the Meq ORF and has been associated with the attenuation of MDV. Previous studies found deletions in four out of six attenuated strains examined. Jarosinski *et al*., subsequently demonstrated that the virulent pRB-1B BAC became attenuated after deletion of RLORF4 with increased virus replication and plaque sizes *in vitro* and decreased replication and tumor development *in vivo*

(36). RLORF4 is not essential for transformation, but does affect replication *in vivo* and moderate the tumorigenic potential of the virus.

UL36 encodes the major tegument protein (MTP) which contains a viral ubiquitin-specific protease (USP) that modifies host cell proteins (53). As a tegument protein UL36 seems to be involved in the structural integrity of viral particles. Therefore, maturation of capsids in the cytoplasm into enveloped virus requires the MTP to generate infectious virus (11). Deletion of the UL36 gene of MDV-1 generated non-enveloped, DNA-filled capsids in the cytoplasm of infected cells (11, 53).

One other essential function of UL36 is its ubiquitin-specific protease (USP/DUB) domain which allows making use of ubiquitin-mediated pathways to enable viral replication. DUBs catalyze the removal of ubiquitin from proteins and other molecules that affects several cellular processes and substrates and any deficiency in the host cell can result in the pathogenesis (13, 53, 69).

The catalytic site Cys65 is conserved throughout the whole family of Herpesviridae and is essential for the USP activity (25, 53, 69). Moreover, it has been reported that a mutation in the catalytic site (Cys65) of the MDV-USP, reduces virus replication *in vivo*, as well as tumor formation in chickens (25). While the deletion of N-terminal region of the major tegument protein UL36 showed that it plays a structural role after the mutated virus (RB-1BΔUSP) was unable to replicate *in vitro* (69).

1.5 Horizontal Transmission

MDV spreads horizontally among chickens as infectious dander in the environment through the shedding of infectious particles produced in the keratinizing layer of feather follicle epithelial (FFE) cells from the skin of infected chickens. MDV shedding of infected dander occurs between two to four weeks post-infection, prior to the appearance of clinical symptoms of the disease, and can continue throughout the bird life. MDV remains infectious in dander in the environment for long periods of time, until inhaled by an uninfected chicken and the virus replication cycle is repeated within the new host (26). The chicken-to-chicken transmission of MDV requires expression of glycoprotein C (gC) and the UL13 protein kinase (27), as mutations in either of these genes ablates transmission.

1.5.1 Glycoprotein C (UL44)

Originally identified as the "A antigen", gC is the major precipitating MDV antigen found in serum of infected chickens. gC is encoded by the UL44 homologue of HSV and has a molecular weight of 57 to 63 KDa (64) due to glycosylation. MDV gC expression occurs in two forms: one as transmembrane protein with a hydrophobic C-terminal expressed on the surface of virions and infected cells, and two, as a secreted protein from infected cells. Secreted gC can be generated by either expression of splice variant forms or from the proteolytic-cleavage of the surface form (39).

gC is involved in the primary attachment of cell-free virus to heparin- and chondroitin-like glycosaminoglycans on surface of cells (64). gC is nonessential for replication in cell culture and *in vivo* , however, its deletion does cause mild attenuation and loss of horizontal transmission (64). In cultured cells, gC expression is

lost during serial passage and MDV plaque size increases after the loss of gC expression (24). Forced over-expression of gC actually decreases MDV plaque size in culture.

A primary function of gC is during *in vivo* infection in which gC is involved in blocking the activation of complement through binding complement protein C3b which thereby provides a mechanism of immune evasion to the virus (64). Secreted gC can be detected in culture and sera of infected birds, even though its expression is dramatically reduced after serial passage in culture (64, 72). In one study, viruses with low and high gC expression were compared in the nucleotide sequence of UL44 or its promoter region and no difference was found.

The function of gC during replication and transmission of MDV *in vivo* has been studied by deletion of gC from MDV strain RB1B (27). An MDV strain lacking of gC showed enhanced replication in cultured cells and little to no effect on the replication and pathogenicity in inoculated chickens. However, horizontal transmission was blocked which indicated its functional importance in transmission. Despite loss of horizontal transmission, the gC deletion mutant traveled to the skin of infected chickens and established latency and induced tumors to comparable levels of its parental virus (27).

1.5.2 UL13 Protein Kinase

Another gene recognized to be significant for horizontal transmission was the UL13 serine/threonine protein kinase (PK) (27). Since the UL13 genes of herpesviruses are highly conserved, this kinase appears to play an important role in herpesvirus replication by phosphorylating cellular and viral proteins. The UL13 PK

of HSV-1 phosphorylates the U_S3 kinase, the US1-encoded protein, VP22, gE and gI (which form an Fc receptor), and also is capable of autophosphorylation. The VZV *UL13* ortholog is required for infection of T lymphocytes in the SCID-hu mouse model (26). Moreover, UL13 seems to be a factor important to egress of virus particles from the nucleus.

The importance of the UL13 PK during replication and transmission of MDV *in vivo* has been studied by Jarosinski *et al*. (26, 27). The initial pRB-1B-BAC reported by the Nair laboratory was infectious, pathogenic and oncogenic in inoculated but not contact-exposed chickens (48). In follow-up work, the Spatz laboratory sequenced the genome of this clone and found frame-shift mutations in the glycoprotein C and UL13 PK ORFs (60).

In this subsequent work, Jarosinski *et al*., found that the MDV UL13 protein kinase is essential for horizontal transmission by mutating an invariant lysine within the catalytic site of the UL13 ORF in an infectious pRB-1B clone (26, 27). They found that there was no significant difference in the plaque sizes for the mutant and parental viruses. However, the U_L13 kinase mutant did not spread to contact chickens indicating that the ability of horizontal transmission of MDV between chickens had been eliminated (27). Mechanistically, however, it is not clear if the result of the UL13 mutation acted directly (affecting virus egress in the FFE) or indirectly (via other viral or cellular substrates).

1.5.3 Glycoprotein D (US6)

The MDV glycoprotein gD homologue is encoded by the US6 gene. MDV gD is synthesized into a 42 to 53 kDa protein due to posttranscriptional glycosylation. The

MDV gD contains four *N*-linked glycosylation sites and seven cysteine residues of which six are conserved among gD of herpes viruses (51). In HSV-1 infection, gD is essential for the entry of virus into susceptible cells through the binding to a cellular receptor, HVEM (for herpesvirus entry mediator), a TNFR-family member (1, 61). Some of the roles of gD of HSV-1 is entry into susceptible cells, receptor binding, cell fusion and neuroinvasion. (1, 62). The MDV gD homologs are HSV-2, pseudorabies virus (prV), bovine herpesvirus (BHV-1), and equine herpesvirus. MDV gD expression is blocked in cell culture, and it is not essential for MDV replication in cell culture, pathogenesis *in vivo*, or in the horizontal transmission of MDV (1, 62). However, *in vivo* gD is expressed at high levels in the feather follicle epithelium cells or in chickens (1, 62).

1.5.4 The UL47 tegument protein

The tegument protein encoded by UL47 gene is dispensable for MDV replication in the skin cells of infected chickens (22) and for the production of infectious virus and horizontal transmission. The UL47-encoded VP13/14 tegument proteins of HSV-1 have homologs in the genomes of all three MDV serotypes. The MDV UL47 (MDV060) is a tegument protein of molecular weight of 91.9 kda. The UL47 tegument protein is 808 amino acids long and is believed to bind to various RNA transcripts (12, 32). UL47 gene seems to be involved in virus-encoded RNA due to its high affinity for polyadenylated transcripts (12).

Deletion of UL47 from the genomes of HSV-1, MDV, infectious laryngotracheitis virus (ILTV), pseudoravies virus (PRV), and bovine herpesvirus 1 (BHV-1) negatively affects viral replication in vitro, and attenuates these viruses in

vivo (52). Jarosinki *et al*., (22, 27) generated a recombinant MDV having a green fluorescent protein fused to the C terminus of UL47 in order to analyze its expression *in vitro* and *in vivo*. He found that UL47 is expressed at low levels *in vitro* and in lymphocytes, *in vivo*. He found that the MDV UL47 protein was highly-expressed, however, in the FFE. UL47-expression was undetectable in transformed cells in tumors, suggesting that it was only associated with lytic infection. Interestingly, UL47 is mainly expressed in the nucleus of infected CEF in culture, but in the cytoplasm in the FFE *in vivo*. This difference in localization may indicate that this tegument protein is important for the site-specific production of cell-free virus in the skin of MDV-infected chickens (22). This work also provides a tool for the study of MDV-associated FFE-specific expression.

1.6 Control of MDV

1.6.1 Vaccines

After identification of the MDV as the causative cell-associated herpesvirus in 1967, vaccination against MD was introduced in the 1970s by using an attenuated serotype MDV-1 (HPRS-16 strain) (59, 74). In 1970, a herpesvirus of turkeys (HVT) was isolated from healthy turkeys, and was found to elicit protection to chickens against challenge with MDV-1 strains. This HVT-based vaccine became the most commonly-used vaccine, first as a cell associated vaccine and later as cell-free, lyophilized vaccine (3, 47). By the early 1980s, field strains evolved that overcame HVT-elicited protection (73). MD caused by these more virulent field strains could be

controlled with a bivalent vaccine consisting of HVT and the non-oncogenic MDV-2 strain (SB-1) (47, 57, 59).

In the early 1990s, an attenuated vaccine strain of serotype MDV-1, CVI988 was introduced in the United States (47). The CVI988 strain, also called the Rispens strain, had been used to control MD since 1972 in the Netherlands. In the US, CVI988 is used either alone or in combination with HVT. Currently, a bivalent vaccine with HVT and Rispens is used for longer lived birds (Layers, Broiler Breeders). In areas of high challenge, trivalent vaccines are used with HVT/SB-1 being administered *in ovo*, followed by CVI988 at hatch. Worldwide, HVT only or HVT/SB1 remain the most widely used MD vaccines (5). Even though the Rispens vaccine provides protection against vv+MDV strains, which are strains that overcome bivalent vaccines, no vaccines are 100% effective, and the persistence of MDV in vaccinated chickens is increasing virulence even in birds vaccinated with CVI988 (36).

MD vaccines are produced in two types: one, as a cell-associated vaccine (wet vaccine), which is administered as an infected cell suspension (HVT, HVT/SB1 and CVI988), and as a cell-free virus (dry vaccine, HVT only), which is a lyophilized form necessary for transport to areas lacking cold storage for the cell-associated vaccine (5). Cell-associated vaccines are used primarily in poultry production, since they have good efficacy in the presence of maternal antibodies (20). The vaccine is administrated either at 18 days of embrynation *in ovo* or at hatch via subcutaneous (subQ) or intramuscular (IM) routes (5).

1.6.2 MDV evolution of virulence

MD had been successfully controlled by vaccination with attenuated and non-pathogenic MDV strains since the 1970s. The virulence of MDV field strains has increased, overcoming the prevailing vaccines used over time. The MD vaccines appear to play a major role in driving the evolution of MDV strains. Field strains have been classified in virulence from mildly virulent (m) to virulent (v), very virulent (vv) and very virulent plus (vv+).

In 1907, Josef Marek described a polyneuritis affecting mostly older chickens with low morbidity and mortality. Until the 1950s, MDV strains caused a mild paralysis with few lymphomas, principally in peripheral nervous tissue and mortality was relatively rare. These strains then were considered "classical MDV strains" because they represented the pathogen described by Marek. However, a first shift in MDV virulence was observed during the late 1950s and early 1960s by the poultry industry. This new form of MD was caused by "acute" or virulent MDV (vMDV) strains that affected not just the nervous system but also caused greater number of tumors in visceral organs (16). The isolation of HPRS-16 in the UK represented one of these acute MDVs, and due to its increased replication in chickens, was able to be readily isolated in cell culture.

Following the attenuation of HPRS-16 and the isolation of HVT, MD was largely controlled by the introduction and widespread use of these viruses as vaccines. A second increase in virulence was observed in the early 1980s, when MDV was able to break through the protection induced by the HVT vaccination, and losses from MD began increasing again due to very virulent (vv) strains of MDV (16). These strains are very lymphomagenic and immunosuppressive MDVs that can cause tumors in birds in less than 6 weeks. A new, bivalent vaccine was introduced to control losses

due to vvMDVs, consisting of HVT and naturally non-oncogenic serotype MDV-2 strains (HVT/SB1).

During the early 1990s, another increase in MDV field strain virulence was observed when outbreaks of MD in flocks vaccinated with HVT/SB1 occurred. These strains were termed very virulent plus (vv+), and were able to cause MD in >35% of chickens vaccinated with HVT/SB1 (74). The vv+MDVs not only rapidly cause lymphomas in chickens, but are associated with MD in adult birds, and profound neurological lesions and stunting in younger birds (16, 74).

