CHARACTERIZATION OF THE MEQ ONCOPROTEINS OF MAREK'S DISEASE VIRUS VACCINE STRAIN CVI988/RISPENS

A Dissertation

by

DHARANI KUMAR AJITHDOSS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2009

Major Subject: Poultry Science

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Approved by:

Chair of Committee,	Blanca Lupiani
Committee Members,	Steve A. Maxwell
	Sanjay M. Reddy
	Van G. Wilson
Head of Department,	John B. Carey

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ABSTRACT

Characterization of the Meq Oncoproteins of

Marek's Disease Virus Vaccine Strain CVI988/Rispens. (May 2009)

Dharani Kumar Ajithdoss, B.V.Sc., Tamil Nadu Veterinary and Animal Sciences

University; M.V.Sc., A.N.G.Ranga Agricultural University

Chair of Advisory Committee: Dr. Blanca Lupiani

Marek's disease virus serotype-1 (MDV-1) causes T cell lymphomas in chickens. Vaccines prepared from attenuated CVI988/Rispens MDV-1 strain currently offer the best protection. Although attenuated CVI988 is non-oncogenic, it codes for two forms of the MDV-1 oncoprotein Meq (CVI-Meq and CVI-L Meq). In this study, both CVI-Meq proteins, like the Meq protein of Md5 (a very virulent oncogenic strain), transformed Rat-2 and NIH3T3 cells. Both CVI-Meq and CVI-L Meq proteins activated the *meq* promoter only in the presence of chicken c-Jun (CK-Jun) whereas Md5-Meq activated the same promoter irrespective of CK-Jun co-expression. However, all three Meq proteins bound the *meq* promoter regardless of whether CK-Jun was co-expressed. We constructed three chimeric Meq proteins, namely, Md5-CVI-Meq, CVI-Md5-Meq, and Md5-CVI-L by exchanging domains between Md5 *meq* and CVI *meq* genes. Although these chimeric Meq proteins transactivated the *meq* promoter, the activation was significantly less than Md5-Meq. The current study indicated amino acid residues at positions 71 and 320 were important for Md5-Meq increase transcription of its own

promoter. All three Meq proteins activated the MDV *gB*, *MMP-3* and *Bcl-2* promoters and suppressed transcription from the MDV *pp38/pp14* bidirectional promoter.

CVI-Meq protein in the context of other Md5 genes caused tumors only in 6% of chickens when compared to parental rMd5 (a very virulent strain), which induced lymphomas in 100% of chickens, (Reddy and Lupiani, unpublished data). Taking advantage of these two different phenotypes, we constructed two chimeric Meq proteins, Md5/CVI-Meq and CVI/Md5-Meq, by exchanging DNA binding and transactivation domains between Md5-Meq and CVI-Meq to understand the role of the DNA binding and the transactivation domains of Meq in transformation. rMd5-Md5/CVI-Meq virus caused 100% mortality in chickens and T lymphomas were found at high frequency in the peripheral nerves and various organs such as the heart, spleen, kidney, and gonads. On the other hand, rMd5-CVI/Md5-Meq induced disease in 36% of chickens on average and lesions were primarily in the nerves. Very rarely, lesions were present in the spleen and heart and no tumors were present in the kidney or gonads. Our results suggest that both the DNA binding domain and transactivation domain of Meq could cooperatively determine the nature of lymphomas in chickens.

DEDICATION

To my parents,

Mr. Ajit doss Krishnaswamy and Mrs. Pavannammal Ajit doss

Their love and sacrifice have given me the world

ACKNOWLEDGEMENTS

I wish to place on record a deep sense of gratitude that I have for my major advisor Dr. Blanca Lupiani. Her dedicated guidance in all aspects helped me in completing this work successfully. I register my profound gratitude to my committee members, Drs. Reddy, Wilson and Maxwell, for their counsel, co-operation and precious suggestions rendered throughout my research work. I benefited to the highest degree from their teachings.

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NOMENCLATURE

bZIP	Basic leucine zipper
CEF	Chicken embryo fibroblast
CHIP	Chromatin immunoprecipitation assay
CK-Jun	Chicken c-Jun
CtBP	C-terminal-binding protein
DBD	DNA binding domain
DEF	Duck embryo fibroblast
EARC	Ellipsoid-associated reticular cells
ECM	Extracellular matrix
FFE	Feather follicular epithelium
GaHV	Gallid herpesvirus
gB	Glycoprotein B
HSV-1	Herpes simplex virus type 1
IR_L	Internal repeat long
IR_S	Internal repeat short
LAT	Latency associate transcript
MDV	Marek's disease virus
Meq	Marek's EcoQ fragment
MERE	Meq response element
MMP-3	Matrix metallo-proteinase-3

NLS	Nuclear localization signal
NoLS	Nucleolar localization signal
Ori	Origin of replication
PCR	Polymerase chain reaction
REV	Reticuloendothelial virus
SEM	Standard error of mean
SPF	Specific pathogen free
TAD	Transactivation domain
TR_L	Terminal repeat long
TR_S	Terminal repeat short
U_L	Unique long
U_S	Unique short
vLIP	Viral lipase
vTR	Viral telomerase RNA gene

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1. INTRODUCTION

1.1. History of Marek's disease

Marek's disease (MD) is a common, highly contagious, malignant lymphoma of chickens characterized by lymphoid infiltrations in peripheral nerves, visceral organs, eye, muscle, and skin, contributing to significant economic losses to the world chicken industry. MD was named in honor of the Hungarian veterinarian, Joseph Marek, who first described the condition in four adult male chickens in 1907 (Biggs, 1961; Marek, 1907). He described the disease as a "polyneuritis" or a "neuritis interstitialis", characterized by paralysis of the legs and wings. Grossly, the sacral plexuses and spinal cords were thickened, and microscopically, many of these nerves had mononuclear infiltration. In America, the disease was first reported as " paralysis of the domestic fowl" (Kaup, 1921). Soon after, a similar condition, named as " neuromyelitis gallinarium" was reported in The Netherlands (Van der Walle and Winkler-Junius, 1924). At that time, the disease was commonly known as "fowl paralysis", "range paralysis" or " Marek's paralysis". In addition to the peripheral nerves, mononuclear infiltration was also found in visceral organs in 10% of chickens that showed paralysis (Pappenheimer et al., 1929). Although most common in the ovary, tumors were also found in the liver, kidneys, lungs, adrenals, and muscle. The authors named this condition as " neurolymphomatosis gallinarium". Because of lymphoid tumors and difficulty in differential diagnosis, the disease was often confused with lymphoid

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This dissertation follows the style of Virus Research.

leukosis, also a neoplastic disease caused by avian leukosis virus (Biggs, 1961; Campbell, 1961; Ellermann, 1922). Based on susceptibility, organs affected, and histopathogenesis, the disease can be distinguished from lymphoid leucosis and a new name, Marek's disease, was proposed (Biggs, 1961). Later, it was clearly established that these two diseases are separate and caused by two different agents (Biggs and Payne, 1964).

1.2. Etiology of Marek's disease

When chickens were inoculated with blood and tumor cell suspensions obtained from diseased birds, both inoculated and uninoculated contact chickens developed MD, indicating that the disease is readily transmissible (Biggs and Payne, 1963; Sevoian et al., 1962). These, and other studies have revealed that MD causative agent is highly cell-associated as cell free suspension failed to cause cytopathic effect in cell culture as well as disease in chickens (Churchill, 1968; Solomon et al., 1968). By identifying transformed cells were of host origin through the use of sex chromosomes markers, the possibility of cell transplantation causing the disease was ruled out (Owen et al., 1966). Electron microscopic examination of chicken kidney cells infected with blood and tumor cells revealed virus particles, whose morphology resembled those of herpesviruses (Churchill and Biggs, 1967). Subsequently, presence of herpesvirus like particles in infected duck embryo fibroblasts was also reported (Nazerian and Burmester, 1968). These studies have established that a herpesvirus, called as Marek's disease virus (MDV), is the etiology of the disease. Vaccines prepared by virus attenuation in cell culture (Biggs et al., 1970; Churchill et al., 1969a; Churchill et al., 1969b) and from

MDV related virus from a turkey, herpesvirus of turkeys (HVT) (Okazaki et al., 1970) protected chickens against MD; these studies provided conclusive evidence that MDV is the etiological agent of MD.

1.3. Classification

Presently, MDV serotype 1, officially known as *Gallid herpesvirus 2* (GaHV-2), is classified as a member of the order *Herpesvirales*, family *Herpesviridae*, subfamily *Alphaherpesvirinae* and genus *Mardivirus* (Davison et al., 2008). Two other members in the genus *Mardivirus* are *Gallid herpesvirus 3* (previously MDV-2), and *Meleagrid herpesvirus 1* (previously MDV-3 or HVT) (Davison et al., 2008).

Three methods are widely used to classify MDV isolates (Fig. 1). In the beginning, MDV isolates, which caused lesions only in nerves and lesions in both nerves and visceral organs were classified as classical and acute, respectively (Biggs et al., 1965). Based on the antigenic differences, MDV was later classified into three serotypes: MDV serotype 1 (MDV-1), MDV serotype 2 (MDV-2), and MDV serotype 3 (MDV-3 or Herpesvirus of Turkeys, HVT) (Bulow and Biggs, 1975a; Bulow and Biggs, 1975b). Among these three serotypes, only MDV-1 causes disease in chickens. The virulence of MDV seems to increase over a period of time with the introduction of new vaccines; it is speculated that intense vaccination is one of the reasons for continuous virus evolution. Based on the lesions such as increased cytolytic infection, uncommon cell tropism, severe lymphoid organ atrophy, early mortality, severe suppression of immune system, and aggressive tumors in chickens that are vaccinated with turkey herpesvirus (HVT)

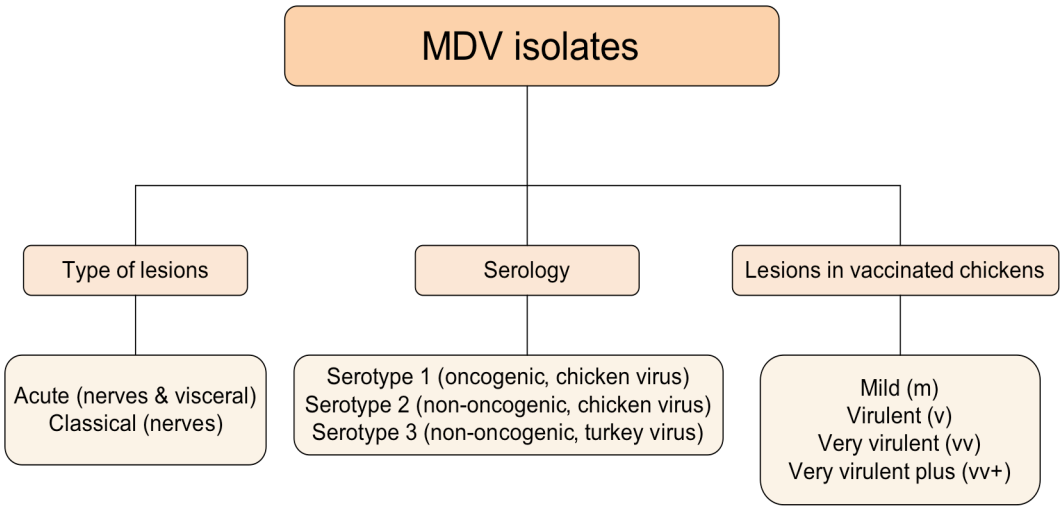

Fig. 1. Classification of MDV isolates.

and bivalent (HVT + SB-1), MDV isolates are classified into mild (m), virulent (v), very virulent (vv), and very virulent plus (vv+) pathotypes (Witter, 1997). Though 15 X 7 chickens available in Avian Disease and Oncology Laboratory, USA were initially used, this classification can also be done by using specific pathogen free (SPF) chickens (Dudnikova et al., 2007).

1.4. Genome and its organization

The genome of MDV is large, linear, double-stranded DNA with a buoyant density of 1.715 gm/cm^2 (Lee et al., 1971) and represents class E, identical to herpes simplex virus type 1 (HSV-1), in genome organization. It comprises of two unique DNA segments called a unique long (U_L) and a unique short (U_S), which are flanked by large inverted repeated sequences (Fig. 2). The flanking DNA sequences of U_L are called as terminal repeat long (TR_L) and internal repeat long (IR_L) and that of U_S are known as terminal repeat short (TR_S) and internal repeat short (IR_S).

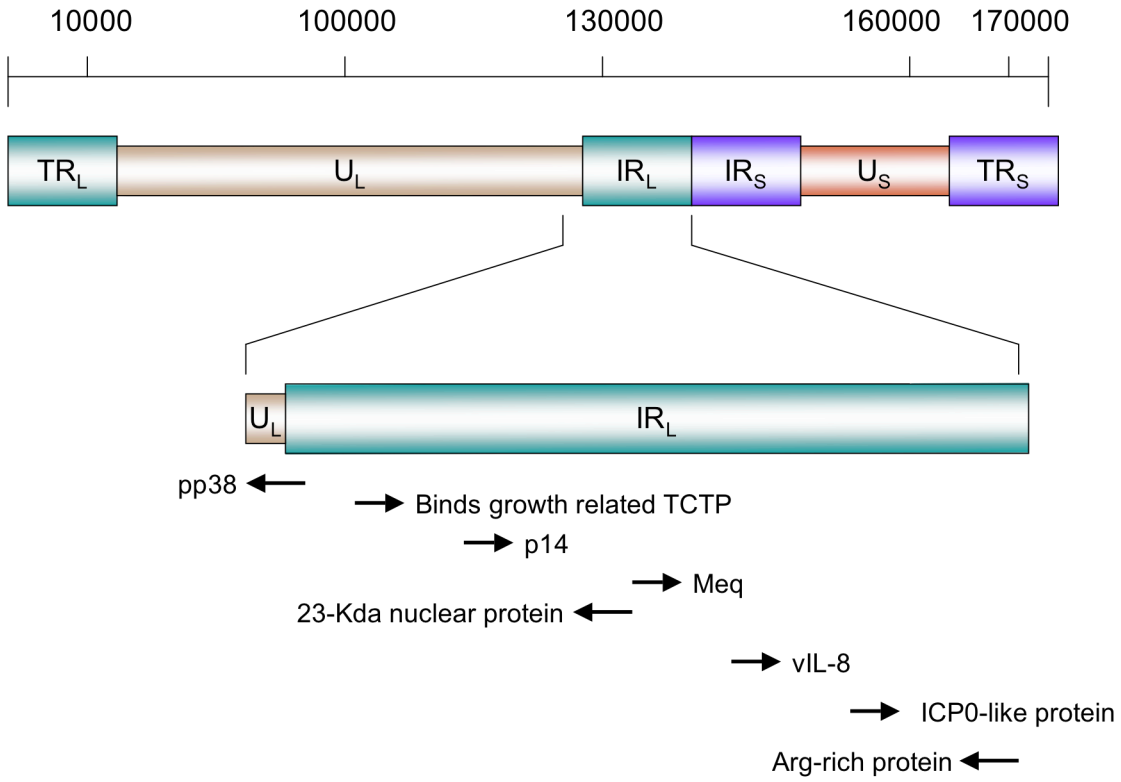

Fig. 2. MDV genome. It is composed of unique long (U_L) and unique short (U_S) segments, flanked by inverted repeats. The inverted repeats are: TR_L: terminal repeat of the long segment; IR_L: internal repeat of the long segment; IR_S: internal repeat of the short segment; and TR_S: terminal repeat of the short segment. Most of unique genes are located in the repeat long regions. The direction of transcription and relative positions of genes transcribed in this repeat are noted. Among these, Meq is of particular importance due to its requirement for transformation.

These flanking sequences can invert their orientation relative to unique segment, thus producing four isomeric forms during virus replication. The complete nucleotide sequences for many MDV strains are currently available. The genome of a very virulent strain of MDV-1, Md5, contains 177, 874 bp, which is predicted to code for at least 103 proteins (Tulman et al., 2000). The 64 genes located in U_L region are important for virus

replication and virus assembly. Most of these genes are highly homologous and corresponds to those present in U_L region of human alphaherpesvirus, herpes simplex virus type 1 (HSV-1). Among six genes that are unique to MDV, viral lipase (*vLIP*) has been identified as one of the virulent factors of MDV (Kamil et al., 2005). Out of nine genes in the U_S region, seven are found in HSV-1 U_S DNA segment. Two genes that are unique to MDV are MDV090 and MDV093 with presently unknown functions. The short repeat regions (TR_S, and IR_S) contain 12 genes; many of them including immediate early proteins, ICP4, ICP22, and ICP27 are found in HSV-1. Unlike unique regions and the short repeat regions, 16 genes located in the long repeat regions (TR_L, and IR_L) are exclusively present in MDV-1 genomes and are not found in other MDV serotypes or non-avian herpesviruses. Many of these genes (*meq, pp38, pp24, vTR,* and *vIL-8*) are associated with MDV pathogenesis (Brown et al., 2006; Cui et al., 2004; Lupiani et al., 2004; Parcells et al., 2001; Reddy et al., 2002).

The GA (a virulent MDV strain) genome is 174, 078 bp long, which unlike Md5 strain, contains U3 sequences from reticuloendothelial virus (REV) near the U_L/ IR_L junction (Lee et al., 2000a). The significance of this retrovirus insertion remains to be studied.

The genome size of another MDV virulent strain, Md11, is 170,950 bp (Niikura et al., 2006). Interestingly, the terminal short region in this genome is completely replaced with a portion of duck chromosome 19. The complete DNA sequence vaccine of strain CVI988, a non-oncogenic MDV strain, consists of 178,311 bp (Spatz et al., 2007a). When compared with genomes of MDV oncogenic strains, there are several

differences present in the CVI988 genome. These include a 177 bp insertion in the *meq* region, deletions in ORFs 49.1, 5.5, and 3.0 (*vIL-8*), nucleotide changes in UL36 and UL49 (tegument genes), and absence of ORF6.2.

In addition to MDV-1, the complete genome sequences of MDV-2 and HVT are also available. Its organization, similar to MDV-1, is collinear with and closely related to HSV-1 genome. The genome of HPRS16, MDV-2 strain, is 164,270 bp in length and contains 91 ORFs. HVT strain FC-126 genome size is 159,160 bp or 160, 673, which is estimated to code for 99 proteins (Afonso et al., 2001; Kingham et al., 2001). Sixteen genes that are present in MDV-1 strains are absent in HVT genome including *meq* (MDV oncogene), *pp24* and *pp38* (MDV early replication proteins), and *vIL-8* (MDV chemokine), which are important for MDV pathogenesis. Conversely, 13 genes of HVT are absent in MDV-1 genomes. Notably, HVT contains NR-13 gene, a Bcl-2 homologue of quail and has been shown to inhibit apoptosis in the DT40 (a B cell line) (Ewert and Duhadaway, 1999).

1.5. Stages of MDV infection

Infectious virus is shed along with the debris of dead stratified feather follicular epithelial cells, which serve as a source of infection for chickens. The infection is usually acquired through inhalation of infected poultry house dust and chicken dander (Beasley et al., 1970). The virus soon reaches the lung through unknown mechanisms. According to 'Cornell Model', MDV pathogenesis in chickens is divided into four phases: an early cytolytic phase, a latent phase, a late cytolytic and immunosuppressive phase, and a transformation phase (Calnek, 1986) (Fig. 3).