With the evolution of vv+MDV field strains, the attenuated serotype MDV-1, CVI988 was introduced as a vaccine, and it has been the most effective vaccine for protection against MD (5). Since field strains of MDV persist even in vaccinated hosts, it is likely that MDVs will evolve and overcome the protection conferred by CVI988, as well.

1.6.3 CVI988 BAC

A relative low pathogenicity strain of Marek's disease virus was isolated in the Netherlands and described by Bart Rispens in 1972 (50), a strain designated CVI988 (from the Central Veterinary Institute, Lelystad, the Netherlands). After serial passage in cell culture, the CVI988 strain was tested for safety and was found to be highly efficacious as a vaccine (50). Due to its efficacy, CVI988 has been licensed for production by several vaccine companies with varying degrees of success. Production of CVI988 in cell culture results in the generation of multiple species that are readily detectable by PCR, and can be isolated by plaque purification (56), and (Parcells, unpublished).

To generate molecularly-cloned CVI988 MDVs that can be selected for increased vaccine efficacy, one approach has been the generation of bacterial artificial chromosome (BAC)-based infectious clones via insertion of the mini F sequence from *E. coli* into the MDV genome (54). A widely used and effective vaccine against MD has been cloned as a infectious BAC clone and has showed significant stability during both *in vitro* and *in vivo* passages (47, 63). Schumacher *et al.,* were able to generate a DNA vaccine comprised of the infectious BAC20, based on the 584A strain of MDV (63).

Since CVI988 vaccines are known for providing a high level of protection against MD, an analysis of the protective ability of the pCVI988-BAC virus against the virulent RB1B strain would be a contribution to the MD vaccines. Petherbridge *et al.,* compared the CVI988 vaccine and a pCVI988-BAC clones and showed similar morphology and size of the plaques *in vitro* (47) and found that birds vaccinated with the BAC-derived CVI988 virus did not developed MD. These results demonstrated that the recombinant vaccine was able to induce protection against infection by the virulent MDV strain similar to the parental vaccine virus (47). As CVI988 is not typically required for a vvMDV challenge, but for a vv+MDV challenge, however, it was not clear if the pCVI988 and the commercial CVI988 provided equivalent protection.

The efficacy of CVI988-based vaccines varies according to manufacturer and passage history of the vaccine (16). In our laboratory, we were able to generate a bacterial artificial chromosome (BAC)-based infectious clone of a low passage of CVI988 (~p19) by transfection of virus into chicken embryo fibroblasts (CEF) with transfer vector pDSHA-1, followed by four rounds of positive selection, re-

transfection of DNAs into CEF to select for clones that regenerated infection, and finally, electroporation into *E.coli* to isolate pCVI988-BAC clones (Pennington and Parcells, unpublished). To provide a basis for the selection of protective pCVI988-BACs, we attempted to reisolate virus from PBMC and spleen cells from visibly-protected broiler chickens at 7 weeks post-challenge with TK, a vv+MDV. One clone (clone 2), isolated from the spleen cells of bird #699, regenerated infection in CEF and was termed CVI988-699-2. This clone was tested with and without deleted BAC sequences (CVI988-699-2Δ) and provided superior protection compared to several commercial CVI988 vaccines (16, 33).

1.6.4 Deletion of BAC sequences on pCVI988-699-2 and safety issues

To evaluate the efficacy and safety of pCVI988-699-2, we performed a number of vaccine efficacy trials and also a safety back-passage trial (33) . As this work had been sponsored by a commercial vaccine company, it has yet to be published. To remove the BAC sequences from CVI988-699-2, the DNA of this BAC was co-transfected with a Cre recombinase expression vectors (pBKCMV-Cre). The BAC sequences (aka mini-F plasmid) were flanked by loxP sites in the same orientation. Consequently, expression of the Cre recombinase would result in the deletion of the BAC leaving only 110 nt of foreign sequence at the US2 locus. Stocks of CVI988-699-2Δ were prepared using CEFs.

CVI988-699-2Δ was used in a series of shedder-based vaccine trials and found to provide protection levels superior to commercial CVI988 products (33). To evaluate the safety of CVI988-699-2Δ, a serial back passage study was performed. Twenty SPF leghorn chickens were inoculated with a standard vaccine dose (~2,500 PFU/bird) and five were bled at one-week post-infection. Their blood was pooled and

used to infect a second hatch of twenty chickens. This process was repeated to complete five sequential passages with the evaluation of viremia from PBMC and spleen cells at each passage level. The remaining chicken were kept in isolation for an additional 6 weeks, euthanized, and scored for MD lesions.

CVI988-BAC, 699-2Δ retained some level of pathogenicity upon serial back passage, in that tumors were identified in one or two birds at back-passages 3 and 5 (33). In addition, paralysis was noted also in a few birds at these passages. To determine if genetic changes had taken place in CVI988-699-2Δ during back passage, or if a minority population of pathogenic virus had been present in the original stocks of CVI988-699-2, chickens were inoculated with a 10X dose (~25,000 PFU) of CVI988-699-2 and placed with contact chickens. BAC-containing virus was reisolated from a small kidney-localized lymphoma in a chicken. Essentially, tumor cells were co-cultivated with CEF and virus was amplified for a few passages, DNA was isolated and electroporated into *E. coli*. , This tumor cell-derived virus was termed CVI988-699-2-RV for reverted-to-virulence.

The original objectives of this project were to make select mutations in the CVI988-699-2 BAC clone in order to decrease its virulence and increase its efficacy as a vaccine. In collaboration with Dr. Stephen Spatz (USDA-SEPRL), the entire genome sequence of CVI988-BAC-699-2 was determined by Ilumina 454 sequencing. Following the isolation of CVI988-699-2-RV, the sequence of this virus was performed at the University of Delaware using the Pacific Biosciences (PacBio) instrument and was also performed at the USDA-SEPRL by Dr. Stephen Spatz using the Ilumina 454 pyrosequencing. The comparison of these sequences and the cell culture biological characteristics of these viruses comprise the body of this research.

1.7 Hypotheses of Research

Our initial hypothesis was that the targeted deletion of the glycoprotein C gene from the genome of CVI988-699-2 would result in mild attenuation of the virus to the extent that this vaccine would then be safe. As gC expression is apparently essential for horizontal transmission (26), its deletion would generate a non-spreading vaccine. We further hypothesized that this virus would be able to carry immunoregulatory genes to the skin of vaccinated chickens and their keratinocyte-specific expression may elicit responses that would block the transmission of field strains, *in trans*.

Since we were unable to generate the recombinant MDVs for addressing these hypotheses, we focused on the genetic sequence of these two strains, CVI988-699-2 and CVI988-699-2-RV, in order to possibly identify specific mutations that are associated with the MDV reversion to virulence. Most work of this nature has focused on the loss of MDV gene products and mutations that accumulate during serial passage in cell culture (69). Our work will look at those genetic changes that correlate with the acquisition of virulence, not its loss. As the vaccine strain CVI988-699-2 was largely attenuated in broiler chickens and SPF leghorns, and the acquisition of virulence required very high dosage (10X), or serial back-passage in cell culture, it seems likely that the genetic changes between these two strains will provide direct insight into genes important to MDV virulence. Based on the comparison of CVI988-699-2 and CVI988-699-2 RV sequences, we have developed several aims to be tested.

1.7.1 Aim 1: Genetic Changes between CVI988-699-2 and CVI988-699-2-RV

The genomic organization and gene content of these two strains, CVI988-699-2 and CVI988-699-2 RV, can indicate what changes are associated with reversion to virulence. Therefore we are seeking to analyze what genes are involved in the

reversion of the efficacious vaccine to a virus that can maintain some level of virulence (CVI988-699-2 RV) by comparison with the original CVI988-699-2 vaccine strain. Our first hypothesis is that the CVI988-699-2-RV sequence (performed at UD using the PacBio instrument) will show genetics changes compared to the previously-sequenced CVI988-699-2 in collaboration with Dr. Stephen Spatz (USDA-SEPRL).

In addition to having the entire genome of CVI988-699-2-RV sequenced here at the University of Delaware, Dr. Stephen Spatz has also performed the sequencing of CVI988-699-2-RV using Ilumina 454 sequencing. In comparing the two genomes (CVI988-699-2 and CVI988-699-2 RV) by these two sequencing methods (PacBio and 454), 51 genetic changes were noted using the PacBio sequence, while only 5 changes were noted in the Illumina 454-generated sequence. Some of these changes are single nucleotide polymorphisms (SNPs), while others are insertions, deletions (INDELs), and reiterations of blocks of sequence. We will confirm these differences through the direct Sanger resequencing using the BAC templates or through targeted PCR amplification and sequencing to determine whether these changes are in fact present in the virus, or are sequence assembly artifacts.

1.7.2 Aim 2: Changes Affecting Expression of Specific MDV Genes

Our second hypothesis is to the observed genetic mutations affect expression of associated MDV gene products, and confers the increased pathogenicity phenotype to the virus. As part of our comparison of these viruses, we will perform growth curve, and plaque area analyses to study the effects of these genetic changes on the cell culture replication of these viruses.

Chapter 2

MATERIALS AND METHODS

2.1 Cells

For the propagation of MDV strains (CVI988-699-2 and CVI988-699-2 RV), we used secondary chicken embryo fibroblasts (CEF) prepared freshly from 10-day-old specific-pathogen-free (SPF) single comb white leghorn (SCWL) embryos (Sunrise Farms, Inc.). CEF were grown in Medium 199 (M199) supplemented with 3% calf serum, 4 mM L-glutamine, 1X Penicillin/Dihydrostreptomycin/Neomycin (PSN) and 1X fungizone (Life Technologies).

Primary chicken keratinocytes were initially prepared from neural crest cells of three-day-old embryos by Dr. Toyoko Akiyama, a visiting scientist in the laboratory of Dr. Parcells in 2001. These cultures initially contained mixes of melanocytes and keratinocytes; however upon thawing from cryopreserved stocks, primarily keratinocytes grew. Keratinocytes were grown in F-12 medium supplemented with 10% fetal bovine serum, 4 mM L-glutatmine, 2 mM sodium pyruvate, 1X PSN, 1X fungizone and 10 ng/ml rat endothelin 3 (Sigma Chemical Co.).

For expression analysis, chicken macrophage cell line (HTC) (49), and chicken Leghorn Male Hepatoma cell line (LMH) (29, 49) were used. HTC cells were propagated in DMEM medium supplemented with 10% FBS, 4 mM L-glutamine, 1X PSN and 1X fungizone. LMH cells were growth in Waymouths medium supplemented with 10% FBS.

2.2 Viruses

The CVI988-699-2 and CVI988-699-2 RV stocks were prepared by transfection of virus DNAs using the calcium phosphate method (34). All stocks were titrated on secondary CEF prior to use.

2.3 Deletion of gC of CVI988-669-2

To address our initial hypothesis that deletion of the glycoprotein C gene from the genome of CVI988-699-2 RV would result in decrease of its pathogenicity and an increase vaccine strain safety, we attempted Red-mediated recombination method (68). The gC gene (1150 bp) would be replaced by a kanamycin resistance (Kan^R) cassette obtained by PCR amplification using primers flanking the UL44 (gC) gene and the pLAY-2 plasmid as a template plasmid (26).

2.3.1 PCR Reactions and preparation of DNA samples:

For deleting gC, the Kan^R cassette from plasmid pLAY-2 was amplified by PCR using the primers listed in Table 1. The PCR reaction was performed in 25 µl with the following conditions: 1 cycle for 94˚C for 5 min, 35 cycles of 94˚C for 1 minute, 55˚C for 1 minute, 68˚C for 1.5 minutes, followed by a final extension of 68˚C for 10 minutes using AccuprimeTM *pfx* DNA polymerase (Invitrogen). The PCR product was gel purified with QIAquick gel extraction kit (QIAGEN, Inc.) and cloned into the *SmaI* restriction site of plasmid pUC19 (*New England Biolabs Inc*) via T4 ligase. Positive clones were selected on LB agar plates with ampicillin (100µg/ml) and kanamycin (50µg/ml).

2.3.2 Preparation of electrocompetent cells, and electroporation of DNA

For preparation of competent cells, we used two types of electrocompetent *E. coli* cells for BAC mutagenesis: (1) DH10β cells harboring the pGETrec plasmid, and (2) the *E.coli* strain GS1783 cells, which harbor a temperature-inducible recombinase and L-arabinose-inducible restriction enzyme (*Sce* I).

The electrocompetent DH10β cells were initially transformed with the pGETrec plasmid. Both cells and plasmid were prepared as described in (65). The pGETrec plasmid encodes the the *E.coli recE* and *recT* genes which are regulated by an *araC* (arabinose-inducible) regulon and allow homologous recombination by RecET recombinase (37). DH10β/pGET electrocompetant cells were induced with 1% L-arabinose for 40 min, then they were electroporated with the 100ng of gel purified PCR product and pCVI988-699-2RV DNA. The electroporation was performed in a 1mm gap electroporation cuvette by using PEPTM (Personal Electroporation Pak Electroporator - BTX® Genetronics Inc) with the following parameters, 15kV/cm, 25μF and 200Ω. Following electroporation, SOC medium was added to each cuvette and bacteria were transferred to snap-cap tubes for growth. After 1 - 2 hours of incubation at 37˚C, 100 μl cells were plate on LB agar selective plates having 50 μg/ml of kanamycin (KAN) and 30 μg/ml of chloramphenicol (CAM). The positive clones were selected for resistance to KAN and CAM, and subsequently for AMP sensitivity via replicate plating.

Overnight cultures of GS1783 and CVI988-699-2 BAC clones were grown in LB broth with 30 μg/ml chloramphenicol in a bacterial shaker at 32°C. GS1783 cells were electroporated with the approx. 100 ng of PCR product. The DNA/bacteria mix was transferred to chilled electroporation cuvette and immediately electroporated with 15 kV/cm using settings of 25μF and 200Ω. Following electroporation, cuvettes were

placed on ice and 450 µl of SOC medium was added. Cultures were transferred to snap-cap tubes and grown at 32˚C with agitation for 1 – 2 hours. Following this incubation, 100µl cells were plate on LB agar selective plates having 50 µg/ml of KAN and 30 µg/ml of chloramphenicol (CAM).

Selected colonies were grew in 3 mls of LB + CAM (25 µg/ml) for mini-prep purification of BAC DNA and positive clones were screened by *Bam*HI digestion to ensure that positive clones did not contain contaminating plasmid DNA (our usual result). Clones that contained only BAC DNA were further screened for proper KAN cassette insertion by Southern blot hybridization analysis using enzymes that cut on either side of the gC locus.

After greater than 1 year of attempting these procedures, no site-specific recombinants were generated.

2.3.3 Keratinocyte-specific expression

2.3.3.1 Subcloning of the US6 (gD) promoter.

The US6 region of the MDV genome is obtained from plasmid PMD100 by digestion with restriction enzymes *Ase* I – *Avr* II. This 871 bp fragment of gD was gel purified via the QIAquick gel extraction kit (QIAGEN, Inc.). The purified gD fragment was cloned into the *Ase* I – *Nhe* I sites of vector peYFP-N1, thereby replacing the CMV promoter with the promoter sequences of US6 (gD). Positive clones were confirmed by DNA sequencing analysis.

2.3.3.2 Sub cloning of the UL47 and Keratin-5 promoters.

For constructing kerotinocyte-specific expression vectors, we amplified type II keratin-5 (Ker-5) and MDV UL47 promoters, using the oligonucleotides primers listed in Table 2 (22, 76). Each of these were designed for insertion of *Ase* I and *Nhe* I restriction sites for directional cloning into CMV promoter-containing vector. The RB-1B strain of MDV was used as a template to amplify the UL47 promoter, and secondary chicken embryo fibroblasts (CEF) DNA was used as a template for amplification of Ker-5 promoter in specific PCR reactions. The amplified PCR products of UL47 and Ker-5 promoters, 251 bp and 404 bp respectively, were used for TOPO cloning and transformation. For each promoter construction, two TOPO clones were submitted for DNA sequencing to confirm that no mutations were introduced during the amplification reaction.

In order to construct vectors with fluorescent proteins as a marker for gene expression, each promoter was then sub-cloned into eYFP-N1, eCFP-N1 and monomeric eRFP expression vectors. The UL47 and Ker-5 promoters were released from the TOPO vector using the restriction enzymes *Ase* I - *Nhe* I and ligated into the *Ase* I – *Nhe* I sites of vectors peYFP-N1, peCFP-N1 and peRFP, thereby replacing the CMV promoters in these plasmids. Finally, constructs were sequenced to confirm the presence of the insert with the right fluorescent protein before performing transfection into cells.

2.3.3.3 Transfection with gD, UL47 and Ker-5 promoter expression vectors

To determine the tissue specificity of the chosen promoters, gD-, UL47- and Ker-5-promoter expression vectors were transfected into four cell types: CEF, HTC (macrophages), LMH (hepatocytes), and keratinocytes. For transfection, 24-well

dishes were plated with rows of each cell type with ~5 x 10^4 cells per well. Each cell type was plated in one row of 6 wells and incubated overnight.

Prior to transfection, cells were switched to medium without serum and antibiotics and incubated for 30 minutes, as plasmid DNA/liposome mixes were prepared. For transfection, Lipofectamine 2000 was used (Invitrogen) and each well was transfected with 200 ng of test plasmid, 50 ng of control plasmid, ± 125 ng of CVI988-699-2 DNA . The vector eCFP-N1 was used as transfection efficiency control. DNA/liposome mixes were added on the serum-starved cells and incubated for 4 hours, after which, fresh growth medium was added and cells were incubated overnight.

The next day, we observed for each well for transfection efficiency and expression of the fluorescence vectors under 488 nm illumination with inverted-stage epifluorescence microscope (Nikon Inc., Tokyo, Japan). Cells were fixed using 2% Paraformaldehyde in 1X PBS and incubated for 30 minutes at room temperature. Following fixation, cells were washed three times with 2mLs of 1X PBS to each well. Finally, cells were leave in 1X PBS, 10% glycerol + 3 μM 4',6-diamidino-2-phenylindole (DAPI). Each well was imaged at the respective wavelengths using a Nikon TE2000 epi-fluorecent scope with NIS-Elements software.

2.4 DNA Sequencing Analysis

In collaboration with Dr. Stephen Spatz (USDA-SEPRL) we have determined the full genome sequence of CVI988-699-2 using Ilumina 454 pyrosequencing with an average coverage of ~5,000 reads per nucleotide. The genome sequence of CVI988-699-2 RV was determined using the Pacific Biosciences (Pac-Bio) polymerase-based

method, which yields up to 100 independent reads per nucleotide at the University of Delaware. In addition, Dr. Stephen Spatz also determined the DNA sequence of CVI988-699-2 RV via 454 pyrosequencing.

To analyze genetic differences between CVI988-699-2 and CVI988-699-2 RV primers were designed to further confirm each change noted. Targeted PCR amplification was carried out at the Sequencing and Genotyping Center of the Delaware Biotechnology Institute (DBI) with the correspondent oligonucleotide pairs. DNA sequences were aligned and analyzed with MAFFT software (http://mafft.cbrc.jp/alignment/server/) and compare to other herpesvirus or cellular proteins via the BLAST tools at GenBank (NCBI, http://www.ncbi.nlm.nih.gov) and the Viral Pathogen Resource Center (ViPRC, http://www.viprbrc.org).

2.5 MDV Growth Curves

Growth curves of CVI988-699-2 and CVI988-699-2 RV were performed in cell culture. Secondary CEF (2.0 x 10^6 cells/dish) were plated in (48) 60 mm tissue culture dishes and inoculated with CVI988-699-2 and CVI988-699-2-RV at approximately 200 plaque-forming units (PFU) per dish. At days 1, 2, 3, 5 and 7 post-infection, duplicate 60 mm dishes were harvested and titrated on fresh CEF at three serial dilutions. The day 0 time point was included in the initial plating and incubated for 6 days prior to counting viral plaques. At days 1 and 2, titration dishes were plated as 1:10, 1:100 and 1:1,000 in duplicate, whereas for days 3, 5 and 7, titration dishes were plated as 1:100, 1:1,000 and 1:10,000 dilutions. Titration dishes were similarly incubated at both 37°C and 41°C. All titration dishes were counted at 6 days p.i and the mean plaque number per time point was calculated (± SD).

2.6 Plaque Area Analysis

For plaque area determinations, we used CVI988-699-2 and CVI988-699-2 RV-infected 60-mm dishes from the growth curve titration dishes. Titration monolayers were fixed with cold 95% ethanol (ETOH), and rehydrated with 0.45 μM-filtered 1X PBS, pH 7.4. Monolayers were then stained using the T65 monoclonal antibody (anti-pp38 of CVI988), as the primary antibody at 1:1000 dilution and goat anti-mouse FITC (Sigma Chemical Co.) as secondary antibody at 1:100 dilution. Dishes were stained for 2 hours with primary antibody, washed three times with 5 mls wash buffer (1X PBS, pH 7.4, 1% BSA, 1% FBS, 0.1% sodium azide) and incubated for 1 hour in secondary antibody. Finally, dishes were washed three times with wash buffer and 5 ml of 1X PBS was added to each dish. Virus plaques were examined using a Nikon TE2000 epi-fluorescence inverted-stage microscope (Nikon Inc., Tokyo, Japan), fitted with a filter for observing FITC fluorescence. For both viruses, plaque areas of 250 randomly-selected plaques were determined using NIS-Elements Imaging software (Nikon). Statistical analyses of plaque sizes were performed using an unpaired Student's t-test.

2.7 Statistical Analysis

The statistical significance of differences observed in growth curves, and plaque areas data was assessed using paired Student's t-test. The differences were considered significant at the level of $p < 0.05$.

Chapter 3

RESULTS

3.1 Deletion of gC

To decrease the virulence observed over the course of the back-passage of the CVI988-699-2, we attempted to delete the gC gene from this infectious clone of MDV via Red-mediated mutagenesis (64). After electroporation into competent cells and selection for Chloramphenicol and Kanamycin resistance, several colonies were obtained after 24 hours post-plating. In order to determine if the recombination was site-specific, the DNA of several clones was isolated and digested with *BamH* I to assess whether the entire BAC sequence was present and further characterized by Southern blotting to determine if the Kanr cassette was inserted at the gC locus. After screening numerous clones, we were not able to identify clones that had inserted the Kanr cassette at the deleted gC locus within the MDV genome. In follow-up work to this, Dr. Benedikt Kaufer's laboratory continues to work on this project.

3.2 Subcloning of the US6 (gD) Promoter.

We generated two clones of gD promoter-eYFP constructs to determine if the MDV gD promoter was active in cell culture with or without co-expression of MDV genes. Transfection of CEF, and Kerotinocytes cells was performed with each clone. We used the peCFP-N1 plasmid as transfection efficiency control (CMV driven

expression of eCFP), and US6 (gD) promoter-eYFP reporter plasmids with and without co-transfection of CVI988-699-2 (MDV) of each cell type.

In HTC cells, the CMV promoter was active for eCFP, eYFP and Meq-eYFP (fusion of eYFP to the C-terminus of the Meq oncoprotein) (Figure 2). We did observe expression of eYFP driven by the gD promoter in these cells, but the expression level was lower than that of the CMV promoter (Figure 2, row 2).

However, we did not find US6 (gD) promoter to be active in any of the other cell types which suggests that the gD promoter is only active *in vitro* in the HTC cell line, albeit somewhat decreased compared to the CMV promoter and despite previous published findings suggesting kerotinocyte-specific expression (58, 62). The identification of US6 (gD)-promoter activity in HTC (macrophage) cells suggests that it would be unsuitable as a kerotinocyte-specific promoter for immune modulatory proteins. Moreover, we did not observed expression from the gD promoter in keratinocytes (Figure 3B).

3.3 UL47 and Ker-5 Promoters

We were able to generated eYFP, eRFP, and eCFP expression vectors driven by both UL47 and Ker-5promoters. Expression from these constructs was compared in different cell types (CEF, HTC, LMH, and keratinocytes), all of chicken origin. The different cell types allow us to study their tissue-specific expression. We detected slight expression of RFP with the UL47 promoter in HTC cells, and CFP with UL47 promoter in LMH cells (Figure 4 and 5). However, we did not observe expression of UL47 promoter -eYFP and -eCFP in CEF and HTC cells. Interestingly, there was no detectable expression of the UL47 promoter with fluorescent proteins in keratinocytes

cells despite previous reports which indicated that UL47 is present mainly in infected FFE cells (22) , as shown in Figure 6.

Weak expression of vector of Ker-5-eCFP was observed in CEF, HTC, and LMH cell lines. We did not observe any Ker-5-mRFP and Ker-5-eYFP expression in the keratinocytes cells, since they are from the same origin (Figure 7 and 8).

3.4 Biological Characterization

To determine whether *in vitro* growth of CVI988-699-2RV was comparable to CVI988-699-2 virus, plaque sizes and virus growth curves at 37°C and 41°C were preformed. As shown in Figure 9, both viruses, CVI988-699-2 and CVI988-699-2RV exhibited growth characteristics that were essentially identical. These growth curves indicated that the genetic changes did not affect the ability of the replicate to grow in CEF at either temperature.

To further compare the replication of these viruses in cell culture, we measured the plaque areas of both CVI988-699-2 and CVI988-699-2 RV viruses at 6 days post-infection. We randomly-selected 250 plaques for each virus and measured their area by auto-tracing the plaques using NIS-Elements imaging software, and means plaque area was determined for each virus. The mean values of plaque areas were 36,945 sq μm and 46,306 sq μm of CVI988-699-2 and CVI988-699-2RV, respectively. These data demonstrated that CVI988-699-2RV induced slightly larger plaques in cell culture than did the CVI988-699-2 virus (t test: 1.03×10^{-30}; p value < 0.0001), despite identical replication curves (Figure 10).