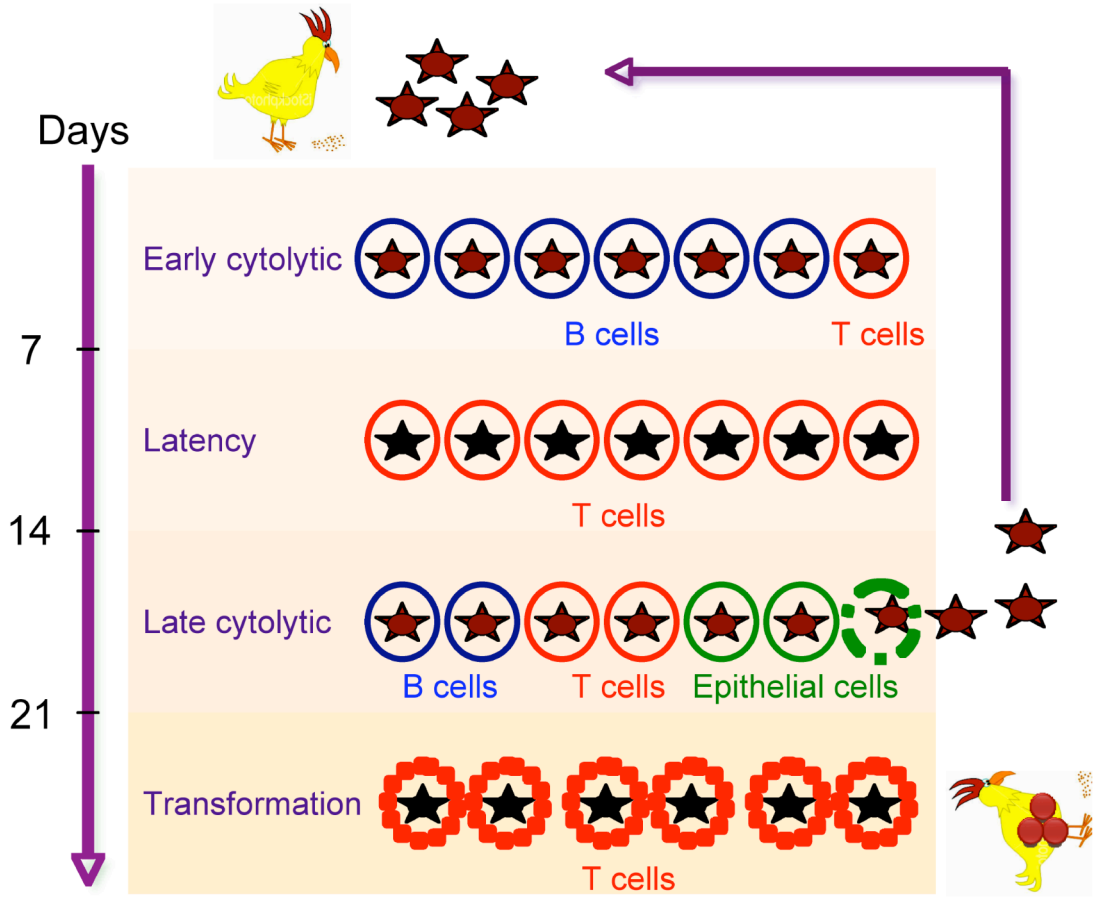

Fig. 3. Biology of MDV. Following inhalation, the virus reaches the lung. Macrophages are believed to carry the virus to lymphoid organs (spleen, thymus and bursa), where it undergoes cytolytic infection mostly in B cells and in few T cells. Thereafter, the virus establishes latency only in T cell at 7 dpi. After a week, second cytolytic infection is seen in lymphoid organs as well as in epithelial tissues such as feather follicular epithelial cells (FFE). Cell-free virus is released only from FFE in chickens, which is the source of infection for susceptible chickens. The hallmark of MDV infection is the transformation of latently infected T cells, occurs as early as three weeks.

1.5.1. Early cytolytic phase

It is commonly believed that MDV is transported from the lung to the spleen and caecal tonsil by macrophages. Expression of MDV antigens such as pp38 (an immediate

early protein) can be demonstrated in the spleen 4-6 days post-infection (Baigent et al., 1998) and not at or after 8 dpi, coinciding with the onset of virus latency (Baigent and Davison, 1999). This productive-restrictive phase is characterized by genome expression and production of intracellular virions; but cell-free infectious virus particles are not produced. There is evidence that the virus interacts with antigen presenting cells such as ellipsoid-associated reticular cells (EARCs) after entering into the spleen (Jeurissen et al., 1989). In the spleen, 95% of MDV infected lymphocytes are B cells, which are in close contact with EARCs, while 4% are double positive $CD4^+$ $CD8^+$ T cells (Baigent et al., 1996). As the name indicates, the outcome of this infection is destruction of cells. Additionally, splenomegaly due to reticular hyperplasia commonly occurs. It is noteworthy that resting T lymphocytes are resistant to cytolytic infection. However, activation of these resting cells occurs at the end of this phase, more likely, due to relocalization of T cells into the proximity of infected B cells leading to infection (Baigent and Davison, 1999). Interestingly, the spleen is not essential for MDV pathogenesis since neonatal splenectomy did not affect the viremia in chickens (Schat et al., 1980). Besides the spleen and caecal tonsil, virus replication at lower level can also occur in the thymus and bursa. It is speculated that the source of infection for these organs is the infected B-lymphocytes from the spleen and caecal tonsil. Marked reduction in the size of the bursa was noted in chickens infected MD agents, JM and Conn-A (Jakowski et al., 1969) . Further, necrosis of follicles and loss of follicles in many bursas were observed microscopically. Similar lesions were also reported in the thymus (Purchase and Biggs, 1967) .

1.5.2. Latent phase

MDV enters into latency 7 dpi. Like in other herpesviruses, there is no expression of viral protein or infectious virus production during MDV latency. However, viral genomes and few latency-associated transcripts (LATs) including two MDV small RNAs (0.9 and 0.75 kb) and a 10-kb RNA can be found in latently infected cells (Cantello et al., 1994). In stark contrast to the early cytolytic infection, the majority of latently infected cells are T lymphocytes, with few B cells (Calnek et al., 1984; Shek et al., 1983). These latently infected T lymphocytes retain the virus life long, serving as a virus reservoir. Because of lack of suitable experimental systems that differentiate latently infected cells from transformed cells, mechanisms of latency are yet to be understood. In this regard, it is of interest to note that cell-mediate immune response, not humoral response, can influence the development and maintenance of latency (Buscaglia et al., 1988; Schat et al., 1980).

1.5.3. Late cytolytic phase and immunosuppression

At 14-21 dpi, virus replication resumes in the thymus and bursa (Calnek, 1986). At the same time, virus replication also occurs in the kidney, esophagus, feather follicular epithelium (FFE), adrenal gland and proventriculus. At present, knowledge regarding the source of infection for these epithelial tissues is lacking. Replication in FFE is of particular importance since it is the only site, where productive infection occurs resulting in the release of cell-free virus along with dead epithelial cells into the environment (Calnek et al., 1970). Immunosuppression involving both humoral and CMI response is associated with this phase as demonstrated in several studies (Calnek et al.,

1979; Friedman et al., 1992; Lee et al., 1978; Rivas and Fabricant, 1988), and is apparently due to the cytolytic infection in the thymus and bursa.

1.5.4. Transformation

Transformation of latently infected T lymphocytes occurs at 21-28 dpi and is the hallmark of MDV infection. The phenotype of these neoplastic cells, which resembles that of activated T helper-2 cells, is $CD4^{+}$, TCR $\alpha\beta^{+}$, $CD30^{hi}$, $CD28^{lo/-}$, MHC class I^{hi}, MHC class II^{hi}, IL-$2\alpha^{+}$, MDV $pp38^{-}$, and MDV gB^{-} (Burgess and Davison, 2002). Besides transformed T lymphocytes, other cells such as B lymphocytes, macrophages, and reticular cells are also present in the lymphomas albeit at very low percentage. As early as 3 weeks, neoplastic cells can be found in peripheral nerves, muscles, skin, and visceral organs such as the adrenal gland, heart, intestine, kidney, liver, lung, ovary, proventriculus, spleen, eye, and thymus. Factors such as virus serotype and pathotype (Witter, 1997), chicken genotype (Baigent and Davison, 1999), age at infection (Calnek, 1973), maternal antibodies (Calnek, 1972), and vaccination (Witter, 1997) greatly influence the tumor incidence. It is still unclear whether latently infected cells become transformed following infiltration or alternatively, cells that are transformed elsewhere infiltrate these sites.

It was first proposed that MD tumors were monoclonal in nature because latently infected lymphocytes collected from multiple organs of the same chicken had identical pattern of integration (Delecluse et al., 1993). Recent study, in contrast, suggests that they are polyclonal as the population contain both $TCR2^{+}$ and $TCR3^{+}$ cells, products of two different chicken TCR variable beta chain genes (Burgess and Davison, 2002). Cell

lines have been established from MD lymphomas (Akiyama et al., 1973; Powell et al., 1974). Depending upon the cell lines, MDV genome can be found as episomal (Tanaka et al., 1978) or integrated (Kaschka-Dierich et al., 1979) or both (Kaschka-Dierich et al., 1979).

1.6. Symptoms in chickens

Clinical signs in chickens vary according to the susceptibility, virus strain, age at infection, maternal antibodies, and vaccination status. Affected chickens exhibit neurological signs such as paresis or paralysis of the legs and wings, which may be either spastic or flaccid, dyspnea, torticollis, and paralysis of the tail and eyelids. Transient paralysis is often seen in young chickens due to temporary vasogenic edema in the CNS (Swayne et al., 1989). Some birds develop blindness because of neoplastic and inflammatory lesions in the eye, commonly referred as “pearly eye” or “gray eye” or “ ocular lymphamatous” (Ficken et al., 1991; Smith et al., 1974). In the early-mortality syndrome, chickens infected with highly pathogenic strains suddenly die with wasting at 12-13 dpi and show no neurological signs (Witter et al., 1980).

1.7. Pathology of MD

Macroscopically, enlargement of the peripheral nerves such as the brachial plexus and sciatic plexuses, and vagus is commonly found. This may be unilateral or bilateral. Further, depending upon the virulence of strains, gross tumors are present in various organs including the ovary, testicles, liver, spleen, kidney, intestines, heart, proventriculus, and eye.

Microscopic nerve lesions in advanced MD cases are classified into three types: 1. Type I: infiltration of small lymphocytes and plasma cells with little edema; 2. Type II: infiltration of few lymphocytes with marked edema and fibrosis; 3. Type III: infiltration of neoplastic lymphoid cells (Wight, 1962). It was noted that neoplastic infiltration was secondary to the inflammation. However, a subsequent study, focused on chronology of disease development in chickens infected experimentally showed that the lesions were due to primary neoplastic process (Payne and Biggs, 1967). The nerve lesions were classified into 3 types: (a) Type A: increased proliferation of lymphoid cells and Schwann cells, and demyelinization seen in 14 to 21 dpi; (b) Type B: a diffuse infiltration of lymphoid cells and some edema see in later stage of disease; (c) Type C: a little infiltration of lymphoid cells seen in clinically normal, mature chickens. Furthermore, the nerve lesions based on electron microscopic observations were classified into 3 types: (1) Type 1: infiltration of small lymphocytes; (2) Type 2: infiltration of small, medium, and large lymphocytes; (3) Type 3: infiltration of small lymphocytes, plasma cells and reticular cells (Okada and Fujimoto, 1971). Lesions in visceral tumors were similar to those in peripheral nerves (Nazerian, 1973) .

1.8. MDV vaccines

MD is the first neoplastic disease in the history against which successful vaccines were developed. Vaccines prepared from all three serotypes are being used either alone or in combination for the control of MD around the world.

1.8.1. Serotype 1 vaccines

The oncogenicity of MDV-1 strain HPRS-16 has been reported to be lost in chickens following a serial passage for 33 times in the chicken kidney cells, which suggested for the first time that MDV could be attenuated by repeated passage in cell culture (Churchill et al., 1969a). Notably, chickens vaccinated with thirty-first passage of HPRS-16 virus were protected against the challenge by direct inoculation or by contact exposure of fourteenth passage virulent virus (Churchill et al., 1969b). This was the first protection study that demonstrated the feasibility of using attenuated MDV as a vaccine. This type of attenuation is not strain specific as JM strain that was passaged in DEF or CEF for 100 times also failed to cause MD in susceptible chickens (Nazerian, 1970). Interestingly, progeny from a parental stock vaccinated with a cell culture attenuated GA strain were protected against the challenge with the virulent GA strain, indicating the role of maternal antibodies in vaccine-mediated protection (Eidson et al., 1971). The protective efficiency of an attenuated MDV strain in a major field trial that used more than 32,000 commercial birds was reported to be 80 % (Meulemans et al., 1971). Currently, vaccines prepared from CVI988 strain offer the best protection and are widely used in the USA and Europe and the characteristics of this strain will be discussed in detail here. CVI988, a MDV avirulent strain, was originally isolated from MDV positive but MD and ALV disease free eleven-months-old laboratory flock, maintained in semi-isolation at the Central Veterinary Institute, The Netherlands (Rispens et al., 1972). This strain caused neither disease nor death in experimentally inoculated chickens. Importantly, the virus (CVI988-4) that was passaged four times in DEF protected

chickens against a challenge with the strain K, a virulent MDV. In the absence of vaccination, the strain K caused 43% mortality, which was reduced to 1% following CVI988-4 vaccination. In this experiment, CVI988-4 caused C-type microscopic lesions in the nerves in few chickens. To avoid this, CVI988-4 was further passaged in DEF. CVI988 -26 virus at the level of 26th passage did not induce microscopic lesions in the nerves and was completely attenuated. Further, this virus provided 97-100% protection in various genetic chicken lines against the strain K challenge. In DEF, the CVI988-26 formed a syncytial type of microplaque, characterized by rounding and darkening of cells with more than 100 nuclei. Comparable plaques were also observed in CVI 988-26 infected CEF. This strain was safe to use as vaccine for chickens even at the dose of 300,00 PFU. Isolation of CVI988-26 from vaccinated chickens was variable (30-75%). Though the virus spreads to contact birds, the isolation was even lower (5-30%) from them. Vaccinated birds excreted virus by 3rd week. Contact birds were viremic for CVI988-26 by 4th week and virus demonstration in feather follicle was possible by 5th week. Notably, it provided excellent protection (98-100%) against the strain K challenge, irrespective of whether the challenge was done at day-old or 4 weeks post vaccination. This level of protection was effective for 2 years. The virus maintained its avirulence and did not revert back to induce any microscopic lesions. It was apathogenic for Rhesus monkeys and humans handling chickens and vaccines did not show any MDV antibodies. Intramuscular route of vaccination was preferred over intraperitoneal because non-specific mortality was found to be fewer with the former than the later. Minimum dose required for vaccination was determined to be 3,000 PFU. Partial

vaccination at the level of 10 % did not offer good protection. The level of protection offered by CVI988-26 was superior to that of HVT. MD mortality following vaccination with CVI 988-26 and HVT were 1.25% and 5-8.75 %, respectively.

Although several virulent MDV-1 strains have been attenuated over many decades in order to develop them as potent vaccines, the protective efficiency of all of them is inferior when compared to that of CVI988. That being said, rMd5ΔMeq virus has been recently reported to provide better protection than CVI988 in the laboratory chickens (Lee et al., 2008). It is important, however, to evaluate the efficacy of this vaccine in commercial chickens.

1.8.2. Serotype 2 vaccines

The SB-1 strain, originally isolated from 28-week-old unvaccinated, MD free S-strain chickens, was nononcogenic even in immunosuppressed chickens and offered protection against virulent virus (Schat and Calnek, 1978). The strain 301B/1, isolated from MD disease free chickens, provided a protection comparable to that of SB-1 strain (Witter, 1987). These two strains have been approved to use in the USA and are commonly used as a bivalent vaccine in combination with HVT.

1.8.3. Serotype 3 vaccines

The FC126 strain was isolated from 23-week-old turkey flock that had a history of lymphoid tumors and when experimentally inoculated, it caused no disease in chickens (Witter et al., 1970). Chickens vaccinated with FC126 survived 100% the JM strain challenge (Okazaki et al., 1970). Interestingly, cell free virus obtained by sonication of FC126 infected CEF also protected chickens against challenge with

virulent virus (Okazaki et al., 1970). It is noteworthy that cell free virus cannot be obtained with two other serotypes and thus, freeze-dried (lyophilized) vaccines are only available for FC126 strain.

1.8.4. Protective synergism

Although the protective efficiency of SB-1 and HVT was good in chickens that lacked maternal antibodies, it was less efficient in chickens that had maternal antibodies. Interestingly, these chickens were protected better when they were vaccinated with both strains instead of one (Schat et al., 1982). In other words, the degree of protection by one vaccine is increased by the inclusion of a second vaccine. Subsequently, this phenomenon was named as "protective synergism" (Witter and Lee, 1984).

1.9. MDV unique genes and their role in pathogenesis

1.9.1. MDV phosphoprotein 38 (pp38)

The *pp38* gene, which codes for 290 aa long protein, is located in the *Bam*HI-H fragment at the junction of U_L and IR_L regions (Cui et al., 1990). The pp38 antigen is expressed in MDV lymphoblastoid cell lines as well as in tumor samples (Cui et al., 1990) and is essential for the maintenance of transformation phenotype of MSB-1 (Xie et al., 1996). These findings indicated a role for pp38 in transformation process. However, the expression of pp38 is restricted to early cytolytic phase of the MDV infection. Importantly, a direct role of pp38 only in the early cytolytic infection has been demonstrated by using a pp38-null MDV recombinant virus, which did not replicate well but still retained the ability to induce tumors in chickens, albeit at a lower level (Reddy

et al., 2002). Furthermore, MDV strains belonging to all three serotypes encode for the pp38, suggesting that it has no direct role in oncogenesis.

1.9.2. Viral lipase (vLIP)

The viral lipase (vLIP) is a 756 amino acid long protein with 26 % sequence identity to the α/β hydrolase fold of pancreatic lipase (Kamil et al., 2005). The vLIP gene, found in the genome of all three serotypes, is located in the U_L region. The vLIP lacks enzymatic activity and is secreted (Kamil et al., 2005). Tumor incidences were reduced by 53% in chickens infected with MDV virus lacking vLIP while parental MDV caused tumors in 90% of chickens (Kamil et al., 2005). Therefore, vLIP could be involved in the MDV-induced oncogenesis, but not as a major determinant.

1.9.3. vIL-8

The *vIL-8* gene is located within the *Bam*HI-L fragment of MDV-1 genomes and encodes for 134 aa long chemokine that has high homology to human and chicken cellular IL-8 (Liu et al., 1999a; Parcells et al., 2001). This secreted protein attracts PBMC and not heterophils (Parcells et al., 2001). vIL-8 is expressed late in the infection and may play a role in lytic phase of MDV infection through recruiting target cells; however, it is not essential for viral replication, latency, tumor formation and virus transmission (Cui et al., 2004; Parcells et al., 2001). Interestingly, vIL contains a DKR motif instead of the ELR motif present in cellular counter that attracts heterophils. Recombinant MDV carrying DKR-to-ELR mutation functions similar to the parental MDV virus, indicating no disadvantage in pathogenesis for having DKR motif (Cui et al., 2004).

1.9.4. Viral telomerase RNA gene (vTR)

In the course of analyzing the ends of the RB1B genome, a 443-bp long, a novel viral telomerase RNA gene (vTR) was discovered and its sequence was found to share 88% identity with chicken telomerase RNA subunit gene (cTR) (Fragnet et al., 2003). To date, this is the only known TR of viral origin. The telomerase complex, which plays a role in the replication of telomere DNA, comprises of a protein subunit (TERT) with reverse transcriptase activity, and an RNA subunit (TR) containing a short, specific template for TERT. The vTR combines with cellular TERT to form a telomerase complex, which has been shown to be functional (Fragnet et al., 2003). When injected with a vTR-null MDV, the tumor incidence was reduced by 60 % in chickens when compared to wild type MDV, suggesting a role for vTR in oncogenesis (Trapp et al., 2006); however, it may not be major determinant since a non-oncogenic MDV vaccine strain CVI988 also contains this gene.

1.9.5. Meq

The *meq* gene (**M**DV **E**co**Q** fragment) located in the *Bam*I_2-*Bam*Q_2/*Eco*Q fragments within the repeat long regions was first identified based on a observation that the *Eco*Q transcripts are abundantly present in the MDV transformed T-cell lines such as RP1, RP4, and MSB1 as well as in tumors (Jones et al., 1992). Later, expression of Meq proteins was demonstrated in these cell lines (Liu et al., 1998).