3.5 DNA Sequencing

To identify the genetic changes of the original CVI988-699-2, and CVI988-699-2 RV, that could be associated with the observed reversion to virulence, whole genomes were sequenced by 454 pyrosequencing in collaboration with Dr. Stephen Spatz (USDA-SEPRL). Initially, the genome sequence of CVI988-699-2-RV was determined using the Pacific Biosciences (Pac-Bio) polymerase-based method. This sequencing showed 51 changes in CVI988-699-2 RV (Table 3). Targeted PCR amplification followed by Sanger sequencing at the UD sequencing center was also preformed to confirm these genetic changes; however, these genetic changes were not confirmed, indicating that the Pac-Bio sequence had produced false-positive mutations.

After CVI988-699-2 RV DNA was sequenced by using 454 pyrosequencing, both DNA sequences, CVI988-699-2 and CVI988-699-2 RV, were analyzed using MAFFT software and other web-based tools. The results of the whole genome sequencing of both CVI988-699-2, and CVI988-699-2 RV, revealed that there were only five nucleotide changes between the genome sequences (Table 4). The complete nucleotide consensus sequences of the CVI988-699-2 and CVI988-699-2RV genome are 182,025 bp. The unique long (U_L) regions are 113,160 bp in length from position 12,929 to 126,094, and the unique short (U_S) regions are 18,382 bp from position 151,464 to 169,844. The terminal long repeat (TR_L) and internal long repeat (IR_L) are 12,908 bp in length from positions 21 to 12,929 and from 126,095 to 12,908 respectively. The terminal short repeat (TR_S) and internal short repeat (IR_S) are 11,796 bp in length from positions 169,717 to 181,515 and from 139,537 to 151,332, respectively.

The changes in the viral coding sequence were non-synonymous mutations in MDV049 encoding the UL36 gene and MDV080, encoding an arginine-rich ORF on one strand and the virus-encoded telomerase RNA (vTR) on the opposite strand. These are encoded in the TRL and IRL and therefore represent two of the mutations.

The mutation observed in MDV049 (UL36, the Major Tegument Protein) is a thymidine to adenine transversion that causes a non-synonymous mutation of Threonine at 78,785 in the ORF of CVI988-699-2, to serine in CVI988-699-2 RV. Although this causes a mutation in the coding sequence, the substitution is a conservative one (thr→ser).

In addition to this point mutation, we have found that a discrepancy between our Sanger sequencing and both the Pac-Bio and Ilumina 454 sequences of the UL36 gene, in a region known as a hyper-variable region (HVR). By PCR amplification, we have found that a segment reported as being 924 nt in both the CVI988-699-2 and 699-2-RV sequences, and 1257 nt in the Pac-Bio sequence (data now shown). This discrepancy stems from a GC-rich repeat sequence within this region that is repeated a number of times. This sequence is in-frame, however in each of these sequences with varying number of repeats of the amino acid sequence: KPTPAPKPPPASKPKPPPDPDF (22 aa, 66 nt reiterations). The issue of this polymorphism, the actual number within the CVI988-699-2 and CVI988-699-2 RV requires additional work to be resolved.

In the MDV001/080 (vTR) gene that is localized to the repeats flanking the unique-long region (TRL/IRL), there is an adenine to guanine transition resulting in a coding change of leucine at position 509/138,515 of CVI988-699-2 to proline in CVI988-699-2 RV in the MDV001/080 coding sequence. This mutation was observed

as a concomitant thymidine to cytidine transition mutation in MDV001. The non-synonymous mutation is located within the CR2 domain of vTR, one of the conserved regions of this telomere. According to the proposed secondary structure of vTR, the mutation in CVI988-699-2 RV would destabilize the structure of the vTR according with previous published findings (15).

A synonymous mutation was found in the MDV031 (UL19) gene, in which thymidine is changed for cytidine at position 44,716 of CVI988-699-2, resulting in a substitution for proline in CVI988-699-2 RV. Lastly there is one single-nucleotide polymorphism (SNP) between MDV047 (UL34 homolog) and MDV048 (Capsid protein VP26 homolog) genes, close to coding sequence for UL34, where cytidine is changed to thymidine; however it is an intergenic mutation (Table 4).

3.6 Comparison of CVI988-699-2 and -699-2 RV to Other CVI988 Sequences

In addition to a comparison of CVI988-699-2 to CVI988-699-2 RV sequences, we compared these two sequence to other reported CVI988 sequences: CVI988 (Intervet) internal repeat long region [GenBank: DQ534538.1], and CVI988-BP5 internal repeat long region [GenBank: DQ534536.1] (Table 5). This was performed to perhaps associate the SNPs identified with a change in virulence as the reported CVI988 sequences were for attenuated versions of CVI988. An examination of these sequences revealed several SNPs, insertions and deletions (INDELs) between the sequences.

CVI988-699-2, CVI988-699-2 RV and CVI988-BP5 have single nucleotide substitutions in different positions. There is an adenine in CVI988-699-2, CVI988-699-2 RV and CVI988-BP5 except for a [1]CVI988 (Intervet) that has a guanidine in at

the MDV075 gene at positions 127,471 and 127,910 of IRL in CVI988-699-2. CVI988-699-2, and CVI988-699-2 RV have single nucleotide substitutions of guanidine instead of adenine in the MDV075 gene at position 129,310 of CVI988-699-2 in comparison with CVI988 (Intervet) and CVI988-BP5. MDV075 encode R-LORF10 which is involved in MDV virulence and interact with MHC class II cell surface expression (30).

Other substitutions are a thymidine in CVI988-699-2, CVI988-699-2 RV and CVI988-BP5 instead of a cytidine in CVI988 (Intervet) at the position 126,127 of MDV073 in CVI988-699-2. The phosphoprotein pp38 is encoded by MDV073 and it is expressed during lytic replication and associated with the tumor induction (56, 67).

A triplet deletion of (CCA, encoding proline) in the MDV076 (*meq*) gene at position 133,929 of CVI988-699-2, CVI988-699-2 RV and CVI988-BP5 was identified. Meq is the main oncoprotein of MDV, and changes in its sequence have been associated with changes in MDV pathogenicity (31).

Another SNP is a cytidine in CVI988 (Intervet) at position 136,993 and an adenine at position 138,098 in the MDV078 of CVI988-699-2, while CVI988-699-2, CVI988-699-2 RV and CVI988-BP5 have a thymidine and guanidine, respectively. MDV078 gene encodes vIL8 which it has previously been reported to be a virulence factor for MDV (43).

Finally CVI988-699-2 RV has a single nucleotide substitution of guanidine instead of adenine at position 138,515 in CVI988-699-2 of the MDV001/080 genes, as noted above.

3.6.1 Genes associated with reversion to virulence

In this study, we identify two genes MDV049 (UL36) and MDV080 (vTR) that showed the non-synonymous mutations, and both genes could be associated with reversion to virulence of CVI988-699-2 RV. To confirm the observed mutations in the sequences of the MDV049 (UL36) and MDV001/MDV080 (vTR) genes of CVI988-699-2 and CVI988-699-2-RV, we have amplified these sequences via PCR using the primers shown in Table 6.

Sequencing of MDV049 (UL36) gene from CVI988-699-2 and CVI988-699-2 RV provide evidence that there is thymidine to adenine that not only caused a mutation in the coding sequence, but also there is a discrepancy in the sequences of UL36. This polymorphism seems to be related to GC-rich repeat sequence within the region and the repeated amino acid sequence (KPTPAPKPPPASKPKPPPDPDF).

As mentioned above, a SNP was also noted in MDV080 (vTR) gene. However, the outcome of sequencing MDV001/MDV080 genes does not have the predicted mutation, adenine is present in MDV080.

Chapter 4

DISCUSSION

Marek's disease is a serious poultry disease despite its control through vaccination, subtle effects of immune suppression and continued virus shedding of field strains continue to drive the evolution of MDV virulence. The CVI988 vaccine is widely used and is considered to be the most protective vaccine, however vaccine preparations of cell culture-derived vaccines stocks represents populations of virus that can vary considerably in their efficacy. The generation of a molecularly-cloned CVI988 of low passage provides a more defined vaccine population and its propagation as a BAC, allows for a constant, stable source of vaccine stocks.

The initial goal of this research was to diminish the pathogenicity of our molecularly-cloned CVI988 (CVI988-699-2) and to generate a non-spreading vaccine for blocking horizontal transmission of field strains, *in trans*. As we were unable to do this, our secondary goals were to generate skin (keratinocyte)-specific expression vectors for delivery of immune regulatory molecules to the feather follicle epithelium (FFE), potentially eliciting sterilizing protection from field strains by blocking their horizontal transmission. We also sought to identify specific mutations that are associated with the reversion to virulence of CVI988-699-2 when it had been back-passaged in chickens or administered at a high dosage.

Our research, in collaboration with Dr. Stephen Spatz, has shown that only five genetic changes were present between the sequences of CVI988-699-2 and CVI988-

699-2-RV. Furthermore, only two non-synonymous mutations in MDV049 and MDV080/MDV001 (vTR) genes were observed in the actual coding sequences of MDV genes. This work therefore provides the bases for follow-up to determine if these changes are truly responsible for the observed reversion to virulence of CVI988-699-2-RV.

4.1 Generation of Keratinocyte-specific Promoters

Our other goal was to generate vectors for incorporation into CVI988-699-2 that would express immune regulatory genes at the skin of vaccinated chickens and generate an immune response capable of blocking the shed of field strains. The expression of gD and UL47 in the feather follicle epithelium cells in infected chickens was previously correlated with cell-free virus production in the skin of MDV-infected chickens (1, 22, 62). We constructed fluorescent reporter genes (eCFP, eYFP and mRFP) under the control of MDV glycoprotein D (gD), UL47 and chicken cellular Ker-5 promoters. Expression of these vectors was assessed in different cell types (CEF, HTC, LMH and primary keratinocytes) to provide insight into whether their expression would be limited to keratinocytes.

Although we were able to generate these expression vectors, we did not observe expression from the gD, Ker-5 and UL47 promoters in chicken keratinocytes. A problem with our system may have been limited transfection efficiency into the keratinocytes, although in these cultures, we were able to observe expression of the control plasmid (eCFP driven by the CMV promoter) in a few cells. As keratinocyte

cultures are very different from actual skin, and the expression of these promoters in cell culture may be different from the expression seen in FFE cell in chickens.

Whereas the activity of gD, Ker-5 and UL47 promoters are well related to the horizontal transmission of MDV, their lack of activity in keratinocytes cell line is suggests that they would be unsuitable as a kerotinocyte-specific promoter for immune modulatory proteins. This, however, is of considerable importance, as tissue-specific promoters are becoming increasingly important in their effects on gene expression in using genes to treat or prevent disease.

Both the *in vivo* and cell culture studies suggest that tissue-specific promoters can regulate expression differentially in different cell types. *In vivo*, feather follicle epithelial cells (FFE) display expression of gD and UL47 genes with their respective promoters during MDV infection. In cell culture, this observation did not associate with the expression of the fluorescent protein in keratinocytes, and lack of expression in CEF, HTC and LMH cells. In contrast, the CMV promoter of the eCFP-N1 vector, the transfection efficiency control was expressed in all cell types examined. These observations suggest these promoter selection may be unsuitable in regulating immune regulatory genes in FFE cells.

4.2 Comparative Analysis CVI988-699-2 and CVI988-699-2-RV

Since the original CVI988-699-2 was involved in procedures from cells to bacteria, again back to cell to chickens, and recover as a CVI988-699-2-RV that could potentially result in some changes to the viral genome. In this study we compared these two strains, CVI988-699-2 and CVI988-699-2-RV *in vitro*. Our results

demonstrated that there is no a significant difference in their replication. The plaque area measurement and growth curve results reveal that any mutation or change in the sequence of CVI988-699-2 and CVI988-699-2-RV genome has a slight influence on virus cytopathic effect but did not have significant effects on virus grow *in vitro*.

Based on the sequence analysis of CVI988-699-2 and CVI988-699-2 RV sequences, only four genetic changes were identified. These mutations were identified within the genes encoding UL19, UL34 and UL35, UL36 and MDV001/080 genes as single nucleotide polymorphisms (SNP). The SNPs in vTR and UL36 (ubiquitin-specific protease) appeared to be relevant with reversion to virulence, however, we have yet to confirm the mutation in the MDV001/MDV080/vTR gene, although we have had some difficulty in amplifying this region for sequencing.