Meq is a 339 amino acid long protein; contains a DNA binding domain (1-128) with a basic-leucine zipper, similar in structure to c-jun/c-fos bZIP proteins, at its amino terminus and a transactivation domain (129-339) with two and half repeats of proline

rich sequences, closely resembles that of WT-1 (Wilm's tumor suppressor protein), at its carboxy terminus (Jones et al., 1992; Qian et al., 1995) (Fig. 4). The C-terminal domain has transactivation properties, and at least one proline repeat is required for its full transactivation activity (Qian et al., 1995). Furthermore, the last 33 amino acids (307-339), though not adequate, are critical for its function (Qian et al., 1995). As shown in WT-1, proline rich repeats in the transactivation domain of Meq also have transrepression functions when tested in its isolated form (Qian et al., 1995).

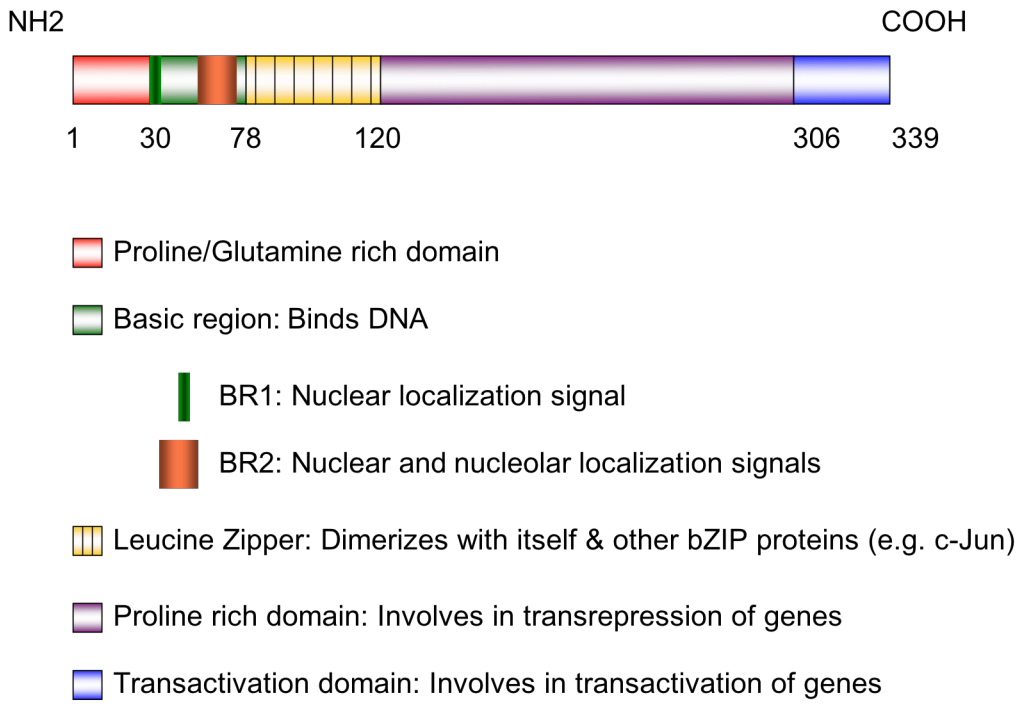

Fig. 4. Structure of Meq. The structure of Meq is typical of a bZIP protein. It contains DNA binding domain (1-120) and transactivation/transrepression domain (121-339). The function of each region is indicated.

Meq has the ability to bind its own promoter, which contains two AP-1 like binding sequences. This binding, as well as transcription from the *meq,* promoter can be increased with the co-expression of c-jun (Qian et al., 1995). Thus, Meq has the ability to form heterodimers with c-jun, a major interacting partner in the cells. There is also evidence that Meq and c-jun colocalize in the cells (Liu et al., 1999a). Chimeric proteins, bZIP-Meq/TA-c-jun and bZIP-c-jun/TA-Meq, have similar properties as Meq and c-jun, suggesting that the bZIP and transactivation domains of these proteins can complement each other in the transformation process (Liu et al., 1999a). Meq/c-jun heterodimer preferentially binds cyclic AMP (CRE)-and 12-O-tetradecanoylphorbol 13-acetate responsive element (TRE)-like sequences, called MERE (**Me**q **r**esponse **e**lement) I such as the one in the *meq* promoter (Qian et al., 1996). Thus, Meq/c-jun heterodimer has stronger activity on the meq promoter when compared to Meq/Meq and Jun/Jun homodimers. The sequence of MERE I is GAGTGA**TGAC(G)TCA**TC (TRE/CRE sequence is indicated in bold). On the other hand, Meq/Meq homodimer preferentially binds a consensus sequence ACACACA, called MERE II (Qian et al., 1996). Meq/Meq homodimers and not Meq/c-jun heterodimer suppress transcription of pp38/pp14, a bidirectional promoter located in the MDV-Ori (Levy et al., 2003b). By CHIP assay, Meq has been shown to bind MDV promoters such as *meq* , *ICP4*, *gB*, and *gD*, which contains MERE I sequence and MDV-Ori, which contains MERE II sequence (Levy et al., 2003b). Furthermore, Meq/jun heterodimer also has the ability to bind AP-1 containing chicken promoters such as IL-2. Though yet to be proven, presence of

potential RNA binding motif in the transactivation domain (315-SGQIYIQF-322) suggests that Meq also binds RNA besides DNA (Lee et al., 2003).

Several studies have pointed out that Meq can interact with cellular proteins in addition to c-Jun. For example, Meq has been shown to interact with the putative tetramerization domain of chicken p53 via its basic region (54-127) (Brunovskis et al., 1996). Additionally, Meq interacts with c-Fos, Jun-B, CREB, ATF-1, ATF-2, and ATF-3. Presence of the RB-binding consensus (LXCXE) at the end of the ZIP domain suggests Meq can potentially interact with RB (Liu and Kung, 2000), but studies are warranted to prove this.

Expression of Meq can be predominantly found in the nucleoplasm as well as the nucleolus during all phases of the cell cycle. Two basic signal sequences, BR1 (30-RRKKRK-35) and BR2 (62-RRRKRNRDAARRRRRKQ-77), present in the N-terminus are required for Meq's nuclear localization (Liu et al., 1997). While either BR1 and/or BR2 function as nuclear localization signals (NLS), BR2 is the sole nucleolar localization signal (NoLS). In addition, Meq can be found in the cytoplasm and colocalizes with CDK2 in the coiled bodies during S phase of the cell cycle (Liu et al., 1999b). Whether Meq causes translocation of CDK2 in to the coiled bodies to cause disruption of the cell cycle remains to be explored. It is noteworthy that this colocalization causes phosphorylation by CDK2 of the serine residue at position 42 of Meq leading to its translocation into the cytoplasm (Liu et al., 1999b). Additional work points out that Meq can also be phosphorylated by PKA, PKC, and MAPK. Further,

colocalization of Meq with PCNA in the nucleoplasm has been reported suggesting a role for Meq in DNA replication (Liu and Kung, 2000).

Meq is required for maintenance of the transformation state since induction of RNA antisense to *meq* in MSB-1, a MDV transformed cell line, results in reduced colony formation in soft agar assay (Xie et al., 1996). It has also been suggested that Meq cooperates with the proto-oncogene c-Myb in the maintenance of transformation phenotype of T9, a MDV transformed cell line that contains a Rous associated virus insertional activation of c-myb (Le Rouzic and Perbal, 1996). Further, transformed CD4 cells carrying AV37 marker in chickens have been found to consistently express Meq (Ross et al., 1997).

When overexpressed, Meq transforms immortalized rat fibroblasts (Rat-2) and immortal chicken fibroblasts (DF-1) (Levy et al., 2005; Liu et al., 1998). Thus far, Meq has not been shown to transform primary fibroblasts, and it causes sarcomas only in 5% of chickens when infected with replication defective retrovirus virus carrying *meq*. These findings suggest that Meq is a weak oncoprotein. Like Jun and Fos, Meq promotes growth of cells in the absence of serum and protects them against apoptosis inducing agents including TNF-α, C2-Ceramide, UV irradiation and serum starvation (Liu et al., 1998). The anti-apoptotic function of Meq might be due its ability to increase *bcl-2* expression and to decrease *bax* expression (Liu et al., 1998).

Recently, the function of Meq in chickens has been examined. The rMd5ΔMeq virus in which the *meq* genes were deleted failed to induce tumors, where as rMd5 virus that had intact *meq* genes caused tumors in susceptible chickens (Lupiani et al., 2004).

This study provided compelling evidence that Meq is essential for transformation of T lymphocytes. Additional evidence that Meq is a major MDV oncoprotein came from the studies of the pRB-1B-Ct20 virus with mutations in the *meq* gene that eliminated the interaction with C-terminal-binding protein (CtBP), which was nononcogenic in chickens (Brown et al., 2006). Meq interacts with CtBP through a motif located in the DNA binding domain (20-PLDLS-24).

Recent evidence suggests that the Meq transforms via v-Jun pathway. These observations are: (1) morphology of Meq transformed DF-1 cells were similar to v-Jun transformed cells, (2) Meq protects DF-1 cells against apoptosis, (3) up-regulation of genes such as JTAP-1, JAC, and HB-EGF, implicated in v-Jun transformation process, and (4) Meq cooperates with c-Jun in the transformation of DF-1 cells (Levy et al., 2005).

In addition to the *meq*, at least three variants of the meq transcripts are reported in the literature: the L-meq, meq/vIL8 and Δmeq. The L- *meq* gene (1,185 bp) was first described in CVI988-infected CEF DNA (Lee et al., 2000b). This has an insertion of 178-bp in the form of proline rich repeat and is absent in CEF infected with other MDV-1 strains including GA, Md5, RB1B or MDV-2 strains including SB infected CEF. The L-*meq* gene can be found in spleen and PBMC collected from chickens infected with CVI988 and JM strains. Recently, this gene has been reported in two more MDV strains, CU-2 and BC-1 (Shamblin et al., 2004). When co-expressed, the L-Meq protein has been shown to decrease the transactivation function of Meq (Chang et al., 2002b).

The Meq/vIL8 protein, first identified as Meq-sp in MKT-1 and MSB-1 cell lines, is a 212 amino acid long splice variant of Meq (Peng and Shirazi, 1996; Peng et al., 1995). The first 100 amino acids of this protein are identical to Meq protein, where as the rest corresponds to receptor binding region of vIL8. This protein lacks transactivation domain and secretor signal of Meq and vIL8, respectively. The Meq/vIL8 could function as a negative regulator of Meq since it is able to bind the *meq* promoter and is also able to interact with c-jun. Furthermore, Meq/vIl8 forms homodimers and localizes in the nucleus, nucleolus and coiled bodies, similar to Meq (Anobile et al., 2006).

Recently, another splice variant of meq, termed Δmeq, was found in MSB-1 cells and CEFs infected with Md5 (Okada et al., 2007). The first 99 amino acids at the N-terminus of the Δmeq protein are identical to Meq, while the remaining 30 amino acids at the C-terminus are from a different reading frame and therefore of different sequence. This novel protein lacks the transactivation domain of Meq, has anti-apoptotic property, dimerizes with Meq and L-Meq and suppresses transcription by both Meq and L-Meq.

2. IN VITRO CHARACTERIZATION OF THE MEQ PROTEINS OF MAREK'S DISEASE VIRUS VACCINE STRAIN CVI988 *

2.1. Introduction

Gallid herpesvirus 2 (GaHV-2), commonly known as Marek's disease virus serotype 1 (MDV-1), causes Marek's disease (MD) in chickens. MDV-1 is classified in the genus *Mardivirus* along with two other non-oncogenic poultry viruses, *Gallid herpesvirus 3* (GaHV-3 or MDV serotype 2) and *Meleagrid herpesvirus 1* (MeHV-1, MDV serotype 3 or Turkey herpesvirus) in the subfamily *Alphaherpesvirinae*. MD is characterized by T cell tumors, partial or complete paralysis of legs and wings, immunosuppression, skin leukosis, depression, and death (Calnek, 2001; Marek, 1907; Pappenheimer et al., 1929). MDV is prevalent in commercial poultry due to its high infectivity and long-term survivability outside its host and is also responsible for significant economic losses worldwide. Based on the severity of the disease in vaccinated chickens, MDV-1 strains in North America are classified as mild (m), virulent (v), very virulent (vv) and very virulent plus (vv+) (Witter, 1997). MDV replicates in B and T lymphocytes during early cytolytic infection and subsequently establishes latency, a common feature of herpesvirus infections, in T lymphocytes. The virus transforms activated T lymphocytes that infiltrate several visceral organs, peripheral nerves, and skin as early as 3 weeks post infection (Calnek, 2001).

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The MDV genome consists of a unique long (U_L), and a unique short (U_S) region flanked by inverted repeat regions, commonly referred to as terminal and internal repeats ($TR_{L,}$ IR_L, IR_S and TR_S) and the sizes vary depending upon the strain; HVT strain FC-126 (159,160-bp)(Afonso et al., 2001), MDV-2 strain HPRS24 (164,270-bp) (Izumiya et al., 2001), MDV-1 strain GA (174,000-bp) (Lee et al., 2000a), and MDV-1 strain Md5 (177,874 bp) (Lee et al., 2000a; Tulman et al., 2000). The organization of the MDV genome resembles that of human herpesvirus 1 (HSV-1) with a co-linear organization of genes in both the unique long and unique short regions. MDV-1 specific genes, such as vIL-8 (Cui et al., 2004; Parcells et al., 2001), viral telomerase RNA (vTR) (Fragnet et al., 2003), viral lipase (Kamil et al., 2005), *meq* (Brown et al., 2006; Lupiani et al., 2004), and *pp38* (Reddy et al., 2002), have been implicated in pathogenesis. Only MDV-1 strains are oncogenic and code for a unique basic leucine zipper (bZIP) protein, Meq (MDV *Eco*RI Q fragment). Two identical copies of the *meq* oncogene are present in the repeat long regions ($TR_{L,}$ and IR_L) and is commonly expressed in MDV lymphoma cells (Jones et al., 1992). Meq has been shown to transform fibroblast cell lines (Rat-2 and DF-1 cells), protect fibroblasts against serum starvation and apoptosis inducers, as well as promote proliferation of cells, all characteristics of oncoproteins (Levy et al., 2003a; Levy et al., 2005; Liu et al., 1998). More importantly, recent work in our lab with a MDV mutant virus lacking both copies of *meq* has conclusively shown that Meq is required for transformation of T lymphocytes, but is not necessary for early viral replication (Lupiani et al., 2004).

Meq is 339 amino acids long and contains a basic amino acid rich DNA binding domain at the amino terminus (1-120) and a proline rich transactivation domain at the carboxy terminus (121-339). Meq also contains both nucleus and nucleolus localization signals (Liu et al., 1997) and co-localizes with Cdk2 in coiled bodies (Liu et al., 1999b)., Like other bZIP proteins, Meq posseses transactivation activity and is capable of forming homodimers with itself as well as heterodimers with other bZIP proteins such as c-Jun. Meq homodimers have been shown to repress MDV early promoters such as *pp38* and *pp14* while Meq/c-Jun heterodimers have been reported to activate the *meq* promoter (Levy et al., 2003b). Meq, like v-Jun, has also been shown to increase transcription of genes involved in growth and anti-apoptosis, suggesting the Jun pathway is involved in MDV transformation (Levy et al., 2005). In addition, Meq has been shown to interact with a cellular co-repressor, C-terminal-binding protein (CtBP), and this interaction is essential for MDV oncogenesis (Brown et al., 2006).

MD vaccines are effective in the prevention of tumor development but not infection. Studies have shown that field isolates continuously evolve towards greater virulence (Witter, 1997), and vaccination has been suggested as one of the driving forces in this evolution (Schat and Baranowski, 2007; Witter, 1998). Consequently, current vaccines may not be effective against MD in the near future. Understanding the molecular mechanisms of T cell transformation by this virus is critical for designing the next generation of vaccines against these evolving MD viruses. Several researchers have explored the use of cell culture attenuated MDV-1 strains as vaccines with little success (Churchill et al., 1969a; Nazerian, 1970; Rispens et al., 1972; Vielitz and Landgraf,

1971; Witter, 1982). Currently, attenuated CVI988/Rispens MDV-1 strain is used as a vaccine in the USA, and in Europe (Baigent et al., 2006) and offers best protection. Interestingly, although attenuated vaccine strains of CVI988/Rispens encode and express Meq, they are non-oncogenic in chickens. Additionally, CVI988/Rispens encode and expresses a longer form of Meq (LMeq, 398 amino acids), which contains 59 amino acids in the form of a proline rich repeat in the transactivation domain (Chang et al., 2002a). Comparison of the predicted amino acid sequences of Meq from the very virulent strain Md5 and the vaccine strain CVI988/Rispens revealed 2 and 4 amino acid differences in the DNA binding and transactivation domains, respectively (Shamblin et al., 2004) (Fig. 5); however, the significance of these differences is currently unknown.

The objective of the current study was to determine how these amino acid differences affect the *in vitro* transformation and transactivation properties of Meq proteins expressed by CVI988/Rispens. Our results indicate Meq proteins from both very virulent Md5 and vaccine CVI988/Rispens strains were capable of transforming Rat-2 and NIH 3T3 cells *in vitro*. Only Md5-Meq but not CVI Meq proteins transactivated the Md5-*meq* promoter in a dose dependent manner. Interestingly, in the presence of chicken c-Jun (CK-Jun), CVI-Meq and CVI-LMeq also activated the *meq* promoter. Chimeric Meq proteins in which DNA binding and transactivation domains were exchanged between Md5 Meq and CVI Meq, significantly transactivated the *meq* promoter. Residues at positions 71 (S) and 320 (T) in Md5 Meq protein were found to be critical for its function. All three Meq proteins suppressed expression from the bidirectional promoter of the MDV (pp38/pp14) and activated MDV late promoter *gB*;

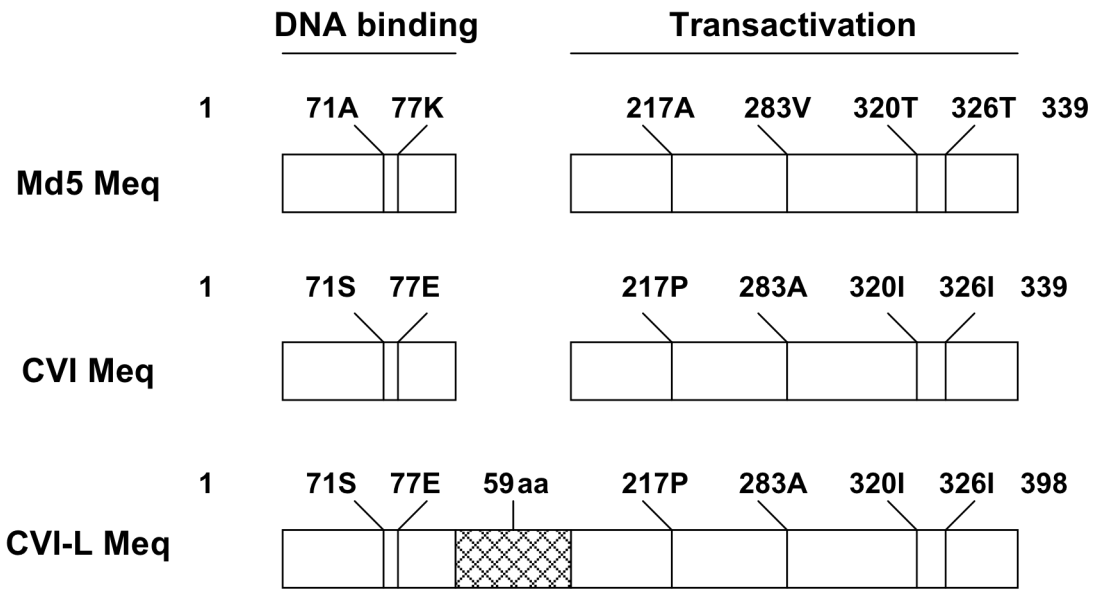

Fig. 5. Structure of the Meq proteins of Md5 and CVI988/Rispens strains. Meq contains an amino-terminal DNA binding domain and a carboxy-terminal transactivation domain. The DNA binding domain contains localization signals for nucleus and nucleolus and a leucine zipper for dimerization with itself and other bZIP proteins. The transactivation domain is rich in proline and is essential for its transactivation function. CVI-Meq proteins have 2 amino acid differences in the DNA binding domain and 4 amino acids differences in the transactivation domain when compared to Md5-Meq. In addition, CVI-LMeq has a 59 amino acid insertion that is rich in proline.

however, in general, higher activation was obtained with Md5-Meq. Similar results were obtained for cellular *MMP-3*, and *Bcl-2* promoters. Our findings indicate Meq proteins from CVI988/Rispens vaccine strain differ from Md5 Meq protein in transactivation activity but not in *in vitro* transformation properties. Based on these results, the lack of oncogenicity of CVI988/Rispens in chickens may be more related to transactivation potential than fibroblast-transforming ability of Meq protein.