The mutation and discrepancy found in the sequence of UL36 in CVI988-699-2 and CVI988-699-2 RV could also contribute to the reversion of virulence. However, it is difficult to estimate how the role of a SNP may have in reactivation of pathogenicity without a more complete genomic approach to describe gene and protein functions and interactions. Since UL36 is not only one of the tegument proteins that mediate egress of infectious virus and, but also it has a viral ubiquitin-specific protease (USP) that modify host cell proteins (53), it seems possible that this mutation could affect ability of the virus to affect cellular immune sensing upon virus entry. There is also the issue of the hypervariable region repeats (see above) that requires additional work for resolution.

Since the UL36 mutation found in CVI988-699-2 and CVI988-699-2 RV is not localized by the N-terminal region of UL36 or by the USP-Cystine active site, we cannot assume it would affect the USP activity of this strain directly. Though Veiga *et*

all., reported that the nucleotide USP sequence of MDV is not important for virus replication *in vitro* after replace it with a synthetic USP (69).

However, UL36 has not only viral ubiquitin-specific protease (USP) activity, but also it has a role tegument structure. During the early infection UL36 dissolves from incoming virus capsid to activate transcription, and deubiquitinate host cell proteins (ostensibly those involved in innate sensing), and during late in infection, UL36 plays a role in the acquisition of the envelop structure to support infectious virus production. It seems possible that this mutation could affect the ability of UL36 to block innate sensing by altering the substrate specificity of its USP (DUB) activity.

The mutation in the MDV001/MDV080/vTR genes is interesting since it is localized in the CR 2 domain (Figure 11). CR2 domain makes part of the pseudoknot domain, a conserved region. The pseudoknot domain contains the site for the TR dimerization and interaction with TERT. The base-paired interaction between CR3 region of a TR molecule to a P3 Helix generated from CR2 region of another TR molecule generates the dimerization interface. Hence, sequences of CR2 and CR3 regions and their secondary structure are important to the purpose of the pseudoknot domain (15). Previously, it has been reported that CR2 region differ in one nucleotide deletion between vTR and chTR and that may contribute to efficiency of vTR activity. Fragnet et all. (15), generated a mutated RB1B-vTR clone consisting in the introduction of a uracil within the CR2 region and compared with the wild type vTR and chTR. This mutation appears to decrease vTR activity at the same level of chTR level indicating that a mutation within CR2 region would destabilize the functional role of the vTR pseudoknot (15).

However, we found that the predicted mutation does not have an adenine in MDV080 of CVI988-699-2 RV. Even though this result requires further confirmation, it would also suggest that CVI988-699-2 had the mutation on the vTR which perhaps decreased its virulence allowing to serve as an apathogenic vaccine. Studies on vTR activity and its efficiency have shown that a mutation in this gene would affect vTR activity. In fact, a deletion of the vTR gene from RB1B strain showed that virus was able to replicate in vivo but this mutation reduced tumors incidence (66). Other in vivo experiment showed that substitution of a single nucleotide in the H-box region of MDV-Rispens strain generated a loss of telomerase activity of vTR (15, 66), and that a mutation in template sequence of RB1B, CR1 domain, decreased tumor formation in infected chickens (15). Moreover, an additional mutation in the secondary structure at CR4-CR5 domain inhibited interaction of vTR with TERT and allowed lymphomas development (28). Therefore, when the CVI-988-699-2Δ was tested for safety by back passage, CR2 sequence may be able to better associate with host telomerase and CVI988-699-2 RV was able to restore telomerase activity and incrementally increase the virulence of this virus.

4.3 Conclusion

Overall, our analysis of these viruses provides clear testable hypotheses for assessing the contribution of genetic changes to the acquisition of virulence. First, the non-synonymous mutation found in the vTR suggest that CR2 sequence from CVI-988-699-2Δ may be able to better associate with host telomerase when this virus was tested for safety by back passage. As a result, CVI988-699-2 RV may have restored telomerase activity and incrementally increase the virulence of this virus. Second, the

non-synonymous mutation in the hyper-variable region of UL36 and the discrepancy found between our Sanger sequencing and both the Pac-Bio and Ilumina 454 sequences of the UL36 gene raises questions about whether this was a sequence assembly error, and why these mutations generate contiguous protein coding sequence?

In the follow-up to this work, characterizing these two viruses *in vivo*, as well as the generation of revertants for each of these loci to assess which, if any, directly increases the virulence of CVI988-699-2 RV.

TABLES

Table 1. Primer Sequences for UL44 (Glycoprotein C gene) Deletion

Primer	Direction	Sequence (5' – 3')
Kan^r	Forward	AAGAGACACcaaacgtaaccctctacatatcttccctcTAATCTCATTGTTA TGTAGTTGTGAAGGATGACGACGATAAGTAGGG
Kan^r	Reverse	AATATGTTTAATaaatcacaactacataacaatgagatTAGAGGGAAGAT ATGTAGAGGGTTACCAACCAATTAACCAATTCTGATTAG

Table 2. PCR primers for UL47 and Ker-5 promoters

Primer	Direction	Sequence (5' – 3')
UL47	Forward (*AseI*)	*ATTAAT*AGGAGGTATTTGTCTATTAG
UL47	Reverse (*NheI*)	*GCTAGC*TCTTCGGTGGAATGTGCTAC
Ker-5	Forward (*AseI*)	*ATTAAT*ATGCTCCAATGGGTGAGCTTGGCACA
Ker-5	Reverse (*NheI*)	*GCTAGC*GGCTCGTGCAGTTGGAAACGCAAAGA

Table 3. Genetic changes by using the Pacific Biosciences (Pac-Bio) polymerase-based method

Mutation No.	Position CVI988-699-2	Type	Variant	Gene affected
1	509	Substitution	T>C	MDV080
2	3041	Insertion	AA	R-LORF4
3	4017	Insertion	AAA	unknown small ORF
4	6750	Deletion	G	Intergenic
5	6752	Insertion	GG	Intergenic
6	6769	Deletion	G	Intergenic
7	9714	Substitution	C>T	MDV006 (lytic protein A)
8	9715	Insertion	C	MDV006 (lytic protein A)
9	9949	Insertion	T	MDV006 (lytic protein A)
10	10267	Insertion	A	MDV006 (lytic protein A)
11	11000	Insertion	T	MDV006 (lytic protein A)
12	14190	Insertion	T	vLIP (viral lipase)
13	44716	Substitution	T>C	MDV031- UL19
14	76828	substitution	C>T	MDV047 - UL34
15	78768	Insertion	*1	MDV049 - UL36
16	128014	Insertion	A	MDV075 ILR
17	128748	Insertion	T	MDV075
18	129064	Insertion	A	MDV075.5
19	129309	Substitution	AG>GA	MDV075.5
20	132255	Substitution	C>T	Intergenic ILR
21	132274	Deletion	C	Intergenic ILR

***1)**GGCTTGGGGGCCGGAGAGGGCTTGGGGGCCGGAGAGGGCTTGGGAGCCGGAGAGGGC
TTGAAATCGGGATCCGGCGGAGGTTTGGGCTTGGGGGCCGGAGAGGGCTTGGGGGCCGGA
GAGGGCTTGGGGGCCGGAGAGGGCTTGGGGGCCGGAGAGGGCTTGGGGGCCGGAGAGGG
CTTGGGGGCCGGAGAGGGCTTGGGGGCCGGAGAGGGCTTGGGGGCCGGAGAGGGCTTGG
GGGCCGGAGAGGGCTTGAAATCGGGATCCGGCGGAGGTTTGGGCTTGGGGGCCGGAGTGG
GCTTGAAATCGGGATCCGGCGGAGGTTTGGGCTTGGAGGCCGGAGGA

Mutation No.	Position CVI988-699-2	Type	Variant	Gene affected
22	133093	Insertion	G	Intergenic ILR
23	133975	Deletion	C	MDV076 (Meq)
24	134996	Insertion	TT	Intergenic ILR
25	135973	Insertion	T	MDV078.2 ILR
26	138405	Insertion	T	MDV080 ILR
27	138515	Substitution	A>G	MDV080 ILR
28	139301	Insertion	G	Intergenic IRS
29	139360	Insertion	*2	Repeats flanking IRL and IRS
30	139388	Insertion	*3	Repeats flanking IRL and IRS
31	139390	Insertion	CC	Repeats flanking IRL and IRS
32	139537	Insertion	*4	MDV081 IRS
33	139728	Insertion	*5	MDV081 IRS
34	139733	Insertion	*6	MDV081 IRS
35	142640	Substitution	C>G	MDV084 (ICP4) IRS
36	149305	Insertion	C	MDV085 - MDV084.5 IRS
37	149813	Insertion	CAA	MDV085.3
38	149815	Substitution	C>A	MDV085.3
39	149834	Deletion	A	MDV085.3

***2)**TAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGG
CCTAGGGTTAGGGTTAG

***3)**GCCTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTA
GGGTTAGGGTTAGGGT

***4)**TAGAGGGCGCGTGCGCAGTCGGAGTTTTTATTTTGATGCTCATGTACGGTGCGCAGTCG
GAGTTTTCCTATTTTCGGCCCCGCGCATGCGCGGTCATGTAGAGGGCGCGTGCGCAGTCGG
AGTTTTCCTATTTTCGGCCCCGCGCATGCGCGGTCATGTAGAGGGCGCGTGCGCAGTCGGA
GTTTCTATTTCGGGCGCATGCGCGGTCATGTAGAGGGCGCGTGCGCAGTCGGAGTTTTCCT
ATTTTCGGCCCCGCGCATGCGCGGTCATGTAGAGGGCGCGTGCGCAGTCGGAGTCTATTCG
GGCGCGCGCGTCATG

***5)** TGCGCAGTCGGAGTTTTCCTATCGGCATGGCGGCGTCGCGTGCGCAGTCGGAGTT

***6)** ATTTTCGGCCCCGCGCATGCGCGGTCATGTAGAGGGCGCGCAGTATTTCCCATC

Mutation No.	Position CVI988-699-2	Type	Variant	Gene affected
40	150304	Insertion	AA	MDV086 (Cytoplasmic protein)
41	151074	Insertion	A	MDV086.2
42	151110	Deletion	C	MDV086.2
43	151125	Insertion	CA	MDV086.2
44	169923	Insertion	TG	MDV097.3 / MDV097.6
45	169939	Deletion	G	MDV097.3 / MDV097.6
46	169967	Insertion	T	MDV097.3 / MDV097.6
47	170735	Insertion	T	MDV098 TSR
48	171214	Deletion	T	MDV098.9
49	171234	Substitution	G>T	MDV098.9
50	171235	Insertion	TG	MDV098.9
51	175023	Insertion	C	MDV100 (ICP4)

Table 4. Genetic changes by 454 pyrosequencing

No.	Position CVI988-699-2	Location	Type	Variant	Gene affected
1	509	MDV001/ MDV080	Substitution	T > C	Virus-encoding telomerase (vTR)
2	44716	MDV031	Substitution	T > C	UL19, major capsid protein
3	76828	MDV047	Substitution	C > T	UL34 and UL35
4	78785	MDV049	Substitution	T > A	UL36, major tegument protein

Table 5. Comparison of the ILR region of CVI988-699-2, CVI988-699-2, CVI988 (Intervet), and CVI988-BP5.

Type	CVI988-699-2 Position	INTERVET	BP	CVI988-699-2	CVI988-699-2 RV	Gene
SNP	126127	C	T	T	T	MDV073
SNP	126396	C	T	T	T	INTERGENIC
SNP	126413	G	A	G	G	INTERGENIC
SNP	126476	T	C	T	T	INTERGENIC
SNP	126614	C	T	T	T	INTERGENIC
INDEL	126787	T	-	T	T	MDV074
INDEL	126788	A	-	T	T	MDV074
INDEL	126789	T	-	A	A	MDV074
INDEL	126790	A	-	T	T	MDV074
INDEL	126791	T	-	A	A	MDV074
INDEL	126792	T	-	T	T	MDV074
INDEL	126793	A	-	T	T	MDV074
INDEL	126794	T	-	A	A	MDV074
INDEL	126795	A	-	T	T	MDV074
INDEL	126796	T	-	A	A	MDV074
SNP	127471	G	A	A	A	MDV075
SNP	127910	G	A	A	A	MDV075
INDEL	127911	C	C	-	-	MDV075
INDEL	128014	A	A	-	-	MDV075
INDEL	128023	A	-	A	A	MDV075
INDEL	128084	T	-	-	-	MDV075
INDEL	128084	T	-	-	-	MDV075
INDEL	128085	T	T	-	-	MDV075
INDEL	128085	T	T	-	-	MDV075
INDEL	128756	T	T	-	-	MDV075
INDEL	129074	A	A	-	-	MDV075
INDEL	129074	A	A	-	-	MDV075
INDEL	129074	A	A	-	-	MDV075
INDEL	129309	G	G	-	-	MDV075
INDEL	129310	A	A	G	G	MDV075
INDEL	132255	-	-	C	C	INTERGENIC

Type	CVI988-699-2 Position	INTERVET	BP	CVI988-699-2	CVI988-699-2 RV	Gene
SNP	132272	-	C	C	C	INTERGENIC
INDEL	132272	-	C	C	C	INTERGENIC
INDEL	132274	-	C	-	-	INTERGENIC
INDEL	132274	-	A	-	-	INTERGENIC
INDEL	132274	-	C	-	-	INTERGENIC
INDEL	132274	-	C	-	-	INTERGENIC
INDEL	132274	-	C	-	-	INTERGENIC
INDEL	132274	-	-	C	C	INTERGENIC
INDEL	133094	-	C	-	-	INTERGENIC
INDEL	133094	G	G	-	-	INTERGENIC
INDEL	133101	G	-	-	-	INTERGENIC
SNP	133554	G	A	A	A	MDV076
SNP	133929	C	-	-	-	MDV076
SNP	133929	C	-	-	-	MDV076
SNP	133929	A	-	-	-	MDV076
SNP	134756	T	C	T	T	INTERGENIC
INDEL	134918	-	-	G	G	INTERGENIC
INDEL	134997	T	T	-	-	INTERGENIC
INDEL	134997	T	T	-	-	INTERGENIC
SNP	135157	A	G	A	A	INTERGENIC
SNP	136077	C	T	C	C	MDV078.2
SNP	136401	C	T	T	T	INTERGENIC
SNP	136993	C	T	T	T	MDV078
SNP	137762	A	G	A	A	MDV078
SNP	138098	A	G	G	G	MDV078.1
INDEL	138414	T	T	-	-	MDV80
INDEL	138414	T	T	-	-	MDV80
INDEL	138414	T	T	-	-	MDV80
SNP	138515	A	A	A	G	MDV80
SNP	138635	T	C	T	T	MDV80
SNP	138992	G	X	A	A	INTERGENIC
SNP	138992	G	X	A	A	INTERGENIC

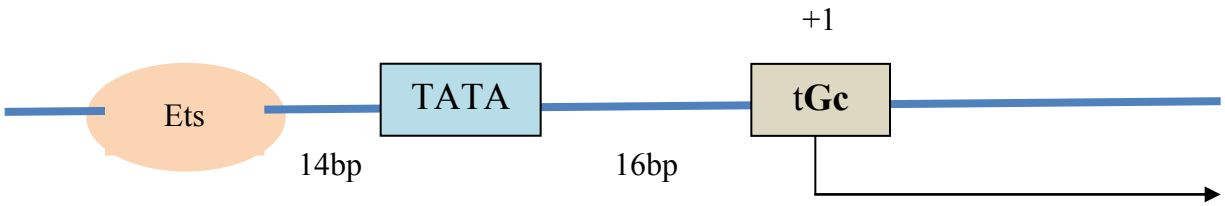
Table 6. Primers for PCR Amplification and DNA Sequencing of Mutation-containing Regions of CVI988-699-2 and CVI988-699-2 RV

Primer	Direction	Sequence (5' – 3')
UL36	Forward	GAAAGCATGTGGGATCGAGTGGT
UL36	Reverse	CTCACTATACGATTCCCTGGGAA
MDV080	Forward (*Spe*I - T7)	GGACTAGTATGGCTAGCATGACTGGTGGACAGCAAATG GGTCGGatgaatgaccgcggagttccaaact
MDV080	Reverse (HA-*Hind*III)	AAGCTTagcgtaatctggaacatcgtatgggtacatCTCACAGAGCCCCG CGCGCGGCTC

FIGURES

A. gD Promoter

B. UL47 Promoter



C. Ker-5 Promoter

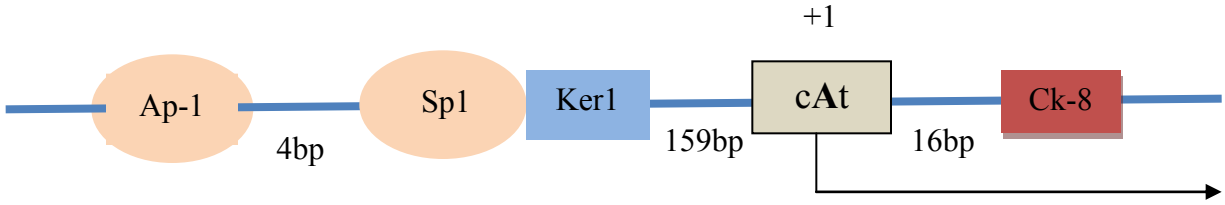


Figure 1. Schematic Diagrams of US6 (gD), UL47 and KER-5 promoters
The gD, UL47 and Ker-5 promoters are depicted showing the transcription start sites (+1) and putative transcription factor binding sites.

A.

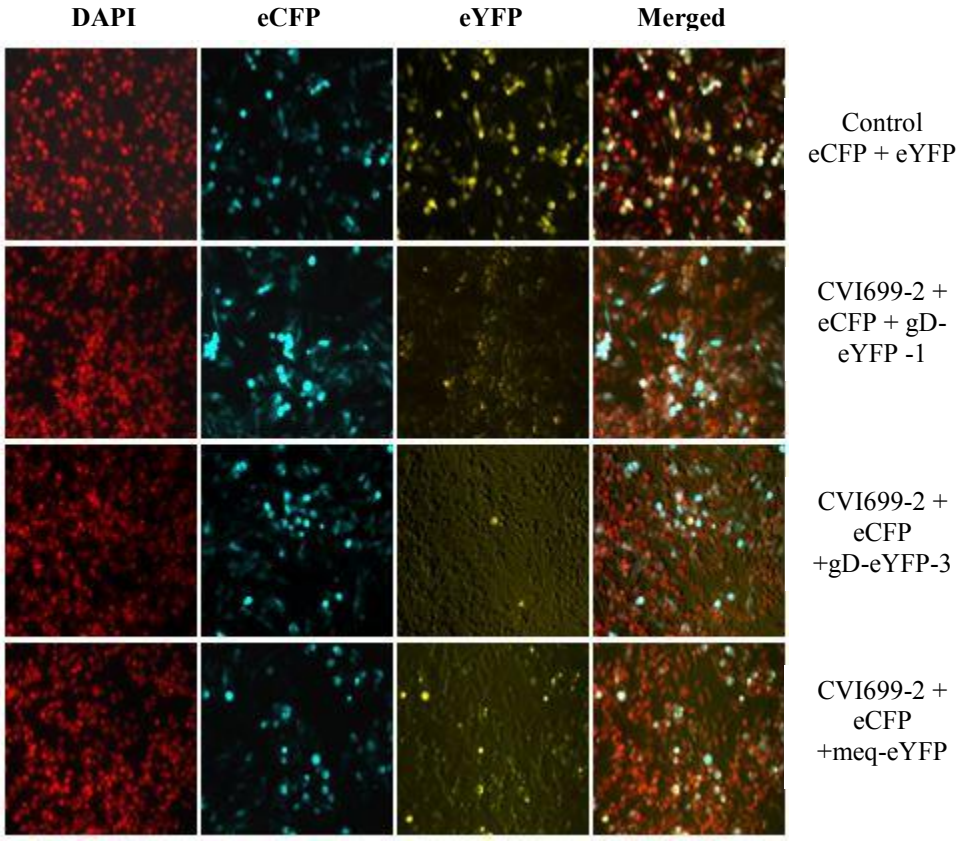
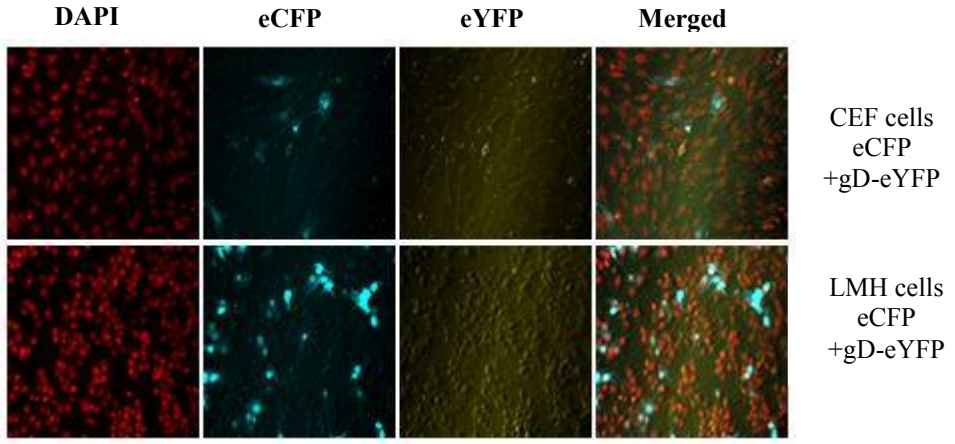


Figure 2. The gD promoter-eYFP expression in HTC cells
The gD promoter-eYFP constructs was found to be active in HTC (macrophage) cell line.

A.



B.

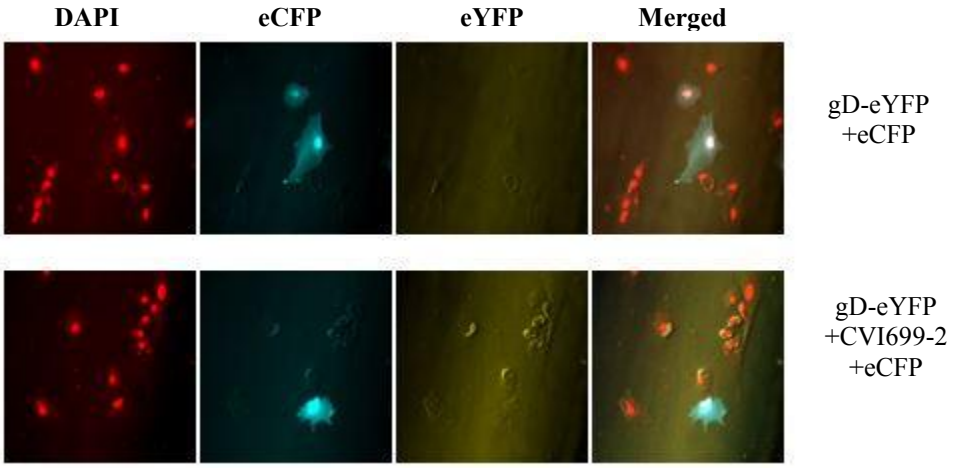
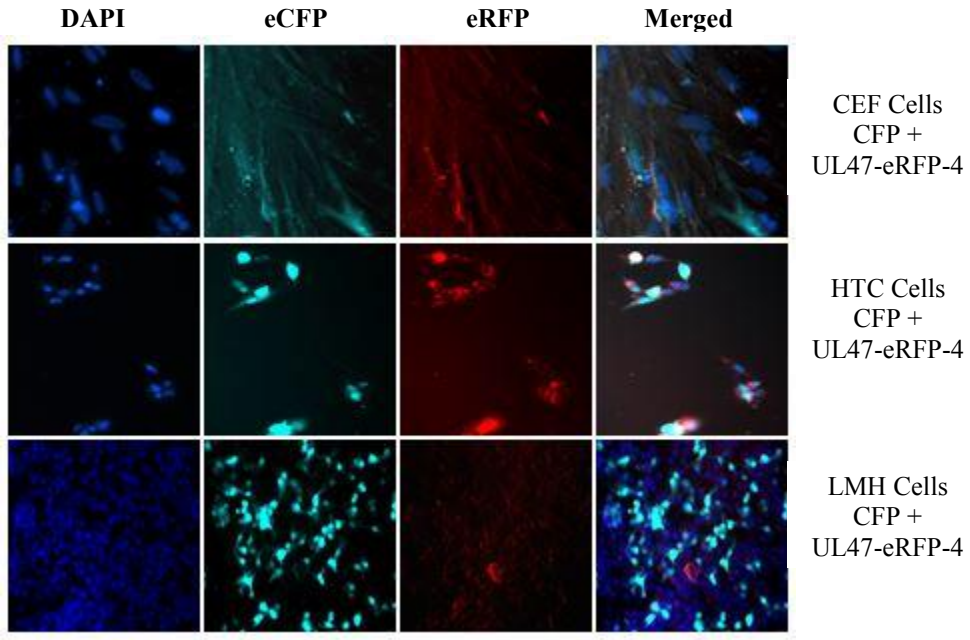


Figure 3. The gD promoter-eYFP constructs transfections.
Panel (**A**) shows transfected CEF and LMH cells, and panel (**B**) shows kerotinocyte cells expressing eCFP and gD-eYFP. The gD promoter-eYFP constructs was found to be active in macrophage cell line HTC but inactive in kerotinocyte as a specific promoter for immune modulatory proteins.

A.



B.