2.2. Materials and methods

2.2.1. Cell culture

DF-1, a chicken embryo fibroblast continuous cell line, was maintained in Leibowitz-McCoy media supplemented with 4% fetal bovine serum (FBS) and penicillin-streptomycin at 37°C. Cell lines 293T (human embryonic kidney cells containing SV40 T antigen), NIH 3T3 (mouse embryonic fibroblast) and Rat-2 (rat embryo) were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10% FBS and penicillin-streptomycin at 37°C.

2.2.2. Cloning of meq and chicken c-jun (CK-jun) genes

The open reading frames of meq gene from Md5 (Md5-*meq*) and CVI988/Ripens (CVI-*meq* and CVI-L*meq*) were PCR amplified from viral DNA using primers (SR1118 forward: 5'-GAT**CCCGGG**GAGATGTCTCAGG AGCCAGAG-3') and (SR1135 reverse: 5'- GAT**CCCGGG**TCAGGGTCTCCCGTCA CCTGGAAACC-3') containing *Sma*I sites (indicated in bold) and cloned into pZero vector (Invitrogen, Carlsbad, CA). Following sequencing, all *meq* genes were subcloned into the *Pme*I site of pcDNA 3.1 (Invitrogen, Carlsbad, CA) to generate pMd5-Meq, pCVI-Meq and pCVI-LMeq vectors for transactivation assays and into *Sna*BI site of pBabe vector (kind gift from Dr. David Everly, University of North Carolina) to generate pB-Md5-Meq, pB-CVI-Meq and pB-CVI-LMeq vectors for transformation assays.

Three chimeric Meq protein expression vectors [pMd5-CVI-Meq (DNA binding domain of Md5-Meq protein and transactivation domain of CVI-Meq protein), pCVI-Md5-Meq (DNA binding domain of CVI-Meq protein and transactivation domain of

Md5-Meq protein), and pMd5-CVI-L (DNA binding domain of Md5-Meq protein and transactivation domain of CVI-LMeq protein)] were constructed by exchanging DNA binding domains. Briefly, pMd5-CVI-Meq was constructed as follows: the *Kpn*I fragment region of pCR2.1-EcoQ-CVI was replaced with the *Kpn*I fragment derived from pCR2.1-EcoQ-Md5 (MDV EcoQ fragment contains the *meq* gene). The chimeric Md5-CVI- Meq gene was then PCR amplified using SR1118 and SR1135 primers and cloned in pZero vector. Following digestion with *Sma*I, the Md5/CVI meq gene was ligated to pcDNA 3.1 vector cut with *Pme*I. The resulting construct was designated as pMd5-CVI-Meq. The pCVI-Md5-Meq and pMd5-CVI-L chimeric vectors were constructed using the same strategy as for the pMd5-CVI-Meq and the accuracy of all the chimeric genes was confirmed by sequencing of these constructs.

Six single amino acid Md5-Meq mutants (**A**71S, **K**77E, **A**217P, **V**283A, **T**320I and **T**326I) were constructed using the Quikchange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). Residues in Md5-Meq and corresponding residues in CVI-Meq are indicated in bold and underlined, respectively. All mutations were verified by DNA sequencing.

The chicken *c-jun* gene (*CK-jun*) was cloned as follows: RNA was isolated from DF-1 cells using the Qiagen RNeasy kit (Qiagen, Valencia, CA) and cDNA of *CK-jun* was made using gene specific primers (BL1227 forward: 5' GA**CCCGGG**ATGAGTCA AAGATGGAGCCTAC-3' and BL1228 reverse: 5'GA**CCCGG G**TCAAAACGTTTGC AACTGTTGTG-3') containing *Sma*I sites (indicated in bold). A full-length *CK-jun*

cDNA was cloned into the *Pme*I site of pcDNA following confirmation by sequencing and the resultant recombinant plasmid was named pCK-Jun.

2.2.3. Retrovirus production and transduction

Replication incompetent murine retroviruses carrying the meq genes of Md5 and CVI988/Rispens were constructed as previously described (Everly et al., 2004). Briefly, 5 x 10^6 293T cells were seeded in a 75 cm^2 tissue culture flask one day prior to transfection and cultured overnight at 37°C. Using siPORT-XP-1 transfection reagent (Ambion, Austin, TX), 6 μg of control pBabe, pB-Md5-Meq, pB-CVI-Meq, or pB-CVI-Meq-L; 6 μg of pG1-VSV-G (VSV-G expression vector); and 6 μg of pGPZ9 (murine leukemia virus Gag-Pol expression vector) were triple-transfected into 293T cells. The culture media was changed the following day and cells were transferred to 33°C**.** Culture supernatants were collected 48 hr post transfection and centrifuged at 1,000 x*g* for 10 minutes. NIH 3T3 cells and Rat-2 cells were transduced overnight with clarified culture supernatants in the presence of polybrene (8 μg/ml) (Sigma, St Louis, MO). Cell lines were established by selecting transduced cells with puromycin (4 μg/ml) (InvivoGen, San Diego, CA) and later maintained in DMEM supplemented with 10% FBS and puromycin (2 μg/ml).

2.2.4. Immunofluorescence

Immunofluorescence was performed as previously described with modifications (Lee et al., 2003). Briefly, transduced cells were washed with PBS and fixed with ethanol:acetone solution (6:4) at 37°C for 10 min. After removing fixing solution, the cells were air-dried, and subsequently incubated with anti-Meq rabbit polyclonal serum

(1:200) for 1 hr at 37°C. Following three washes with PBS, cells were incubated with goat anti-rabbit FITC labeled secondary antibodies (KPL, Gaithersburg, MD) for 1 hr. Cells were then washed 3 times with PBS and examined under a fluorescence microscope.

2.2.5. Western blot analysis

Meq transformed Rat-2 or NIH 3T3 cell lines (0.5 million cells) were mixed with SDS-PAGE sample buffer and heated at 95 °C for 5 min. Samples were size-separated by electrophoresis on a 12% SDS-containing polyacrylamide gel followed by transfer of proteins onto a nitrocellulose membrane. After transferring, the membrane was incubated at room temperature for 30 min with blocking buffer (Invitrogen, Carlsbad, CA) and subsequently incubated with rabbit anti-Meq (diluted 1:2000) for 1 hr at room temperature (Lee et al., 2003). The membrane was washed three times with PBST (PBS plus 0.1% Tween-20), 15 min each and then incubated with anti-rabbit HRP-conjugated antibody (1:2500) for 1 hr at room temperature. Following three 15 min washes with PBST, the membrane was incubated with TMB membrane peroxidase substrate system according to manufacturer's instructions (KPL, Gaithersburg, MD).

DF-1 cells or DF-1 cells transfected with pCK-Jun vector were processed for c-jun expression by Western blot analysis as described above. The primary antibody, rabbit anti-c-Jun, was purchased from Upstate Biotechnology (Lake Placid, NY) and used at a 1:1000 dilution.

2.2.6. In vitro transformation assays

For focus formation assay, 4 x 10^6 puromycin selected Rat-2 or NIH 3T3 cells were seeded in 100 cm^2 dishes and allowed to reach confluency after which they were maintained in DMEM supplemented with 0.5% FBS (Rat-2 cells) or 3% FBS (NIH3T3 cells) for 3-4 weeks. For soft agar assay, 5 X 10^3 puromycin selected Rat-2 or NIH 3T3 cells in 0.3 % agar in DMEM supplemented with 10% FBS per well were seeded on a layer of 0.6 % agar in 6 well plates. Cells were cultured for 3-4 weeks, after which foci were stained with crystal violet and photographed. At least three independent experiments were performed in triplicate.

2.2.7. Cloning of viral and cellular promoters

The Md5-*meq* promoter (nucleotides -355 to -1) was amplified from viral DNA as previously described (Chang et al., 2002b) and cloned upstream of the firefly Luciferase gene in the pGL3 vector (Promega, Madison, WI). MDV early promoters (*pp38* and *pp14*) in pGL2 vector have been previously described (Levy et al., 2003b). The pGL2-3XOri vector containing 5' to 3' three tandem copies of the MDV Ori sequence (TGC TCA TTT GCA T**AC ACA** TCA CGT GAT AGT) (Meq binding sequence is indicated in bold), present in the pp38/pp14 bidirectional promoter, was constructed following self-ligation of three MDV Ori sequences and cloning into the pGL-2 vector. Md5 *gB* promoter (nucleotides -661 to -20 upstream of the translation initiation site) (Sonoda et al., 2000) was amplified from Md5 viral DNA using primers (BL1339 forward: 5'-TC**AGATCT**C AAGTCTCACTC ACA AA-3' and BL1340 reverse: 5'-TC**AGATCT** GCTGTTCATAAATT GTGT-3') containing *Bgl*II sites

(indicated in bold) and following sequencing, the gB promoter was cloned into the *Bgl*II site of the pGL3 vector. Similarly, chicken *Bcl-2* promoter (nucleotides -312 to -1 upstream of the translation initiation site) described elsewhere (Lesault et al., 2002) was cloned into the pGL3 vector following amplification from chicken lymphocyte DNA using primers (BL1338 forward: 5'-TC**A GATCT**GACAGCCAGGAGGAAGCG-3' and BL1340 reverse: 5'-TC**AGATCT**TGGG AGGGGGAGAGGAAG-3') containing *Bgl*II sites (indicated in bold). The cellular promoter *MMP-3* (nucleotides -2264 to +37) in pGL3 vector (Kwak et al., 2007) was generously provided by Dr. Weston Porter (Texas A&M University).

2 2.8. Luciferase reporter assays

For the luciferase assay, DF-1 cells were seeded in 12-well plates (10^5 cells per well) a day before transfection. The following day, 125-500 ng of pcDNA vector, pMd5-Meq, pCVI-Meq, or pCVI-LMeq were transfected along with 250 ng of the indicated promoter reporter vectors using siPORT-XP-1 transfection reagent (Ambion, Austin, TX) according to the manufacturer's instructions. The total amount of DNA transfected was kept constant (750 ng) by supplementing test vectors with appropriate amounts of control pcDNA expression vector. Cells were lysed 24 hr later using lysis buffer (Promega, Madison, WI) and firefly luciferase activity was measured using a luminometer (BioTek Instruments, Winooski, VT). Protein concentration for each transfected sample was measured using the Bradford assay (Bio-Rad, Hercules, CA) and luciferase activity was normalized to protein concentration as previously described (Papin et al., 2003). Fold increase in luciferase activity of the different Meq expressing

vectors relative to pCDNA control vector were expressed as the mean ± standard error of three independent experiments performed in triplicate. Results were analyzed using one-way ANOVA followed by Tukey HSD test in SPSS®, Version 14.0 software (SPSS Inc., Chicago, IL, USA). For all analysis, $P \leq 0.05$ was considered statistically significant.

2.2.9. Chromatin immunoprecipitation assay (ChIP)

DF-1 cells (10^6 cells per well) were seeded on a 100 mm plate were transfected with 3μg of *pGL3-meq* promoter together with 3μg of either pMd5-Meq, pCVI-Meq, pCVI-LMeq or pcDNA control vector using siPORT-XP-1 transfection reagent (Ambion, Austin, TX) as per manufacturer's instructions. In parallel experiments, pCK-Jun was included in the transfection mix. The ChIP assay was performed as previously described (Metz et al., 2006). Briefly, 24 hr following transfection, cells were fixed in 1% formaldehyde for 15 min at 37°C and glycine (125 mM final concentration) was added to stop the cross-linking reaction. Cells were washed three times with PBS by centrifuging at 913 x*g* for 4 min and following the last wash, the cell pellet was resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1). Cells were sonicated 10 times for 10 sec with 3 min cooling on ice in between pulses in order to shear the cross-linked chromatin which would result in an average DNA fragment length of 200-1000 bp. Sonicated cells were centrifuged at 15,700 x*g* for 10 min, and soluble cross-linked chromatin was collected and diluted in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 167 mM NaCl) containing protease inhibitor cocktail. Diluted cross-linked chromatin was subsequently precleared with protein G-agarose beads and blocked with sonicated salmon sperm DNA for 30 min

at 4°C. The protein G-agarose beads were removed by centrifugation and an aliquot of supernatant was collected as "input DNA". The remaining chromatin was immunoprecipitated with anti-Meq rabbit polyclonal serum (Lee et al., 2003; Levy et al., 2003b) or control rabbit IgG antibody at 4°C overnight. The following day, the reaction mixture was incubated with protein G-agarose beads at 4°C for 1 h and centrifuged at 100 x*g* for 1 min. Recovered beads were washed once with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl), once with high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl), once with LiCl buffer (0.25 M LiCl, 1% NP40, 1% deoxycholic acid, sodium salt, 1mM EDTA and 10 mM Tris), and twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA). Immunoprecipitated chromatin was subsequently eluted with elution buffer (1% SDS, 0.1M $NaHCO_3$).The anti-Meq and IgG antibody immunoprecipitated as well as "input DNA" samples were incubated at 65°C for 4 hr in order to reverse the cross-linking. Samples were treated with proteinase K for 1 hr at 45°C, DNA precipitated with 2.5 volumes of 100% ethanol, and resuspended in 100 µl of TE buffer. DNA was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and eluted in 50 µl of sterile water. Processed samples were analyzed by standard PCR with pGL3 vector specific primers (SR3391 forward: 5'- GCTCTCCATCAAAACA AAAC-3' and SR3393 reverse: 5'-GTGGCTTTACCA ACAGTACC-3'), which amplify the *meq* promoter cloned into the pGL3 vector.

2.3. Results

2.3.1. Md5-Meq, CVI-Meq and CVI-LMeq proteins transform fibroblasts

Previous work in our laboratory has demonstrated that deletion of the *meq gene* from Md5 inhibited T cell tumor development in chickens confirming a direct role in MDV oncogenesis (Lupiani et al., 2004). Comparison of the predicted amino acid sequences of CVI-Meq and CVI-LMeq from the vaccine strain CVI988/Rispens with the Meq protein of the very virulent Md5 strain revealed two amino acid differences in the DNA binding domain (positions 71, and 77) and four amino acid differences in the transactivation domain (positions 217, 283, 320, and, 326) (Fig. 5). Additionally, CVI-LMeq had a 59 amino acid insertion corresponding to the proline rich repeat region (Fig. 5). Since attenuated CVI988/Rispens is non-oncogenic in chickens, CVI-Meq and CVI-LMeq were evaluated for transformation capabilities *in vitro*. Rat-2 and NIH3T3 cell lines expressing Md5-Meq, CVI-Meq, and CVI-LMeq proteins were developed using a murine retrovirus system. Integration of the murine retrovirus expression vector in these cell lines was confirmed by PCR and transcription of the *meq* genes was analyzed by RT-PCR using gene specific primers (data not shown). Expression of all three Meq proteins in transduced and selected cell lines was confirmed by Western blot (Fig. 6A) and immunofluorescence (Fig. 6B) assays. Both cell lines expressed Meq abundantly, predominantly in the nucleus. Rat-2 cells expressing all three Meq proteins formed foci only when grown under low serum conditions (0.5%) (Fig. 7). NIH 3T3 cell lines expressing all three Meq proteins formed foci when grown with 3% serum (Fig. 7). Rat-2 cells and NIH3T3 cells expressing all three Meq proteins in anchorage independent

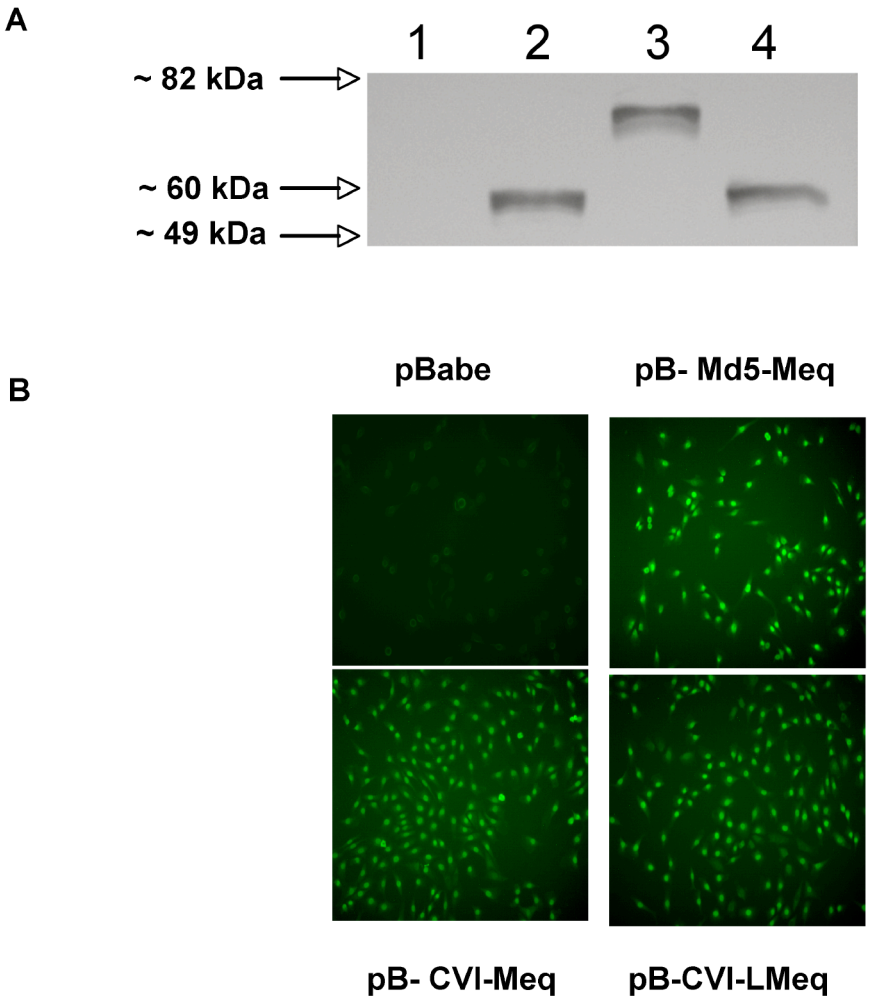

Fig. 6. Meq expression in cell lines. Six microgram of each of three plasmids (pBabe control vector or pBabe expressing Md5-Meq, CVI-Meq or CVI-LMeq proteins, pCMV-Gag-Pol and pCMV-VSV-G) were transfected into 293T cells and the supernatant containing replication incompetent virus was collected 48 hr post transfection. The clarified media was used to transduce Rat-2 or NIH3T3 cells. Following puromycin selection, the expression of Meq under the control of the viral LTR was confirmed in both Rat-2 and NIH3T3 cells by Western blot (A). (1) pBabe empty vector, (2) pB-Md5 Meq, (3) pB-CVI-LMeq, and (4) pB-CVI Meq. Expression of Meq in these cell lines were also confirmed by immunoflurosence (B) using Meq specific rabbit polyclonal sera.

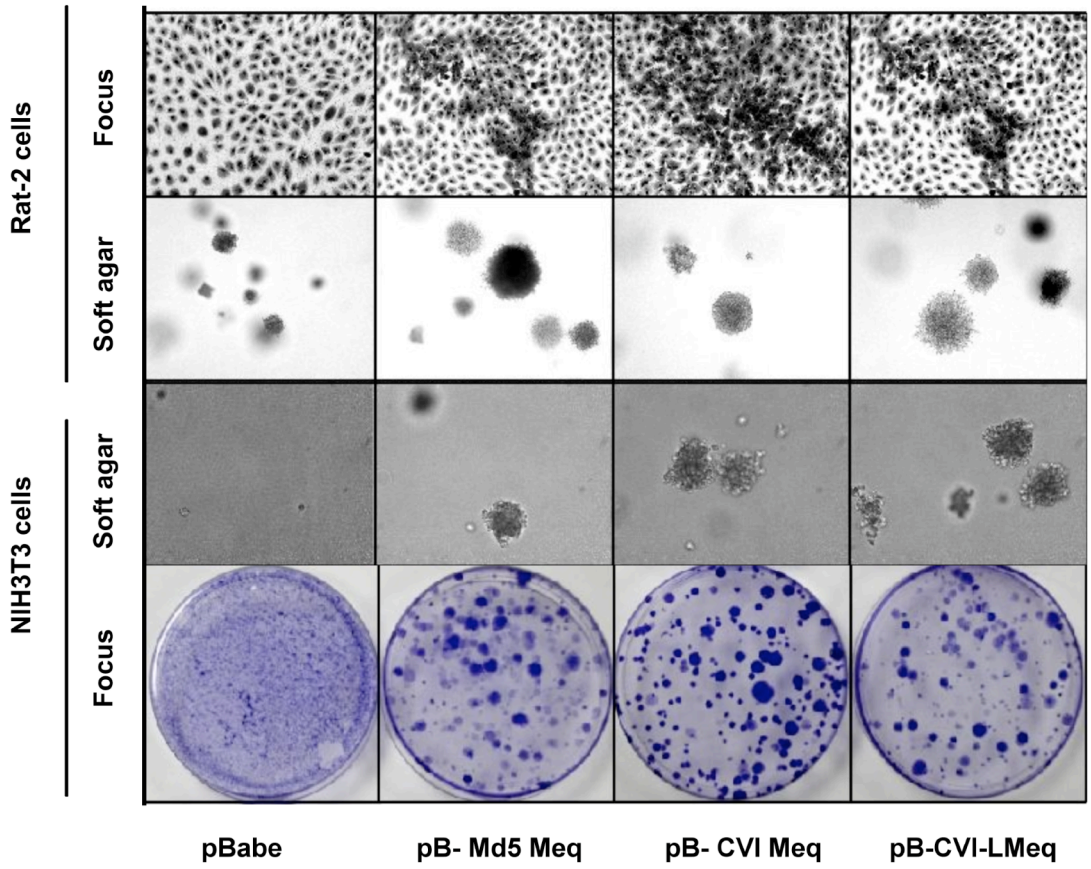

Fig. 7. Md5-Meq, CVI-Meq and CVI-Meq-L transform both Rat-2 and NIH3T3 cell lines. Puromycin selected Rat-2 and NIH 3T3 cells expressing Meq proteins were tested for transformation phenotype in two assays: focus and soft agar. Rat-2 cells expressing Meq proteins formed foci when grown under 0.5% serum and NIH3T3 cells expressing Meq proteins formed foci in 3% serum conditions. Both cell lines expressing Meq proteins formed colonies in soft agar under 10% serum conditions. Foci and soft agar colonies were stained with crystal violet and photographed. Results presented here are representative of three independent experiments.

growth formed colonies under 10% serum conditions (Fig. 7). Fewer background colonies due to control pBabe vector were observed with NIH3T3 cells when compared to Rat-2 cells in the soft agar assay. Our results indicate that Md5-Meq, CVI-Meq, and CVI-LMeq proteins are able to transform fibroblasts *in vitro* despite the amino acid

differences found in the DNA binding and transactivation domains.

2.3.2. Md5 Meq is a potent transactivator of its own promoter

Earlier studies have shown that Meq is a transcriptional activator of its own promoter (Levy et al., 2003b; Qian et al., 1995). Therefore, we wanted to determine if the Meq proteins of CVI988/Rispens have transactivation functions similar to or different from Md5-Meq on the *meq* promoter. Md5-*meq*, CVI-*meq* and CVI-L*meq* were cloned into the pcDNA eukaryotic expression vector, and the expression of Meq in DF-1 cells was confirmed by immunofluorescence (data not shown). DF-1 cells were co-transfected with *pGL3-meq* promoter and pcDNA vector coding one of the three Meq proteins or without any insert. In our preliminary experiments, Meq proteins, expressed from pcDNA vector, influenced the expression of renilla luciferase from pRL-SV40 (normalizing control), probably due to Meq binding to AP-1 sites in SV40 promoter, causing errors in the analysis of the results as previously reported (Huszar et al., 2001). To address this problem, we normalized the luciferase values to the total protein concentration of the cell lysates and expressed the results as fold activation relative to the vector control (Papin et al., 2003).

As shown in Fig. 8A, only Md5-Meq activated the Md5-*meq* promoter in a dose dependent manner while CVI-Meq and CVI-LMeq had no effect (Fig. 8A). Md5-Meq driven activation of the *meq* promoter was statistically significant compared to the control vector and CVI-Meq expressing vectors. These data indicate that Md5- and CVI-Meq proteins differ in their ability to transactivate their own promoter.

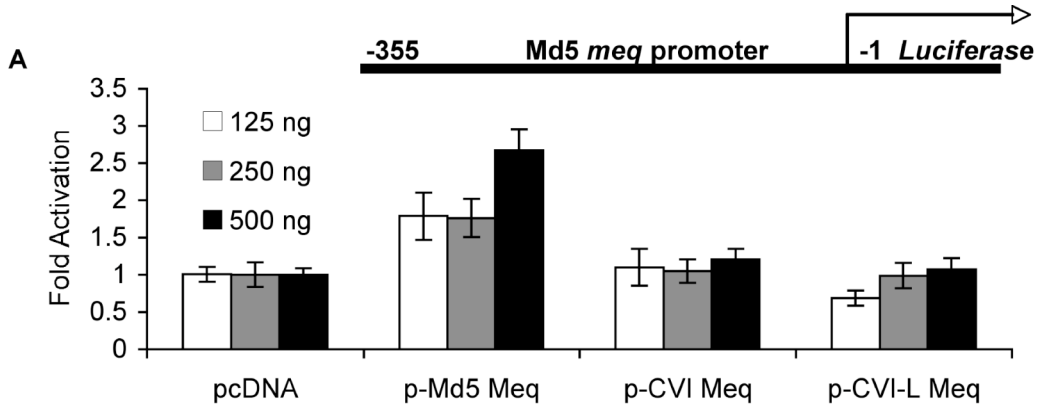

Fig. 8. Md5-Meq is a potent transactivator of its own promoter. (A) Effect of Meq proteins on its own promoter. The *pGL3-meq*promoter construct (250 ng) was co-transfected into DF-1 cells along with 125-500 ng of pcDNA or pcDNA expressing Md5-Meq, CVI-Meq or CVI-LMeq and 24 hr post-transfection luciferase activity was measured. The luciferase values were normalized to total protein concentration and fold activation relative to control pCDNA vector was used to create the graph. The experiment was done three times in triplicates. Error bars indicate SEM. (B) Expression of c-jun. Expression of c-jun from pCK-Jun vector (lane 1) and untransfected DF-1 cells (lane 2) was examined by Western blot using antisera against c-jun. (C) Transactivation of Meq in the presence of over expressed c-jun. Control vector or vectors expressing different Meq proteins (500 ng) were co-transfected along with pGL3-*meq* promoter reporter vector (250 ng) into DF-1 cells. Simultaneously, 0-500 ng of pCK-jun was transfected for a dose response assay. Luciferase activity was measured 24 hr following transfection. Fold activation was calculated relative to control vector. The experiment was done three times in triplicates. Error bars indicate SEM. (D) Transrepression of Md5-Meq when co-expressed with CVI-Meq or CVI-Meq-L. Five hundred ng of pMd5-Meq was co-transfected along with 0-500 ng of pCVI-Meq or pCVI-LMeq long in DF-1 cells together with pGL3-*meq* promoter (250 ng) and 24 hr post transfection luciferase activity was measured. Values were expressed as fold activation relative to pcDNA vector. The luciferase activity of the control vector was assigned a value of 1. The experiment was done three times in triplicates. Error bars indicate SEM.

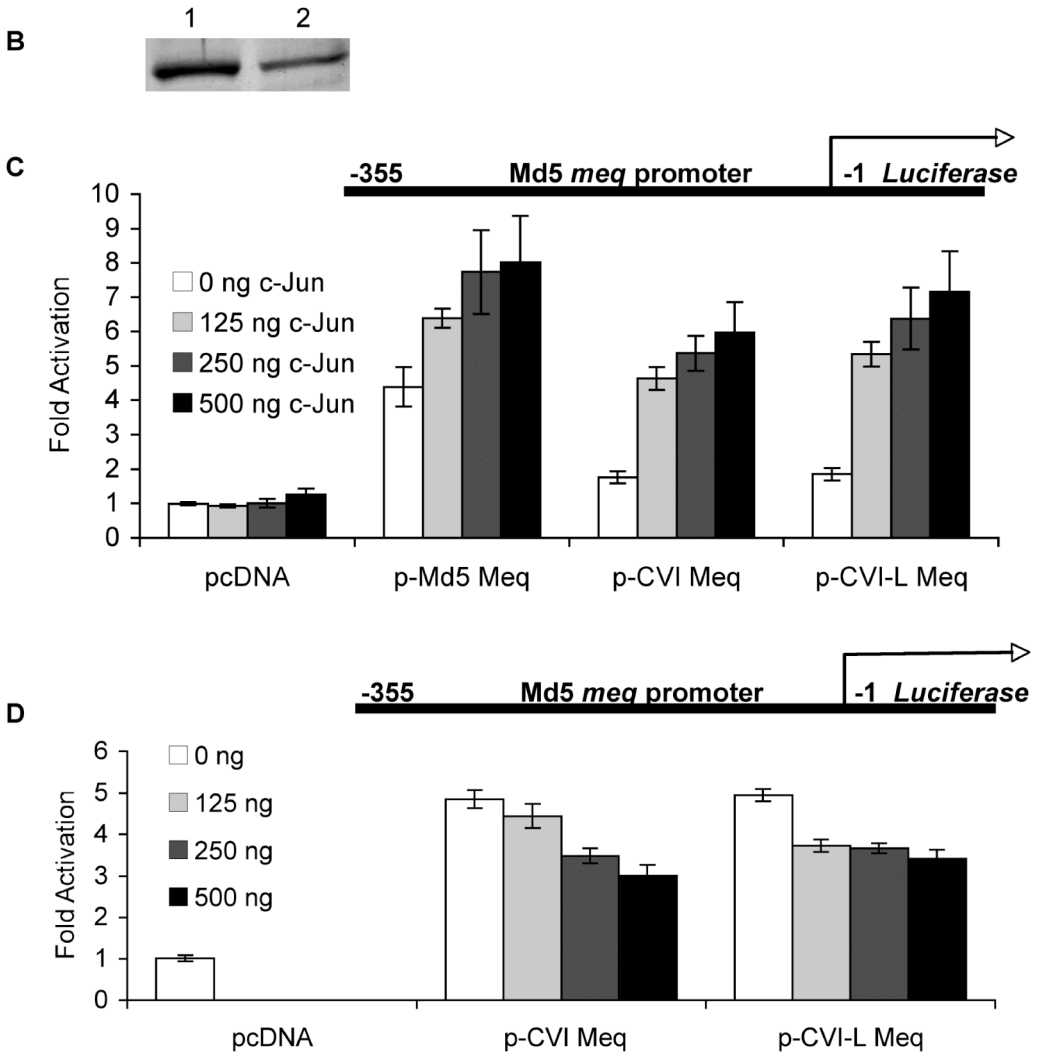

Fig. 8. Continued

Previous studies have shown c-Jun to be a major interacting bZIP protein for Meq and Meq/c-Jun interaction has been shown to increase the DNA binding activity of Meq to the AP-1 like sequences present in the *meq* promoter (Levy et al., 2003b). To test

whether CVI-Meq and CVI-LMeq are able to activate the *meq* promoter in the presence of CK-Jun, a luciferase assay was performed with triple-transfections of *pGL3-meq* promoter, pCK-Jun, and pcDNA vector expressing one of the three Meq proteins or control vector into DF-1 cells. Over expression of c-jun from pCK-Jun vector was confirmed by Western blot analysis (Fig. 8B). The results presented in Fig. 8C indicate that CK-Jun alone did not activate the *meq* promoter in the absence of Meq. Significant activation of the *meq* promoter with Md5-Meq was observed with increasing concentrations of transfected pCK-Jun. Likewise, both CVI-Meq, and CVI-LMeq activated the *meq* promoter in a dose dependent manner in the presence of tranfected pCK-Jun (Fig. 8C). Therefore, cotransfection with pCK-Jun is essential for CVI-Meq proteins but not for Md5-Meq to activate their own promoter.

Because CVI-Meq proteins could not activate their own promoter and thus could interfere with the functions of Md5-Meq in case of mixed infection of CVI988/Rispens and Md5 viruses, we chose to investigate the transactivation function of Md5-Meq in the presence of CVI-Meq proteins. DF-1 cells were triple-transfected with *pGL3-meq* promoter, pMd5-Meq (500 ng), and increasing amounts of pCVI-Meq or pCVI-LMeq vectors (0-500 ng). Cells were collected 24 hr following transfection and lysed in order to determine luciferase activity. Our results show that both CVI-Meq and CVI-LMeq decreased the Md5-Meq mediated activation of the *meq* promoter in a dose-dependent manner (Fig. 8D). These data indicate CVI-Meq proteins could interfere with the expression of Md5-Meq in case of a mixed infection of pathogenic and CVI988/Rispens vaccines in chickens.

Taken together, the above results show CVI-Meq proteins activate the Md5 *meq* promoter only in the presence of CK-Jun, while Md5-Meq activates its own promoter both in the presence or absence of over-expressed CK-Jun. Furthermore, the transactivation function of Md5-Meq is suppressed by CVI-Meq proteins in a dose dependent manner.

2.3.3. All three Meq proteins bind the meq promoter

Because of the observation that CVI-Meq proteins could not transactivate their own promoter in the absence of CK-Jun, we wanted to test whether both CVI-Meq proteins are able to bind the *meq* promoter and whether this binding requires CK-Jun. DF-1 cells were transiently transfected with *pGL3-meq* promoter and pcDNA vector control or vectors expressing Md5-Meq, CVI-Meq, or CVI-LMeq in the presence or absence of pCK-Jun vector. Cells were collected 24 hr post transfection and processed for ChIP assay using anti-Meq polyclonal rabbit serum. “Input DNA” samples and all immunoprecipitates were PCR amplified using primers designed based on sequences external to the Md5-*meq* promoter in the pGL3 vector. As shown in Fig. 9, all three Meq proteins examined bind the Md5-*meq* promoter in the absence (Fig. 9A) or presence (Fig. 9B) of co-expressed CK-Jun. Amplification for the pcDNA vector control was observed in the “Input DNA” but not in the Meq immunoprecipitated sample, indicating that the bands observed in the Meq transfected samples were obtained by specific immunoprecipitation of Meq bound to the *meq* promoter. Samples immunoprecipitated with IgG control antibody were negative thereby confirming the specificity of the Meq antibodies.

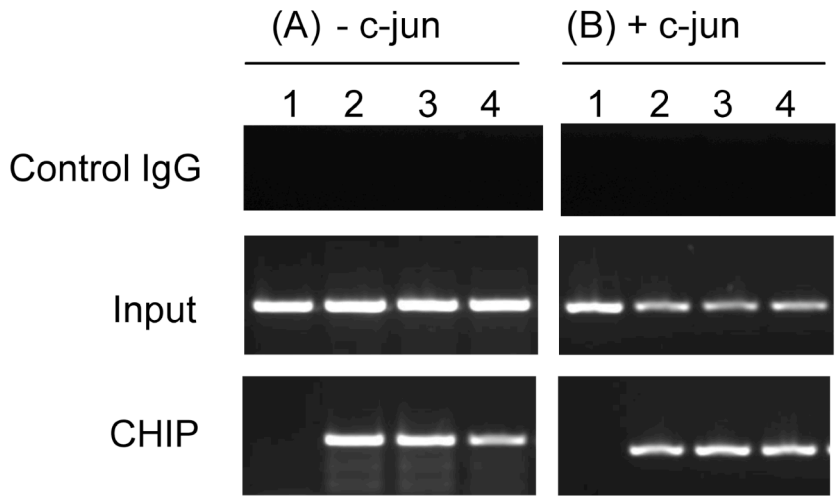

Fig. 9. Md5-Meq and CVI-Meq proteins bind the *meq* promoter both in the presence or absence of c-Jun. DF-1 cells were transfected with pGL3-*meq* promoter, and one of pcDNA Meq expression or control vector in the absence (A) or presence (B) of pCK-Jun. Cells were harvested and processed for ChIP assay as described in materials and methods. Input, rabbit IgG serum immunoprecipitated, and anti-Meq serum immunoprecipitated DNA were PCR amplified using pGL3 vector specific primers. Lane 1, Control vector; lane 2, pMd5-Meq; lane 3; pCVI-Meq; lane 4, pCVI-LMeq.

These findings indicate that the amino acid differences observed in the DNA binding domain of CVI-Meq proteins do not affect DNA binding activity.

2.3.4. Chimeric Meq proteins activate the meq promoter

Since Meq proteins of CVI988 strain did not activate the *meq* promoter, we constructed chimeric Meq proteins by exchanging DNA binding domains among the three Meq proteins to better understand the role of the DNA binding domain and transactivation domain in transactivation. Each of the three chimeric constructs (pMd5-CVI-Meq, pCVI-Md5-Meq, and pMd5-CVI-L) was transfected along with pGL3-meq promoter into DF-1 cells and luciferase activity determined 24 hr post transfection. All

three chimeric Meq proteins significantly increased the *meq* promoter activity compared to control vector and CVI-Meq proteins expressing vectors, although to a significantly lower level than did parental Md5-Meq protein (Fig. 10A). We conclude from these results that neither domain of the Md5-Meq protein is capable of fully restoring the transcriptional activity of CVI-Meq proteins, therefore both domains are required for maximum transactivation function of the Md5-Meq protein.

2.3.5. Residues at 71 and 320 of Md5-Meq protein play a role in transactivation

As shown in Fig. 5, Md5-Meq differs from CVI-Meq protein by six amino acid residues, two in the DNA binding domain and four in the transactivation domain. In order to determine the role of these residues in the transactivation activity of Meq, we used site-directed mutagenesis to generate six Md5-Meq mutants, in which one single amino acid had been replaced with that of CVI-Meq. Transfection of DF-1 cells with each of these six mutants (A71S, K77E, A217P, V283A, T320I and T326I) together with the pGL3-*meq* promoter was performed and cells were collected for assessing luciferase activity. Mutations at residues 77, 217, 283 and 326 did not significantly affect the ability of Md5-Meq protein to transactivate the Md5 *meq* promoter (Fig. 10B). In contrast, like CVI-Meq proteins, Md5-Meq mutants A71S and T320I, failed to transactivate the *meq* promoter (Fig. 10B). These data indicate alanine at position 71 and threonine at position 320 are important for Md5-Meq transactivation of its own promoter.