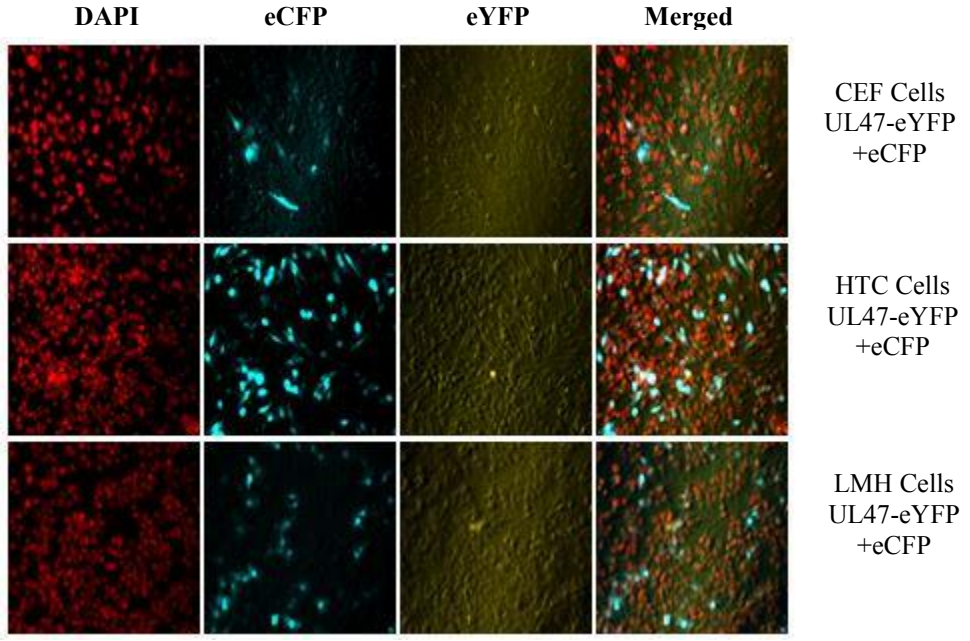


Figure 4. UL47 promoter expression in CEF, HTC and LMH cells.
Expression of (**A**) UL47-eRFP was slightly detected only in HTC cell line, and (**B**) UL47-eYFP was slightly detected only in LMH cell line.

A.

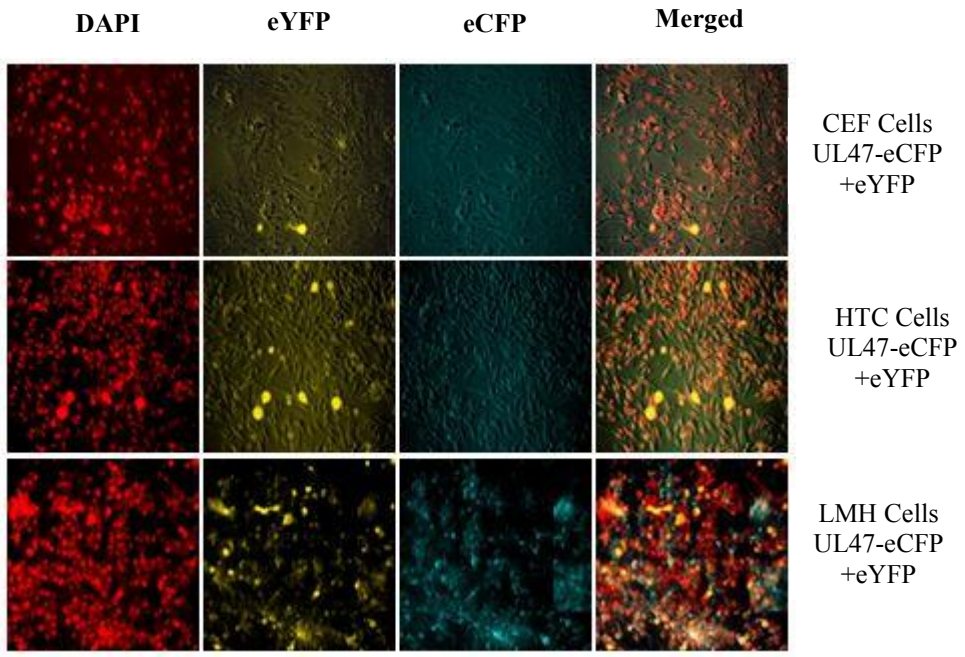
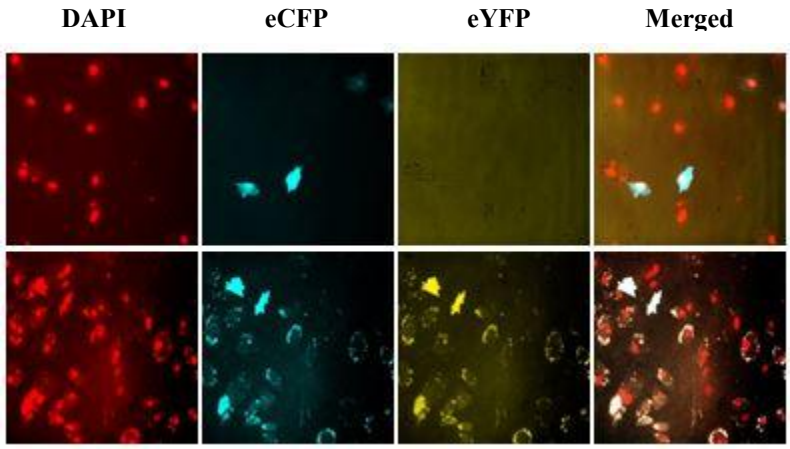


Figure 5. UL47 promoter expression in CEF, HTC and LMH cells.
Expression of UL47-eCFP was slightly detected only in LMH cell line.

A.



B.

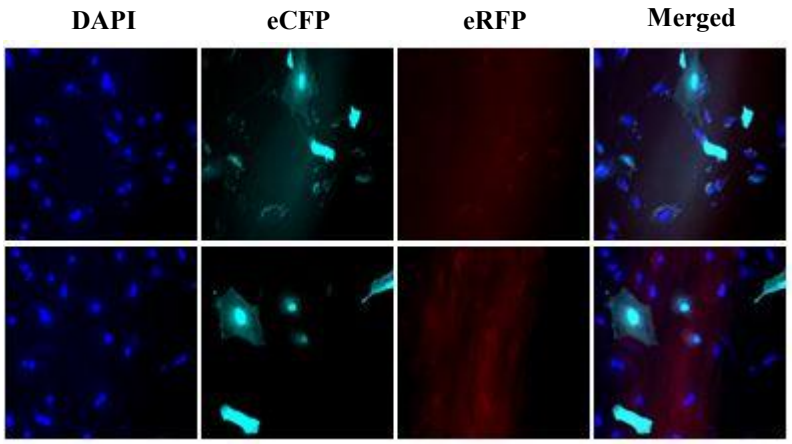


Figure 6. UL47 promoter expression in keratinocytes.
Expression of (**A**) UL47-eYFP and (**B**) UL47-eRFP was not observed in keratinocytes cells.

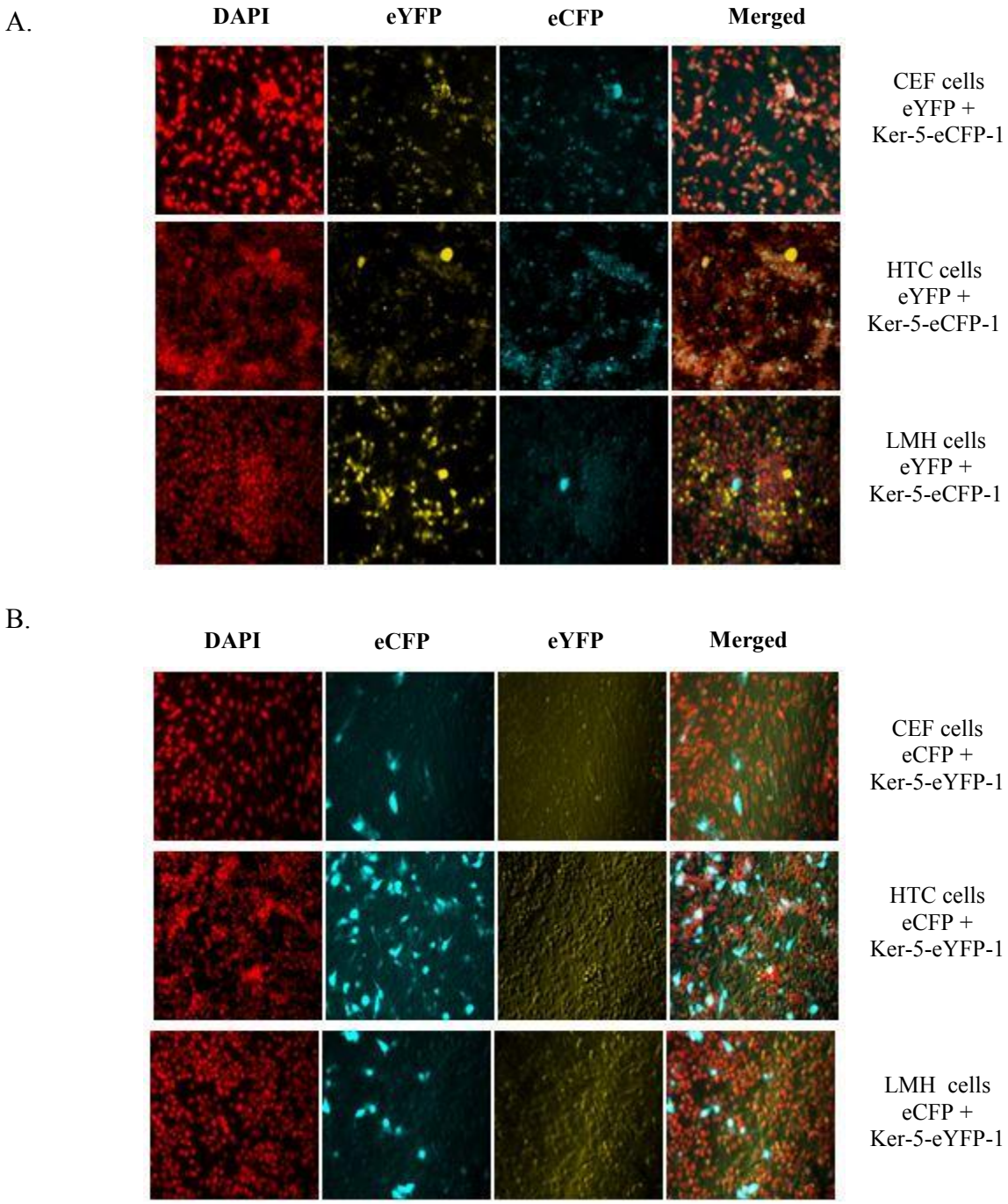
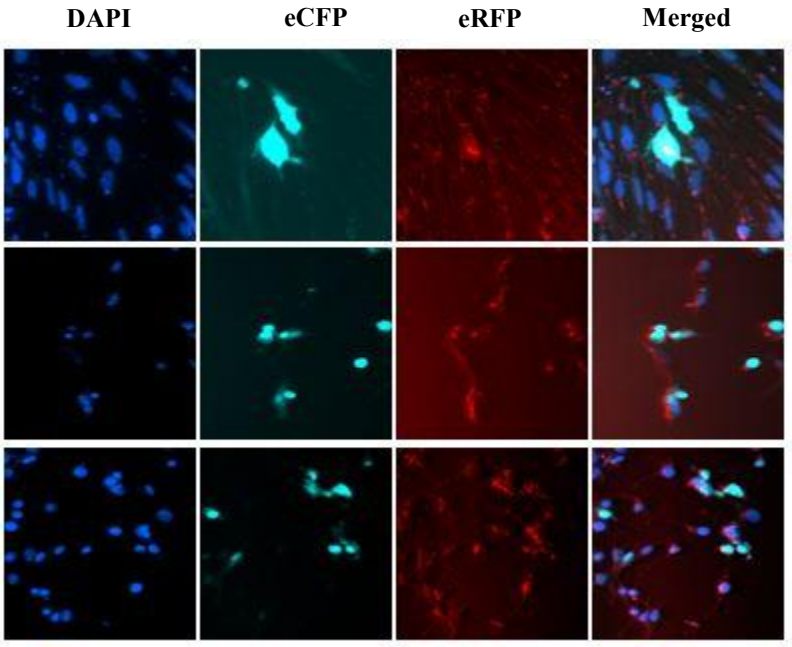


Figure 7. Ker-5 promoter expression in CEF, HTC and LMH cells
Expression of (**A**) Ker-5-eCFP was slightly detected only in HTC cell line and (**B**) Ker-5-eYFP in the cell lines was not observed.

A.



B.