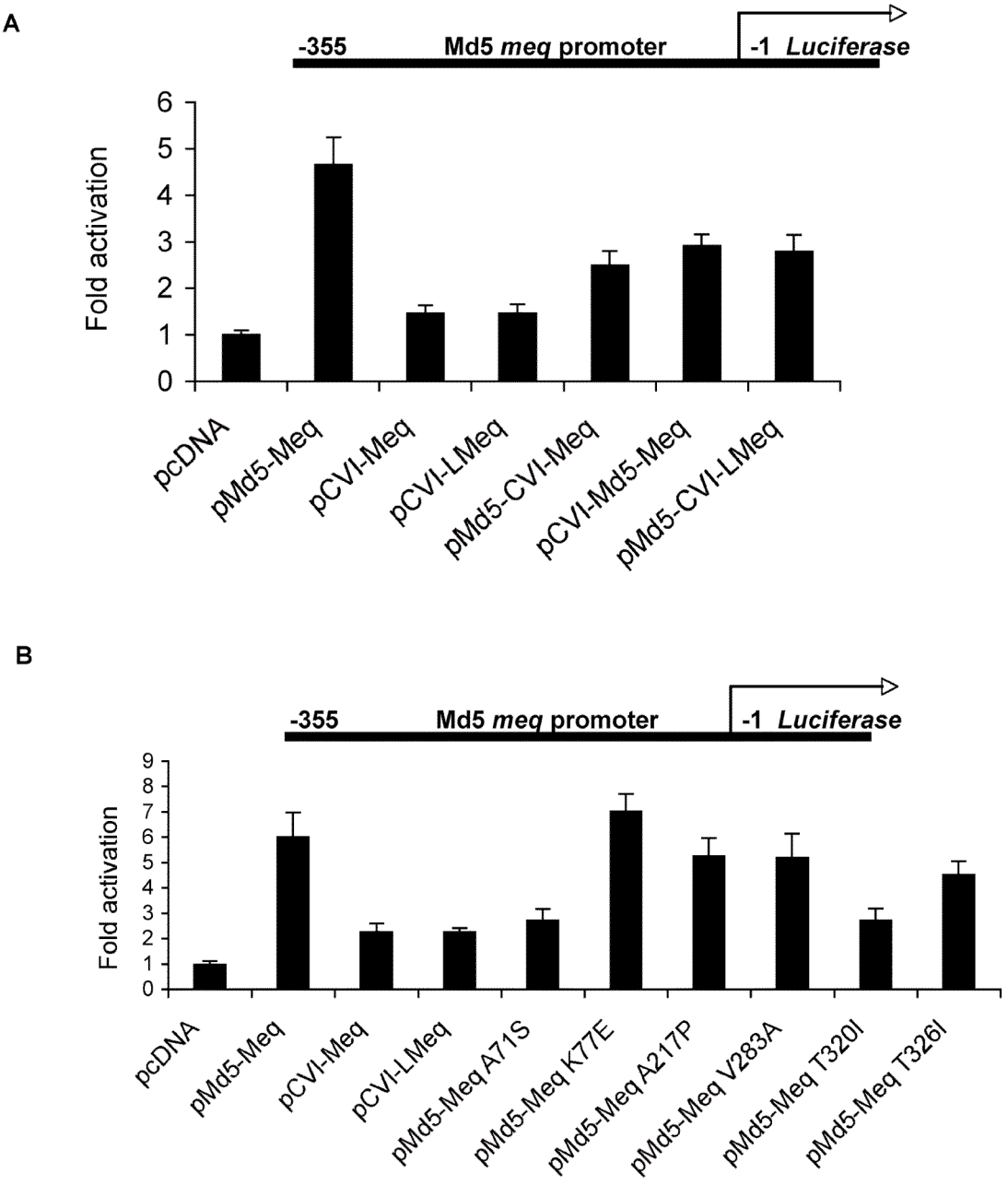

Fig. 10. Transactivation of the *meq* promoter by chimeric Meq proteins and Meq single amino acid mutants. DF-1 cells were co-transfected with either 250 ng of pGL3-meq promoter and 500 ng of one of Chimeric Meq expressing vectors (A) or Meq single amino acid mutants (B). Twenty four hours after the transfection, cells were collected and analyzed for luciferase activity. Values were reported as fold activation relative to the luciferase activity of vector vector. The experiment was done three times in triplicate. Error bars indicate SEM.

2.3.6. All three Meq proteins suppress the MDV pp38/pp14 bidirectional early promoter

MDV early proteins pp38/24 and pp14 are expressed in opposite directions from a bi-directional promoter, which is located in the region of MDV origin of replication (MDV-Ori). Meq/Meq homodimers have been shown to suppress gene expression from this bi-directional promoter (Levy et al., 2003b). We asked whether Md5-Meq, CVI-Meq and CVI-LMeq proteins have the same suppressive effect on this promoter. Transfection of pGL2-*pp38* or pGL2-*pp14* promoters together with pcDNA vectors expressing each of the Meq proteins, was performed in DF-1 cells. Although statistically not significant, our results show that all three Meq proteins suppressed transcription from both *pp38* (Fig. 11A) and *pp14* (Fig. 11B) promoters. Since the Meq binding motif in this region has been identified as an ACACA motif (Levy et al., 2003b), in order to determine whether the suppressive effect of Meq proteins on these promoters is due to binding the ACACA motif, we cloned a 3X MDV Ori sequence into the luciferase reporter vector pGL2 and repeated the luciferase assay with pGL2-3XOri vector. Our results show that all three Meq proteins suppressed transcription from this construct, which was statistically significant when compared to the control vector (Fig. 11C). This indicates that all three Meq proteins are capable of binding to ACACA sequence present in the bidirectional promoter and this interaction might be the cause of transcriptional suppression of this promoter.

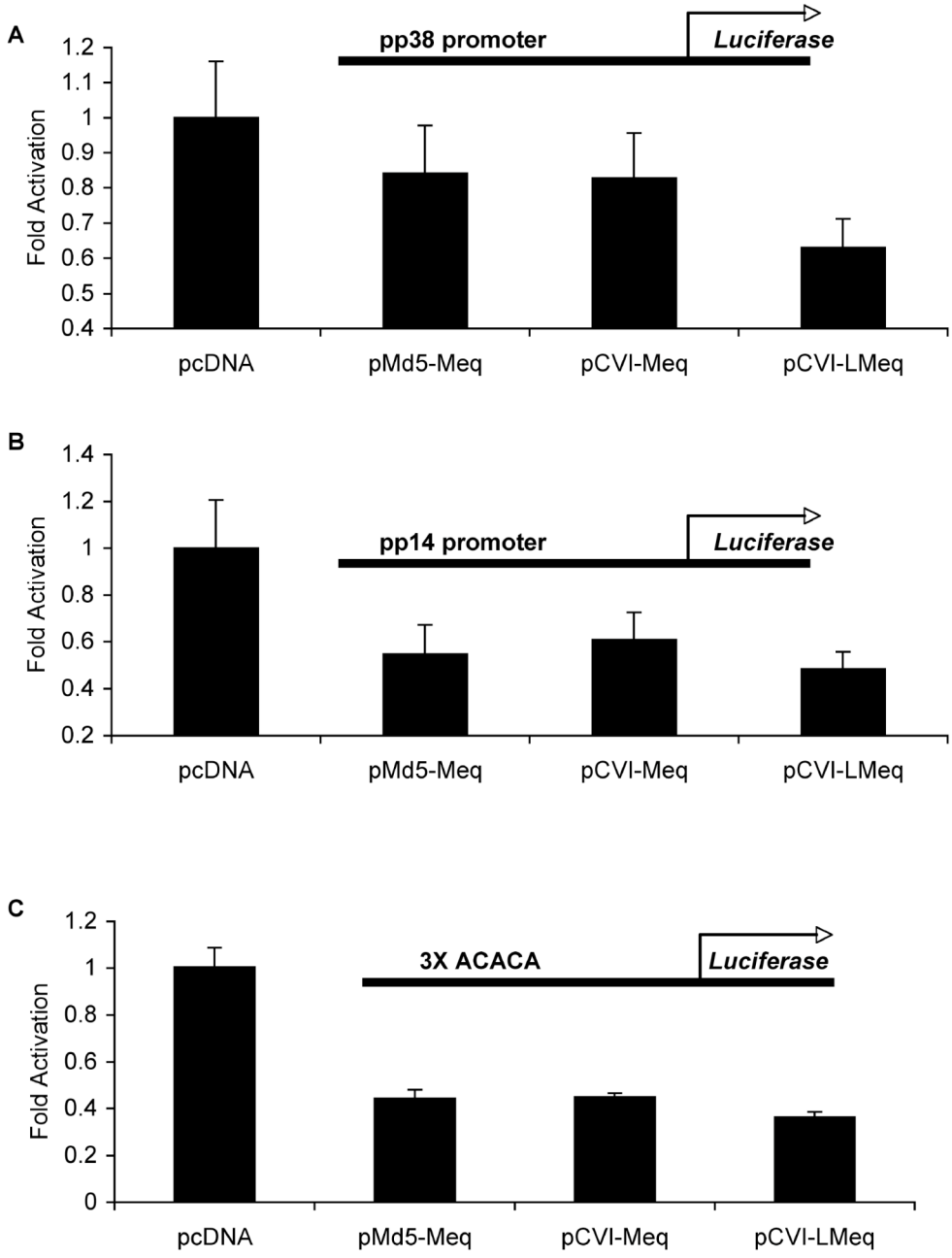

Fig. 11. Transrepression by Meq of MDV-1 bi-directional promoter (*pp38* and *pp14*) and Meq binding motif (ACACA) in MDV-1 Ori. Two hundred and fifty ng of pGL2-*pp38* promoter (A) pGL2-*pp14* promoter (B) or pGL2-3XOri vector (C) were co-transfected with 125 ng of control vector or vector expressing one of three Meq proteins in DF-1 cells. Luciferase activity was measured 24 hr later and fold activation relative to control vector indicated in the graph represents mean of three experiments done in triplicate. Error bars indicate SEM.

2.3.7. Activation of MDV gB late promoter by Meq proteins

Glycoprotein B (gB) is one of the major MDV glycoproteins and has been shown to be essential for viral replication (Schumacher et al., 2000). Using a ChIP assay, a previous study has shown Meq binds to the MDV gB promoter (Levy et al., 2003b). In order to evaluate the functional significance of this binding, we cloned the *gB* promoter of Md5 into the pGL3 luciferase reporter vector. This promoter contains an AP-1 like sequence (AGTCA), where Meq might bind. Plasmids expressing Md5-Meq, CVI-Meq, and CVI-LMeq proteins or control pCDNA vector were co-transfected into DF-1 cells along with the pGL3-*gB* promoter reporter vector and the luciferase activity was determined in the cell lysates 24 h after transfection. As shown in Fig. 12, there was a statistically significant difference between Md5-Meq and CVI-Meq proteins even though all three Meq proteins activated MDV *gB* promoter. Our results show for the first time that Meq is capable of transactivating the MDV *gB* late promoter and thus may play a role in viral replication.

2.3.8. Activation of cellular MMP-3 and Bcl-2 promoters by Meq proteins

T cell tumors produced by Marek's disease are metastatic in nature and infiltrate various internal organs. Breakdown of stromal extracellular matrix (ECM) by matrix metallo-proteinases (MMPs) is critical for tumor invasion and AP-1 factors are known to regulate the expression of MMPs (Westermarck and Kahari, 1999). Since, Meq is related to AP-1 factors structurally and functionally, we wanted to test Meq transcriptional activity on the *MMP* promoter. To achieve this objective, we used a *MMP-3* promoter that contains two AP-1 binding sites.

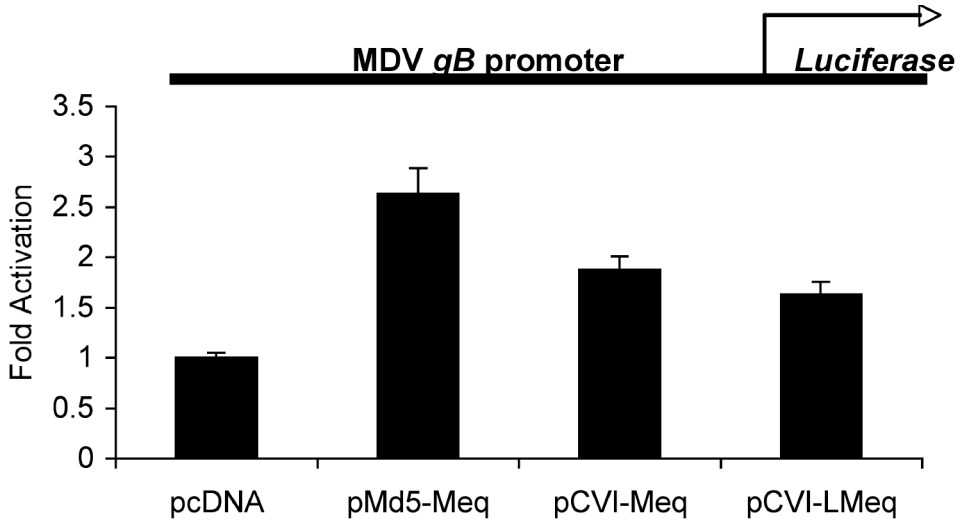

Fig. 12. Transactivation of MDV-1 *gB* promoter by Meq proteins. pGL3-*gB* promoter (250 ng) was transfected together with 125 ng of pcDNA or pcDNA expressing Md5-Meq, CVI-Meq and CVI-LMeq proteins. DF-1 cells were lysed 24 hr after transfection to measure the luciferase activity. Fold activation of the luciferase activity was reported in the graph relative to that of control vector. The experiment was done three times in triplicate. Error bars indicate SEM.

This promoter construct was co-transfected simultaneously along with one of the three pCDNA Meq expressing vectors or control pCDNA vector into DF-1 cells. As shown in Fig. 13A, Md5-Meq, CVI-Meq, and CVI-LMeq transactivated the *MMP-3* promoter. However, these effects were not statistically significant. Nevertheless, these results indicate the Meq protein of MDV might contribute to dissemination of tumor cells to various organs by increasing the transcription of MMP-3.

Meq has also been shown to possess anti-apoptotic function and this function could be attributed to its ability to increase the transcription of anti-apoptotic genes such as *Bcl-2* (Levy et al., 2005). We asked whether this increase could be due to direct transactivation of the *Bcl-2* promoter by Meq. Chicken *Bcl-2* promoter was amplified

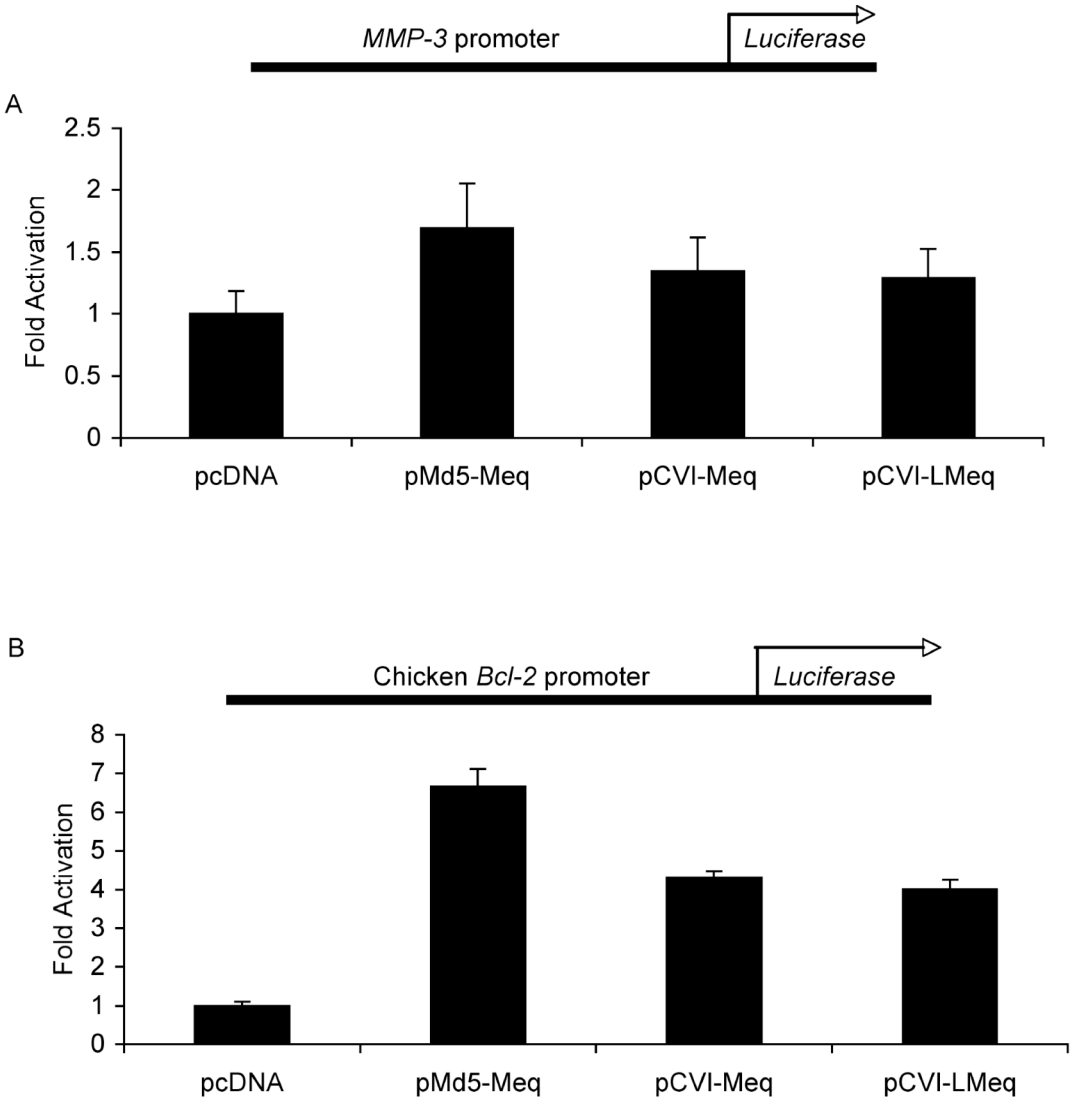

Fig. 13. Md5 and CVI-Meq proteins transactivate cellular promoters *MMP-3* and *Bcl-2*. DF-1 cells were co-transfected with 250 ng of pGL3-*MMP-3* promoter (A) or pGL3-*Bcl-2* promoter (B) along with 500 ng of pcDNA control vector or pcDNA expressing Md5-Meq or CVI-Meq or CVI-LMeq long. Cells were processed 24 hr post transfection for luciferase activity. Activation by each Meq protein was represented as fold activation relative to the activation by control vector. The experiment was done three times in triplicate. Error bars indicate SEM.

from lymphocytes and cloned in a luciferase reporter vector. DF-1 cells were subjected to simultaneous transfection of pGL3-*Bcl-2* promoter and either pcDNA, pMd5-Meq,

pCVI-Meq, or pCVI-LMeq. Meq proteins of both Md5 and CVI988 strains activated the chicken *Bcl-2* promoter (Fig. 13B) and further, the transactivation difference between Md5 and CVI proteins was found to be statistically significant. Therefore, our study suggests that the anti-apoptotic function of Meq might be related to its ability to directly transactivate the *Bcl-2* promoter.

2.4. Discussion

MDV vaccines prepared from attenuated GaHV-2, GaHV-3, and MeHV-1 (MDV serotypes 1, 2, and 3) are largely used to control MD in chickens (Witter, 1998) . Only MDV-1 induces T cell tumors while MDV-2 and MDV-3 do not cause MD in chickens. MDV-1 includes oncogenic strains and can be attenuated into non-oncogenic strains by serial passage in cell culture (Churchill et al., 1969a; Nazerian, 1970; Rispens et al., 1972; Vielitz and Landgraf, 1971; Witter, 1982) or recombinant DNA techniques (Brown et al., 2006; Cui et al., 2005; Hung et al., 2002; Lee et al., 2008; Tischer et al., 2002). CVI988/Rispens, a MDV-1 attenuated non-oncogenic vaccine strain, currentlyprovides the best protection against MD due to its close genetic relatedness to MDV-1 oncogenic strains (Spatz et al., 2007b). The *meq* gene has been implicated in the development of malignant T cell tumors in chickens (Brown et al., 2006; Lupiani et al., 2004). Currently, it is unclear why attenuated CVI988/Rispens does not cause tumors even though it encodes for the oncoprotein Meq. In this study, we attempted to characterize the *in vitro* transformation and transactivation properties of CVI-Meq proteins with reference to the Meq protein of Md5, a very virulent MDV oncogenic strain.