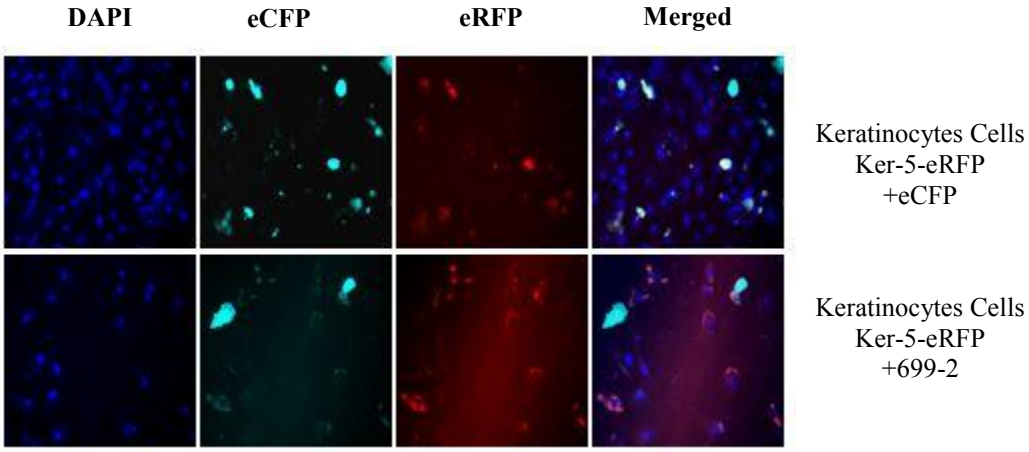
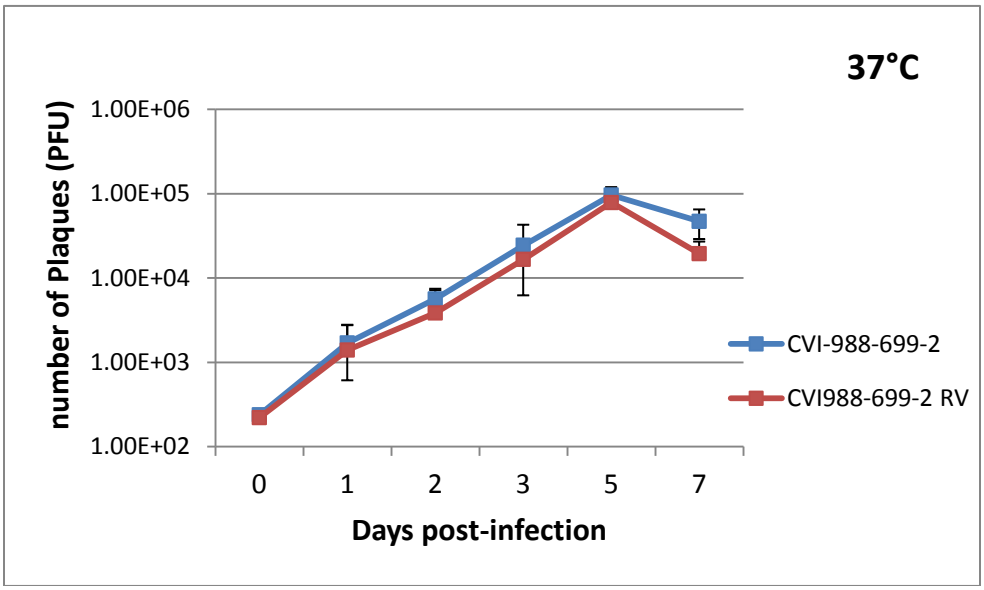


Figure 8. Ker-5 promoter-eCFP expression.
Expression of Ker-5-eRFP was not observed in transfected **(A)** CEF, HTC and LMH cells, and (**B)** kerotinocyte cell.

A.



B.

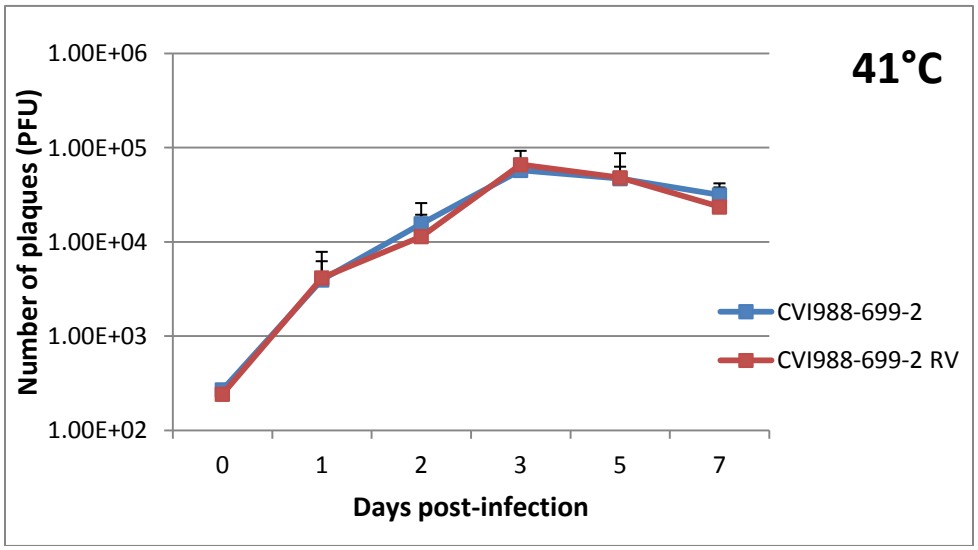


Figure 9. Growth curves of CVI988-699-2 and CVI899-699-2RV.
Comparison of the *in vitro* growth curves of CVI988-699-2 and CVI988-699-2 RV on CEFs at 37°C (**A**) and 41°C (**B**). Each point represents the mean number of plaques counted on total of six titer dishes at 5-6 days post infection.

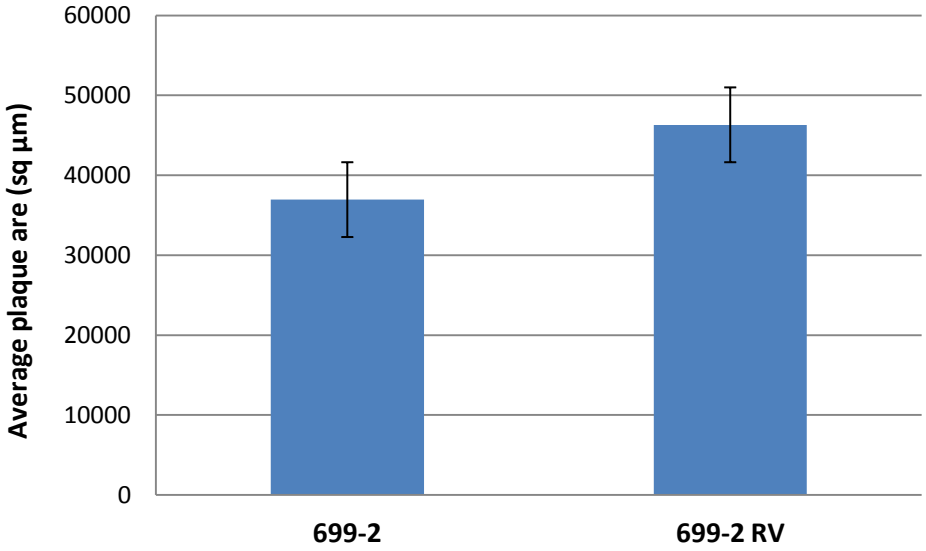


Figure 10. Plaque area measurement of CVI899-699-2 and CVI899-699-2RV on CEFs. The mean and standard deviations of sizes of 250 plaques of each virus were measured with NIS-Elements imaging software. The mean values were 36,945 and 46,306 Sq µm of CVI988-699-2 and CVI988-699-2RV respectively. Standard deviations are shown with the error bars (t test: 1.03×10^{-30}; p value < 0.0001).

A.

CVI988-699-2 GGGCGAAATGAGCGACTGAAAGTCGC

CVI988-699-2 RV GGGCGAAATGAGCGGCTGAAAGTCGC

B.

GG**ACTAGT**ATG**GCTAGC**ATGACTGGTGGACAGCAAATGGGTCGGatgaatgacc

SpeI NheI

gcggagttccaaactcccgcaccggcccctctctgctcgctctcctccccgccgccaatagctacgcggcagcgtacagccc

ggccaataggcgcgcggtgggcgtaggcggaggaagctacaagagccccacgcggggttcccccggcacacgtggcgg

gtggaaggctccgctgtgtctaaccctaatcggaggtattgatggtactgtcgccgcgctccctccg**cccgctgtttactcgc**

CR2

Cg**actttcagcg**ggctaggggagccgccccaggggcgccgcggcggggaggggtggggcggacgcgggagaaa

ggaccgaaaggggctccacggcaaacaaaaaaaaacgtcagcgaggggtcctctcgcccccatccgccctggggtcctcg

cccgcaggccgcggtcggccggcacccgccattgccgccgcgaagagttcgcctctgtcagcctcggcggcgcccgggga

gatgcggcgcgcggccccgcgcccccagcagagcaacacgggagcggcgcccccggggcaacccccgcgcccccctg

cgccgtggggcgcgcggacggcgtcgctcccacacgcgcggccccgcgcgcacgaccgttggagccgttgagccgcgc

gcggggctctgtgagATGTACCCATACGATGTTCCAGATTACGCT**AAGCTT**

HindIII

Figure 11. **MDV001/MDV080 sequence**
(**A**) alignment of CR2 Domain in vTR of CVI988-699-2 and CVI988-699-2-RV and (**B**) MDV080 sequence of PCR product.

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Appendix

PROMOTER SEQUENCES

gD Promoter

ATTAATAAATGGACCTTCGATGCAGGCAGAGGACCCTAAAAGTGTTTTT**TATAAA**
TATA Box
GTTCGTAAGCCTGACCGAAGTC**G**TGATTTTTCATGGCAAAATCTGAACTCCCATG

GCAATAGTGGTCTACGTCGTGAAAAATATATACGTTCCTCTAAGAGGCGATGGAA

G**A**ATCCCGAGATATTTAAGGTATCTTTGAAATGTGAATCAATTGGCGCTGGTAAC

GGAATAAAAATTTCATTCTCATTTTTCTAACATTATAATATATCAGATCGTTTCTTA

TATACTTATTTTCATCGTCGGGA**TATGACTAACG**TATACTAAGTTACAAGAAACA
AP-1
ACTGCTTAACGTCGAACATAACGGAAATAAAAATATATATAGCGTCTCCTATAAC

TGTTAT**A**TTGGCACCTTTTAGAGCTTCGGTATGAATAGATACAGATATGAAAGTAT

TTTTTTTTAGATATATCTCATCCACGAGAATGATTCTTATAATCTGTCTACTTTTGG

GAATTGGGGACATGTCCGCAATGGGACTTAAGAAAGACAATTCTCCGATCATTCC

CACATTACATCCGAAAGGTAATGAAAACCTCCGGGCTACTCTCAATGAATACAAA

ATCCCGTCTCCACTGTTTGATACACTTGACAATTCATATGAGACAAAACACGTAAT

ATATACGGATAATTGCAGTTTTGCTGTTTTGAATCCATTTGGCGATCCGAAATATA

CGCTTCTCAGTTTACTGTTGATGGGACGACGCAAATATGATGCTCTAGTCGCATGG

TTTGTCTTGGGCAG**AGCATGTGGG**AGACCAATTTATTTACGTGAATATGCCAACT
Myc
GCTCTACTAATGAACCATTTGGAACTTGTAAATTAAAGTCCCTAGG

UL47 Promoter

AGGAGGTATTTGTCTATTAATATGATGGTGAGTTGGCAGAACGAAACTAT

ACTTAAATGATATTATACGTAAATGTGACGTTTAATAAACGAATTTGTCGT

ATTCGCTTTGTCTTTGTTTCTCACGTCCCATCAATGCTGTAATATTATTGAT

GCAATTACACTTCCGCCGTTAGATCAATACATTATAAAACGGCGTAGTT

Ets TATA box

TTGATAACAGTATGCTGGTAGCACATTCCACCGAAGAGGCTAGC

Ker-5 Promoter

attaataTGCTCCAATGGGTGAGCTTGGCACAGGGAGCAGGATCTCCTGGGGC

TCTGGAAGCTTCGTGCTGTGTCCTGCTCGTGCCGATCCCGTCCAGTTCTCCT

TCAGTGACTCAGGGCTCTGGGGGCGGGGGGAGCTCCCTTGCTCCGTTA

AP-1 Sp1 Ker 1 Site

CTTTCTTGCCTGCAAACAATGCATTTTTCAGCGCCTCGCCGTAGCCTAGGT

GTGTTTGACAAGGCAAGTTGCTGCAAGGCACGTCAAGTTCTGAATCCCCGT

GCCAAGTTTTCCAAGCAGATAAAAGAGGAGGCTCCCAGGCTCTCCCATCA

CAGGGGTTTCTGTCTCCGTTGCCCTCTGCTGCTTTCCTGCTGCTCAGCTTC

CK-8

TATCACCAGCTCTTTCCCCCGTTCCTGTTCTTTGCGTTTCCAACTGCACGAG

CCgctagc