Due to the lack of an *in vitro* T cell transformation system to study transformation by Meq, experiments have been performed with fibroblasts. We previously showed that Meq is able to transform rat and chicken fibroblast cell lines (Levy et al., 2005; Liu et al., 1998). Our results here confirm that Md5-Meq is an oncogene and that it transforms fibroblast cell lines such as Rat-2 and NIH3T3. In addition, CVI-Meq proteins are capable of transforming both Rat-2 and NIH3T3 cell lines indicating that the amino acid differences observed between Md5 and CVI-Meq proteins have no effect on their *in vitro* transformation properties. Recently, Meq has been shown to transform DF-1 cells by up-regulating genes such as *JTAP-1*, *JAC*, and *HB-EGF*, which are implicated in the transformation pathway of v-Jun (Levy et al., 2005). By knocking down the expression of c-Jun, Levy et al (2005) showed that this pathway is essential for the growth of Meq expressing cells. It should be noted here that there are no differences in the leucine zipper region of Md5-Meq, CVI-Meq, and CVI-LMeq proteins. Hence, all should interact with c-Jun through the leucine zipper in a similar manner and possibly Meq/c-Jun interactions might have lead to the transformation of fibroblasts by CVI-Meq proteins. Logically, the next step would be to determine the transformation properties of CVI-Meq proteins *in vivo*. However, our attempts to study the transformation properties of CVI-Meq proteins in chickens using replication competent avian leukosis viruses were unsuccessful due to the instability of the insert and truncation of *meq* genes in this vector system during virus replication. In previous studies, replication defective virus expressing Meq induced only 5% sarcomas in chickens (Liu et al., 1998) and thus far, Meq has been shown to transform only

fibroblast cell lines but not primary fibroblasts. These findings imply that Meq is a weak oncoprotein.

Meq is a bZIP protein that structurally and functionally resembles proteins of bZIP family such as c-Jun and c-Fos. In one study, DNA binding and transactivation domains of Meq were shown to be interchangeable with those of c-Jun resulting in no changes to their transactivation and transformation functions (Liu et al., 1999a). Additionally, Meq has been shown to function as a transcriptional factor for its own promoter. Meq dimerizes with itself or with c-Jun *via* the leucine zipper present in its DNA binding domain. Meq/c-Jun heterodimers have been shown to carry out stronger DNA binding activity for AP-1 like sequences present in the *meq* promoter (Qian et al., 1995). Data presented herein shows that the *meq* promoter is activated by Md5-Meq and this transcriptional activity is further increased in the presence of over-expressed c-Jun. However, CVI-Meq and CVI-LMeq failed to activate the *meq* promoter. Interestingly, CVI-Meq proteins activated the *meq* promoter in the presence of over expressed c-Jun. These results reinforce c-Jun as a major interacting protein of Meq and also suggest that the CVI-Meq proteins are weak transactivators and only activate the *meq* promoter in the presence of high levels of c-Jun. In addition, the weak transactivation activity of CVI-Meq could in turn cause low levels of Meq expression in CVI vaccinated chickens and in the absence of sufficient Meq, development of T cell tumors might fail to occur. Studies quantifying the expression of Meq in chickens vaccinated with CVI988/Rispens will answer whether low-level expression is the cause for non-oncogenicity of CVI.

Md5 and CVI-Meq proteins bind to the *meq* promoter irrespective of whether CK-Jun is co-expressed. Thus, amino acid differences found in their DNA binding domains had no effect on their transactivation properties. When co-expressed, CVI-Meq proteins reduced activation of the *meq* promoter by Md5-Meq. Findings presented here are in agreement with studies by Chang et al (2002b) who also showed that CVI-LMeq suppressed activation of the *meq* promoter by Md5-Meq. In a mixed infection of CVI988/Rispens and Md5 or other virulent viruses, CVI-Meq proteins may compete for the binding sites in the *meq* promoter and subsequently suppress expression of Meq.

Luciferase assays with DNA binding domain chimeric Meq proteins indicated that both DNA binding and transactivation domains are required for full activation of the *meq* promoter by Md5-Meq protein. Even though, both DNA binding and transactivation domains of Md5-Meq protein increase the transactivation activity of chimeric Meq proteins on the *meq* promoter, this activity is far less compared to Md5-Meq protein. We expected differences in transactivation between Md5-Meq and CVI-Meq proteins would be due to differences in the transactivation domain because both proteins are capable of binding the *meq* promoter. But both Md5-CVI-Meq and Md5-CVI-LMeq chimeric proteins, with transactivation domain from CVI-Meq proteins, showed activation comparable to CVI-Md5-Meq chimeric protein, which has the Md5-Meq transactivation domain. Our study identifies residues at positions 71 and 320 are important for full functionality of Md5-Meq as transcriptional factor for its own promoter. However, further studies are required to understand the significance of these residues on Meq function.

Whether Meq plays a role in virus replication and latency, in addition to its direct role in transformation, is unclear. Meq has been shown to be highly expressed in latently infected transformed T lymphocytes (Qian et al., 1995) and is also expressed, as an early gene, during lytic infection (Parcells et al., 2001). An Md5 virus in which both copies of the *meq* gene were deleted replicated similar to parental Md5 *in vitro* (Lupiani et al., 2004). Interestingly, replication of the *meq* mutant MDV was reduced at the time of latency and/or reactivation but not during the early cytolytic phase of the infection in chickens (Brown et al., 2006, Lupiani et al., 2004). These studies suggested that Meq could play a role in latency and/or reactivation. The MDV-1 *pp38/pp14* bi-directional promoter includes the MDV-1 origin of replication. It was initially thought that pp38 protein played a role in T cell transformation (Xie et al., 1996). However, our recent work with a pp38 deletion mutant virus showed that pp38 is non-essential for transformation but essential for lytic infection (Reddy et al., 2002). It has been shown Meq/Meq homodimers but not Meq/c-Jun heterodimers suppress transcription from this bi-directional promoter (Levy et al., 2003b). Our results show that not only Md5-Meq, but also CVI-Meq and CVI-LMeq suppress transcription of this bidirectional promoter. In addition, all three Meq proteins suppressed transcription of a promoter construct containing the ACACA sequence present in the MDV-1-origin of replication; it is possible that this transcriptional suppression by Meq may be required to maintain the latency. Additionally, Meq activates MDV-1 gB late promoter and thereby might promote virus replication. However, a previous work suggested Meq is not essential for the expression of gB (Lupiani et al., 2004). Interestingly, multiple spliced variants and

microRNAs have been identified in the region of the *meq* gene. Thus, the role of Meq in the biology of MDV-1 is very complex and Meq may promote latency or reactivation depending upon its dimerization partner, phosphorylation status, level of expression of splice variants, and microRNAs.

Meq binds AP-1 like sequences present not only on its own promoter but also on cellular promoters. For example, studies have shown Meq binds and activates the chicken IL-2 promoter (Levy et al., 2003b; Okada et al., 2007). Recently, Meq was shown to increase transcription of the anti-apoptotic *Bcl-2* gene (Levy et al., 2005). Our study suggests that this increase might be due to the activation of chicken *Bcl-2* promoter by Meq. T cell tumors produced by Meq are malignant in nature and are found in various organs such as liver, spleen, skin, and nerves. Malignant cells disseminate in the host by breaking down extracellular matrix proteins. bZIP proteins such as c-Jun and c-Fos bind AP-1 sequences on the *MMP-3* promoter and up regulate its transcription (Westermarck and Kahari, 1999). Similarly, all three Meq proteins studied herein activated the *MMP-3* promoter. As with most of the promoters analyzed in this study, Md5-Meq activated the *MMP-3* promoter better than both CVI-Meq proteins.

When compared with oncogenic strains, CVI988 encodes truncated ORF49.1, ORF5.5/ORF75.91 and vIL-8, lacks ORF6.2/ORF75.6 and has amino acid changes in UL36 and UL49 (Spatz et al., 2007a); however, whether these changes have an effect on oncogenesis remains to be determined. CVI988 shows high homology with other oncogenic strains for genes that play a role in pathogenesis including pp38, lipase, vTR,

and viral lipase (Fragnet et al., 2003; Spatz et al., 2007a) while it shows several amino acid differences in Meq, suggesting that Meq may play an important role in oncogenesis.

Collectively, our data indicate that CVI-Meq proteins, like Md5-Meq, transform fibroblasts. However, CVI-Meq proteins differ from Md5-Meq in their transactivation of the *meq* promoter. While CVI-Meq proteins required c-Jun for the activation of the *meq* promoter, c-Jun was not essential for Md5-Meq mediated activation. Interestingly, chimeric Meq proteins in which the DNA binding domain was swapped among Md5-Meq and CVI-Meq, proteins activated the *meq* promoter but to levels significantly lower than parental Md5-Meq. In addition, amino acid residues at positions 71 and 320 of Md5-Meq were found to be essential for its transactivation on the *meq* promoter. All three Meq proteins (Md5-Meq, CVI-Meq, CVI-Meq-L) activated the *gB* MDV-1 late promoter and cellular promoters (*MMP-3* and *Bcl-2*) whereas they suppressed transcription from the *pp38* and *pp14* MDV-1 early promoters. We conclude that both CVI-Meq proteins are generally weak transactivators compared to Md5-Meq and this might contribute to its non-oncogenicity *in vivo*. It is important in the future to develop recombinant very virulent MDV-1 expressing CVI-Meq protein(s) and conversely, a recombinant CVI988/Rispens virus expressing Meq from a very virulent strain to further elucidate the role of Meq in MDV-1 oncogenesis. Our advancement in understanding the mechanisms of MDV-1 induced oncogenesis will certainly lead to the development of better MDV-1 vaccines.

3. THE DNA BINDING AND TRANSACTIVATION DOMAINS OF MAREK'S DISEASE VIRUS MEQ ONCOPROTEIN COOPERATIVELY DETERMINE THE TRANSFORMATION PHENOTYPE IN CHICKENS

3.1. Introduction

Marek's disease virus serotype 1 (MDV-1) is an important viral pathogen of chickens and is responsible for major economic losses to the world broiler industry. MDV-1, also known as *Gallid herpesvirus 2* (GaHV-2), belongs to the genus *Mardivirus* of the family *Herpesviridae*. MDV-1 causes T cell lymphomas in peripheral nerves and various organs, paralysis of legs and wings, immunosuppression, depression, blindness, dermal lesions, and significant mortality in young chickens (Calnek, 2001). MDV-1 strains vary greatly in their virulence and are classified into mild (m), virulent (v), very virulent (vv), and very virulent plus (vv+) pathotypes depending upon the degree of disease they cause in vaccinated chickens (Witter, 1997). Currently, several vaccines exist that protect chickens against the development of lymphomas, but they do not provide sterilizing immunity; thus field strains are able to replicate in vaccinated chickens leading to evolution of strains towards greater virulence (Witter, 1997). Insights into the mechanisms of transformation by MDV-1 will provide information that could be exploited in the design of better vaccines. In this regard, *meq* (**M**DV **E**co**Q** fragment), the principal transforming gene of MDV-1, is of particular interest for researchers. The MDV-1 genome contains unique long (U_L) and unique short regions (U_S) both of which are flanked by inverted repeat regions called terminal repeat long (TR_L), internal repeat long (IR_L), terminal repeat short (TR_S), and internal repeat short

(IR_S). Two copies of *meq* are located in the *Eco*Q fragments within the repeat long regions (TR_L and IR_L). This oncogene was first identified based on a observation that the *Eco*Q transcripts were abundantly present in MDV transformed T-cell lines such as RP1, RP4, and MSB1 as well as in tumors (Jones et al., 1992).

Meq is a 339 amino acid long bZIP protein, characterized by a DNA binding domain (amino acids 1-120) at its amino terminus and a transactivation domain (amino acids 121-339) at its carboxy terminus (Jones et al., 1992; Qian et al., 1995). The structure and function of Meq closely resembles c-Jun, which is a member of the activator protein 1 (AP-1) transcription factor complex (Jones et al., 1992). Like c-Jun, Meq forms homodimers with itself and heterodimers with other bZIP proteins. Meq binds to the AP-1 like sequences present in its own promoter and the binding affinity and transcription activity from the *meq* promoter can be increased with the co-expression of c-Jun (Qian et al., 1995). Chimeric proteins, bZIP-Meq/TA-c-Jun and bZIP-c-Jun/TA-Meq, exhibit properties similar to those of Meq and c-Jun, suggesting that the bZIP and transactivation domains between them complement in the transformation process (Liu et al., 1999a). Meq/c-Jun heterodimers preferentially bind cyclic AMP (CRE)- and 12-O-tetradecanoylphorbol 13-acetate responsive element (TRE)-like sequences, called MERE (**Me**q **r**eponse **e**lement) I (Qian et al., 1996). On the other hand, Meq/Meq homodimers preferentially bind a consensus sequence ACACACA, called MERE II (Qian et al., 1996). Meq/Meq homodimers but not Meq/c-Jun heterodimers suppress transcription of pp38/pp14, a bidirectional promoter located in the MDV origin of replication (MDV-Ori) (Levy et al., 2003b). By CHIP assay, Meq has been shown to bind MDV promoters

such as *meq*, *ICP4*, *gB*, and *gD*, all of which contain MERE I sequence and MDV-Ori, which contains MERE II sequence (Levy et al., 2003b). Furthermore, Meq/Jun heterodimers bind AP-1 containing chicken promoters such as IL-2. Additionally, Meq has been shown to interact with c-Fos, Jun-B, CREB, ATF-1, ATF-2, and ATF-3 (Brunovskis et al., 1996). The C-terminal domain of Meq, which contains proline repeats, has transactivation properties, and at least one proline repeat is required for full transactivation activity (Qian et al., 1995). Furthermore, the last 33 amino acids (307-339), though not sufficient, are critical for its function (Qian et al., 1995). As in the case of WT-1, proline rich repeats in the transactivation domain of Meq also have transrepression functions when tested in its isolated form (Qian et al., 1995). Though yet to be proven, presence of a potential RNA binding motif in the Meq transactivation domain (315-SGQIYIQF-322) suggests that Meq can also bind RNA (Lee et al., 2003; Liu and Kung, 2000). Meq has also been shown to interact with the putative tetramerization domain of chicken p53 via its basic region (amino acids 54-127) (Brunovskis et al., 1996). In addition, the presence of an RB-binding consensus sequence (LXCXE) at the end of the b-ZIP domain suggests Meq could potentially interact with RB (Liu and Kung, 2000) although this interaction has not been proven.

Meq localizes predominantly to the nucleoplasm and the nucleolus during all phases of the cell cycle. Two basic signal sequences, BR1 (30-RRKKRK-35) and BR2 (62-RRRKRNRDAARRRRRKQ-77), present at the N-terminus are required for its localization (Liu et al., 1997). While either BR1 or BR2 function as nuclear localization signals (NLS), BR2 is Meq sole nucleolar localization signal (NoLS). During S-phase

Meq can be found in the cytoplasm and colocalizes with CDK2 in the coiled bodies (Liu et al., 1999b). It is noteworthy that this colocalization causes phosphorylation of Meq serine at the position 42 by CDK2 leading to its translocation into the cytoplasm. Meq can also be phosphorylated by PKA, PKC, and MAPK (Liu et al., 1999b). Further, colocalization of Meq with PCNA in the nucleoplasm has been reported suggesting a role for Meq in DNA replication (Liu and Kung, 2000).

There are overwhelming evidences that suggest that Meq plays an important role in MDV oncogenesis. Meq is required for maintenance of transformation state since induction of RNA antisense to *meq* in MSB-1 cells, a MDV transformed T cell line, results in reduced colony formation in soft agar assay (Xie et al., 1996). Meq also cooperates with Myb in the maintenance of the transformation phenotype of T9 (an MDV transformed cell line) (Le Rouzic et al., 2002). When overexpressed, Meq transforms rat fibroblasts (Rat-2), mouse fibroblasts (NIH3T3) and immortalized chicken fibroblasts (DF-1 cells) (Ajithdoss et al., 2009; Levy et al., 2005; Liu et al., 1998). Thus far, Meq has not been shown to transform primary fibroblasts, and it causes sarcomas only in 5% of chickens when infected with replication defective retrovirus virus carrying the *meq* gene (Liu et al., 1998). Like Jun and Fos, Meq promotes growth of cells in the absence of serum and protects them against apoptosis inducing agents including TNF-α, C2-Ceramide, UV irradiation and serum starvation (Liu et al., 1998). The anti-apoptotic function of Meq might be due to its ability to increase *bcl-2* expression by directly binding to the promoter and to decrease *bax* expression (Ajithdoss et al., 2009; Liu et al., 1998).

rMd5ΔMeq, a virus in which the *meq* genes were deleted, failed to induce tumors, where as rMd5 virus with intact *meq* genes caused tumors in susceptible chickens (Lupiani et al., 2004). This study provided compelling evidence that Meq is essential for transformation of T lymphocytes. Additional evidence that Meq is involved in transformation came from the studies of a virus with mutation in the Meq that eliminated the interaction with C-terminal-binding protein (CtBP) domain and was found to be nononcogenic in chickens (Brown et al., 2006). It has been suggested that Meq transforms via the v-Jun pathway. Observations that support this hypothesis are: (1) morphology of Meq transformed DF-1 cells are similar to v-Jun transformed cells, (2) Meq protects DF-1 cells against apoptosis, (3) up-regulation of genes such as JTAP-1, JAC, and HB-EGF, which are implicated in v-Jun transformation process, and (4) Meq cooperates with c-Jun in the transformation of DF-1 cells (Levy et al., 2005). Furthermore, a recent work in our laboratory has suggested that Meq/Meq homodimers are insufficient for inducing lymphomas in chickens (Suchodolski et al., 2009).

Analysis of *meq* genes has demonstrated that mutations in Meq correlate with the virulence of MDV strains (Shamblin et al., 2004). For example, low virulence strains have two point mutations (positions 71 and 77) in the N terminus and an insertion in the C terminus. On the other hand, high virulence MDV strains contain point mutations in the proline-rich repeats and the transactivation domain. We have recently shown that Meq proteins from the non-oncogenic, attenuated MDV-1 vaccine strain CVI988/Rispens are in general weak transactivators although they retain the ability to transform fibroblasts (Ajithdoss et al., 2009). Remarkably, CVI-Meq proteins in the

context of other Md5 genes caused tumors only in 6% chickens when compared to parental rMd5 virus, which induced lymphomas in 100% of chickens (Reddy and Lupiani, unpublished observations). It is currently unknown whether the DNA binding domain or the transactivation domain of Meq is critical for transformation. To address this, we constructed two recombinant rMd5 viruses with chimeric Meq proteins, rMd5-Md5/CVI-Meq and rMd5-CVI/Md5-Meq, by swapping the DNA binding and transactivation domains between Md5-Meq and CVI-Meq, and evaluated their pathogenicity in chickens. rMd5-Md5/CVI-Meq caused 100% mortality and tumors were found in the peripheral nerves and various organs such as the heart, spleen, kidney, and gonads. The tumors were bigger in size when compared to tumors induced by rMd5. On the other hand, rMd5-CVI/Md5-Meq caused disease in 36% of chickens and lesions were primarily in the nerves. rMd5-Md5/CVI-Meq caused primarily type A nerve lesions where as rMd5-CVI/Md5-Meq induced predominantly type C lesions in the nerves. Based on these findings, we propose that both the DNA binding domain and transactivation domain of Meq determines the incidence, tissue distribution and size of the lymphomas in chickens.

3.2. Materials and methods

3.2.1. Cell culture

Primary chicken embryonic fibroblasts (CEFs) prepared from specific pathogen free (SPF) chicken eggs were used for transfections to recover recombinant viruses. Primary duck embryonic fibroblasts (DEFs) were used for preparing virus stock, titration, growth kinetics, and reactivation assay. Both CEFs and DEFs were cultured in

Leibowitz-McCoy (LM) medium, supplemented with 5% newborn calf serum and penicillin-streptomycin, at 37°C.

3.2.2. Cosmids

MDV cosmid clones SN5, P89, SN16, A6, and B40 from the very virulent strain, Md5, encompassing the entire MDV genome, were used to recover recombinant Md5 viruses (Reddy et al., 2002) (Figs. 14A & 14B). The EcoQ fragment located in cosmid clones A6 and SN5 contains the *meq* gene (Fig. 14C) and was replaced with Md5/CVI EcoQ, or CVI/Md5 EcoQ using a previously described strategy (Cui et al., 2004; Suchodolski et al., 2009). The DNA binding domain of Meq is coded by the first 360 nucleotides while the transactivation domain is coded by the remaining 657 nucleotides of the *meq* gene. In order to construct Md5/CVI EcoQ, or CVI/Md5 EcoQ fragments, at first, the Md5 EcoQ fragment was released from cosmids A6 and SN5 using the recA assisted restriction endonuclease (RARE) method. Using recA and primers SR1116 (5'-GAA TCG GAT TTG GAA TAA CCG AAT TCG GTG ATA TAA AGA C-3') and SR1117 (5'-GAC ATT ACA AGA ATA GTT TGA ATT CTC GGG ATA ATC TCC C-3'), *Eco*RI sites flanking the EcoQ fragment were protected during the *Eco*RI methylation reaction. The unmethylated *Eco*RI sites were then digested with *Eco*RI and the released EcoQ fragment was subsequently cloned into pCR2.1 vector (Invitrogen, Carlsbad, CA) generating pCR2.1 Md5-EcoQ. The *Eco*RI digested A6 and SN5 cosmids were religated generating A6ΔEcoQ and SN5ΔEcoQ.

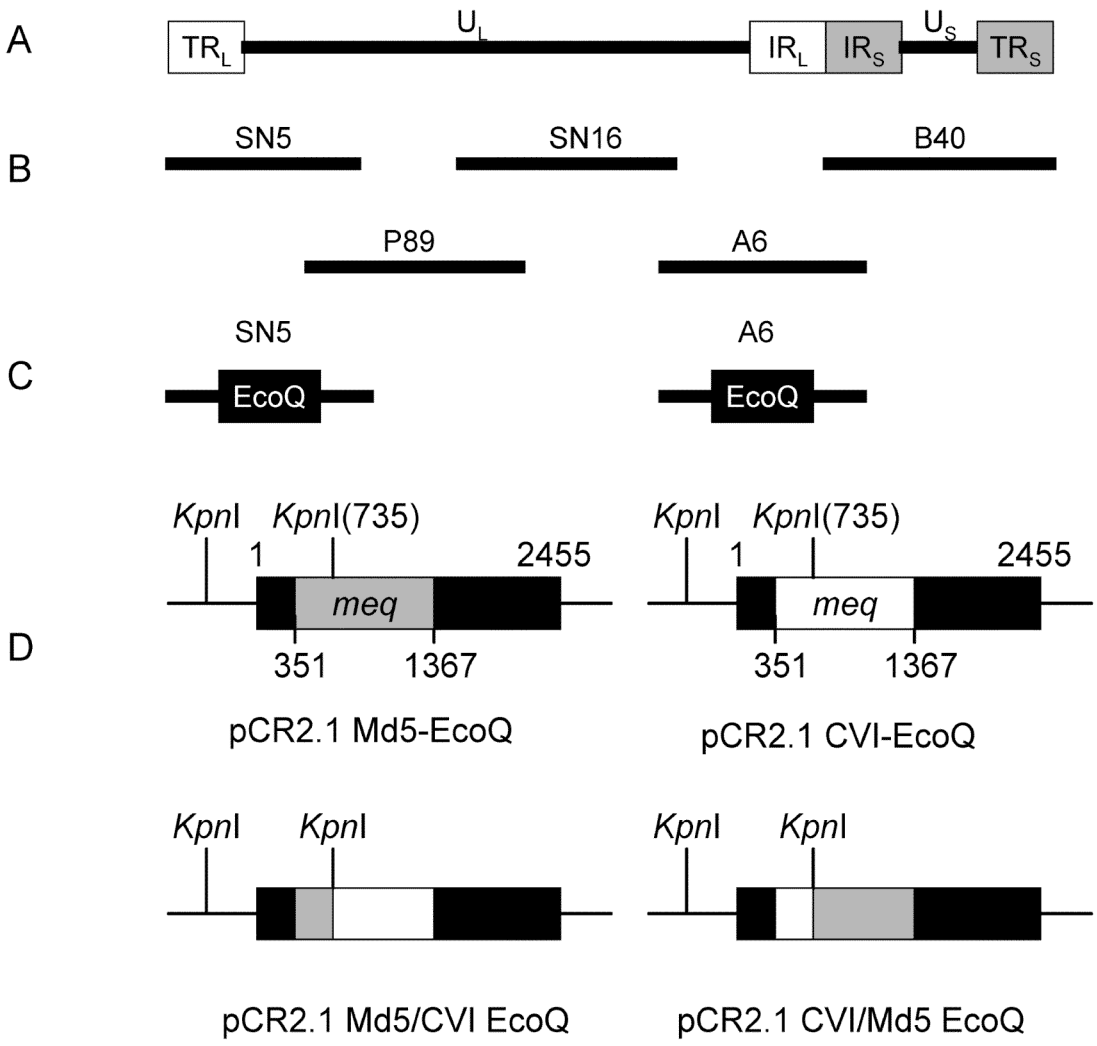

Fig. 14. Construction of chimeric recombinant viruses (A) Organization of the MDV genome. It contains unique long (U_L) and unique short (U_S) regions flanked by repeat regions (TR_L, IR_L, IR_S, and TR_S). (B) Md5 cosmids. Five overlapping cosmids that cover entire MDV genome is used for construction and recovery of chimeric viruses. (C) Location of EcoQ fragments. Cosmids SN5 and A6 contain the EcoQ fragment. The *meq* gene is located in the EcoQ fragment (D) Location of *Kpn*I sites in vectors. The *Kpn*I fragments between pCR2.1 Md5-EcoQ and pCR2.1 CVI-EcoQ vectors were exchanged to generate pCR2.1 Md5/CVI-EcoQ and pCR2.1 CVI/Md5-EcoQ vectors. (E). Construction of chimeric cosmids. The parental Md5-EcoQ fragments in SN5 and A6 cosmids were replaced with Md5/CVI-EcoQ or CVI/Md5-EcoQ fragment.

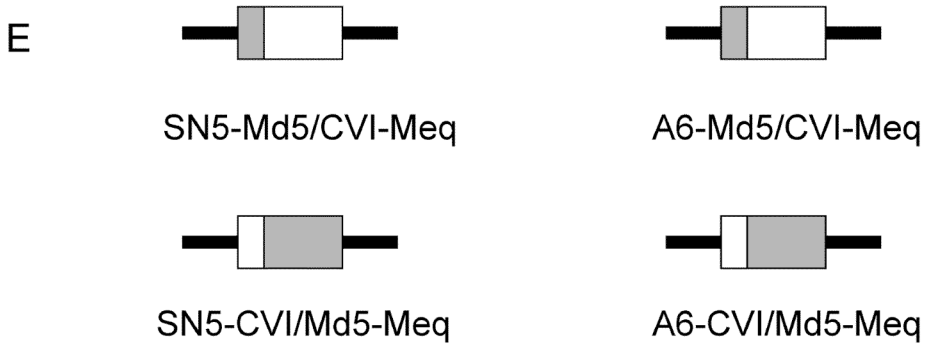

Fig. 14. Continued

The EcoQ fragment from attenuated vaccine strain Rispens/CVI988 was obtained by *EcoR*I digestion of Rispens/CVI988 purified DNA, gel purified and cloned into pCR2.1 vector (Invitrogen, Carlsbad, CA) generating pCR2.1 CVI-EcoQ. As indicated in the Fig. 1D, two *Kpn*I restriction sites, one in the pCR2.1 vector and another in the EcoQ fragment, are present in pCR2.1 Md5-EcoQ and pCR2.1CVI-EcoQ vectors. These two vectors were digested with *Kpn*I enzyme and the were swapped between them to construct pCR2.1 Md5/CVI-EcoQ and pCR2.1 CVI/Md5-EcoQ containing chimeric EcoQ fragments. The integrity of the chimeric EcoQ designated as A6-Md5/CVI-Meq, SN5-Md5/CVI-Meq, A6-CVI/Md5-Meq, and SN5-CVI/Md5-Meq, was confirmed by *EcoR*I digestion.

3.2.3. Recovery of recombinant viruses

The recovery of rMd5-Md5/CVI-Meq and rMd5-CVI/Md5-Meq viruses was done as described (Reddy et al., 2002). Briefly, cosmids SN15, P89, B40, SN5-

Md5/CVI-Meq, A6-Md5/CVI-Meq, SN5-CVI/Md5-Meq, and A6-CVI/Md5 Meq were digested with NotI, phenol-chloroform extracted and ethanol precipitated. To recover rMd5-Md5/CVI-Meq, 500 ng of digested and purified cosmids SN15, P89, B40, SN5-Md5/CVI-Meq, and A6-Md5/CVI-Meq were co-transfected, by the calcium phosphate method (Moriuchi et al., 1992), into 1.2 x10^6 CEF seeded onto 60-mm-diameter dishes. Four days post-transfection, cells were be trypsinized, seeded onto a 100-mm dish and after the appearance of viral plaques, viral stocks were made in DEF. rMd5-CVI/Md5-Meq virus was recovered in a similar fashion by co-transfecting CEF with 500 ng of digested and purified cosmids SN15, P89, B40, SN5-CVI/Md5-Meq, and A6-CVI/Md5-Meq. Additionally, two independent transfections for both chimeric viruses were carried out and these were termed rMd5-Md5/CVI-Meq #1, rMd5-Md5/CVI-Meq #2, rMd5-CVI/Md5-Meq #1, and rMd5-CVI/Md5-Meq #2. Previously described rMd5, rMd5-CVI-Meq and rMd5-CVI-LMeq viruses were used as controls throughout this study (Reddy and Lupiani, unpublished observations) (Reddy et al., 2002).

3.2.4. In vitro growth kinetics assay

DEFs grown to confluency in 35-mm-diameter dishes were infected with 100 plaque forming units (PFU) of all five recombinant viruses (rMd5, rMd5-CVI-Meq, rMd5-CVI-LMeq, rMd5-Md5/CVI-Meq and rMd5-CVI/Md5-Meq). After 2, 3, 4, and 5 days of infection, cells were trypsinized, 10- fold diluted and seeded onto fresh DEF monolayers in 35-mm-diameter dishes. Plaques were counted 7 days post infection. Two experiments were done in duplicate.

3.2.5. Southern blot

DNA from rMd5, rMd5-CVI-Meq, rMd5-CVI-LMeq, rMd5-Md5/CVI-Meq and rMd5-CVI/Md5-Meq viruses infected DEFs was isolated, digested with *EcoR*I, separated on a 1% agarose Tris-borate/EDTA gel, and transferred to a nylon membrane. DNA probes, one from total genomic viral DNA (cosmid DNA) and one from the 2.4-kb EcoQ fragment were labeled with [^{32}P]dCTP by the random priming method (DECAprime II kit, Ambion, Austin, TX) and used to hybridized two independent membranes according to standard protocols (Sambrook, 2001). Briefly, membranes containing denatured DNA were incubated in prehybridization buffer at 43°C for 1 hr. Incubation of membranes with radiolabeled probes was carried out at 43°C overnight. Following hybridization, the membranes were washed three times (1-2 hrs each at 43°C) with wash buffer (0.2 X SSC plus 0.1% SDS) and autoradiographed.

3.2.6. Immunofluorescence (IFA)

Cells infected with recombinant viruses were washed with PBS and fixed with ice cold ethanol:acetone solution (6:4) at room temperature for 10 minutes. Following the removal of fixing solution, the cells were air-dried, rehydrated with PBS and incubated with anti-Meq rabbit serum (Lee et al., 2003) (1:200) or anti-pp38 mouse monoclonal (Cui et al., 1990) (1: 200) for 1 hour at 37°C. Following three washes with PBS, cells were incubated with goat anti-rabbit FITC labeled secondary antibodies for anti-Meq stained cells or with goat anti-mouse FITC labeled secondary antibodies for anti-pp38 stained cells for 1 hour at 37°C. Cells were washed 3 times with PBS and examined under a fluorescence microscope.

3.2.7. Immunohistochemistry (IHC)

Tissues collected from lymphoid organs (thymus, spleen, and bursa of Fabricius) and feather follicles were embedded in optimal cutting temperature compound (Tissue-Tek OCT; Sakura Finetek, Torrance, CA). Tissue blocks were immediately frozen in liquid nitrogen, and kept at –80°C until processed for staining. Eight µm thick sections were prepared from the frozen tissue blocks, fixed with cold acetone at –20°C for 5 min, and air-dried. Immunostaining was carried out using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) as directed by the manufacturer. The H19 pp38 monoclonal antibody (1:3,000) was used as detection antibody.

3.2.8. Revertant virus

To recover a revertant virus containing the parental Md5 *meq* gene, DNA was purified from rMd5-CVI/Md5-Meq and rMd5-Md5/CVI-Meq infected CEF and each was co-transfected with parental Md5 EcoQ fragment in CEF by the calcium phosphate method. Seven days post transfection, transfected cells were overlaid with 0.9% Bacto agar, and individual plaques were harvested by trypsinization. Cells from single plaques were divided into two aliquots: one was used to infect fresh DEF, and the other was used for DNA extraction and PCR analysis. Revertant viruses were identified by using Md5 *meq* mismatch primers (BL1455: 5'- AAA AGG AAT CGT GAC GAC G-3' and BL1456: 5'- AGT ATC CGA GGG AAA CTT AG-3'), which amplified only parental Md5 *meq* and not Md5/CVI *meq* or CVI/Md5 *meq* at an annealing temperature of 60° C. Revertant viruses, rMd5-Md5/CVI-Meq-R and rMd5-CVI/Md5-Meq-R, were then expanded and titrated in DEFs.

3.2.9. Chicken experiments

(a) Experiment 1. Specific-pathogen-free chickens (Charles River SPAFAS, North Franklin, CT) were randomly sorted into seven experimental groups, wing banded and raised in modified Horsfall-Bauer isolators. Five groups of day-old chickens (15 per group) were inoculated subcutaneously with 5,000 PFU of rMd5, rMd5-CVI-Meq, rMd5-CVI-LMeq, rMd5-Md5/CVI-Meq and rMd5-CVI/Md5-Meq viruses. A group of 10 chickens were inoculated with 5,000 PFU of Rispens/CVI988 vaccine and as a negative control, a group of 8 chickens were kept uninoculated. Chickens were monitored for the development of MD for a period of 8 weeks. Chickens that died during the study or that were euthanized at the termination of the experiment were necropsied and nerves were collected for histological evaluation of MD. To examine early viral cytolitic infection, two birds from each group were randomly selected and euthanized at 6 dpi and samples from spleen, thymus, and bursa were collected for immunohistochemistry. To examine the reactivation ability of chimeric viruses, three chickens from each group were randomly selected and bled at 35 dpi. Following centrifugation of heparinized blood at 500 x*g* for 5 minutes, buffy coats were collected and diluted to 10^6 cells/ml. For each sample, 35-mm plates of freshly seeded DEF monolayers were inoculated in duplicates with 10^6 lymphocytes and viral plaques were counted 7 days later. To investigate the ability of chimeric viruses to transmit among chickens, three uninoculated chickens were included in each group at the time of inoculation. Buffy coats were obtained from contact birds at seven weeks and the presence of virus in lymphocytes was determined by PCR using Meq specific primers

(SR1118: 5'-GAT CCC GGG GAG ATG TCT CAG GAG CCA GAG C-3' and SR1135: 5'-GAT CCC GGG TCA GGG TCT CCC GTC ACC TGG AAA CC-3'). In addition, DEFs were co-cultured with 1 x10^6 lymphocytes and examined by IFA for pp38 at 7 dpi.

(b) Experiment 2. Nine groups of day-old chickens (15 per group) were inoculated subcutaneously with 5,000 PFU of rMd5, rMd5-Md5/CVI-Meq, rMd5-Md5/CVI-Meq #1, rMd5-Md5/CVI-Meq #2, rMd5-CVI/Md5-Meq, rMd5-CVI/Md5-Meq #1, rMd5-CVI/Md5-Meq #2, rMd5-Md5/CVI-Meq-R and rMd5-CVI/Md5-Meq-R viruses. A group of 10 chickens were kept uninoculated to serve as negative control. Chickens were monitored for MD for a period of 8 weeks.

3.3. Results

3.3.1. Construction of chimeric Meq viruses: rMd5-Md5/CVI-Meq and rMd5-CVI/Md5-Meq

Previous work in our lab has demonstrated a direct role for Meq in MDV oncogenesis as a Meq null virus (rMd5ΔMeq) failed to cause tumors in chickens (Lupiani et al., 2004). Intriguingly, attenuated vaccine strain CVI988/Rispens, although it codes for Meq, does not induce tumors. CVI-Meq proteins (CVI-Meq and CVI-LMeq) contain two and four amino acid substitutions in the DNA binding domain and transactivation domains, respectively relative to Md5-Meq. In addition, CVI-LMeq contains a 59 amino acid insertion in the transactivation domain. Like Md5-Meq, CVI-Meq proteins also transform fibroblasts *in vitro*, but are generally weak transactivators when compared to Md5-Meq (Ajithdoss et al., 2009). Furthermore, rMd5-CVI-Meq and rMd5-CVI-LMeq viruses, in which Md5-*meq* was replaced by CVI-*meq* or CVI-L-*meq*

caused tumors in 6% and 20% of chickens, respectively while parental rMd5 with Md5-*meq* gene induced tumors in 100% of chickens (Reddy and Lupiani, unpublished data). This study demonstrated that CVI-Meq proteins are very weakly oncogenic in the background of other Md5 genes and thus can be used to define the role of the DNA binding domain (DBD) and transactivation domain (TAD) of Md5-Meq in oncogenesis. To this end, we generated two chimeric rMd5 viruses: rMd5-Md5/CVI-Meq (DBD of Md5 and TAD of CVI) and rMd5-CVI/Md5-Meq (DBD of CVI and TAD of Md5) by exchanging domains between Md5- and CVI-Meq proteins. The amino acid differences in the DBD and TAD among Md5-Meq, CVI-Meq, Md5/CVI Meq and CVI/Md5 Meq proteins, are noted in Fig. 15. To obtain chimeric Meq rMd5 viruses, *Kpn*I digested DNA fragments of pCR2.1 Md5-EcoQ and pCR2.1 CVI-EcoQ vectors were exchanged to create pCR2.1 Md5/CVI-EcoQ and pCR2.1 CVI/Md5-EcoQ vectors (Fig. 14C). Subsequently, these chimeric EcoQ fragments were cloned in both A6ΔEcoQ and SN5ΔEcoQ cosmids (Fig. 14D). rMd5-Md5/CVI-Meq virus was recovered by co-transfection of A6-Md5/CVI-Meq, SN5-Md5/CVI-Meq, SN16, B40, and P89 cosmids while rMd5-CVI/Md5-Meq virus was generated by transfecting A6-CVI/Md5 Meq, SN5-CVI/Md5 Meq, SN16, B40, and P89 cosmids in CEF. To confirm that no unforeseen gene rearrangements occurred during the generation of viruses by homologous recombination of the five cosmids, the integrity of the viral genomes was confirmed by Southern blot analysis. As shown in Fig. 16, rMd5, rMd5-CVI-Meq, rMd5-Md5/CVI-Meq and rMd5-CVI/Md5 Meq viruses showed identical *EcoR*I digestion patterns. As anticipated, except for a large size EcoQ fragment due to

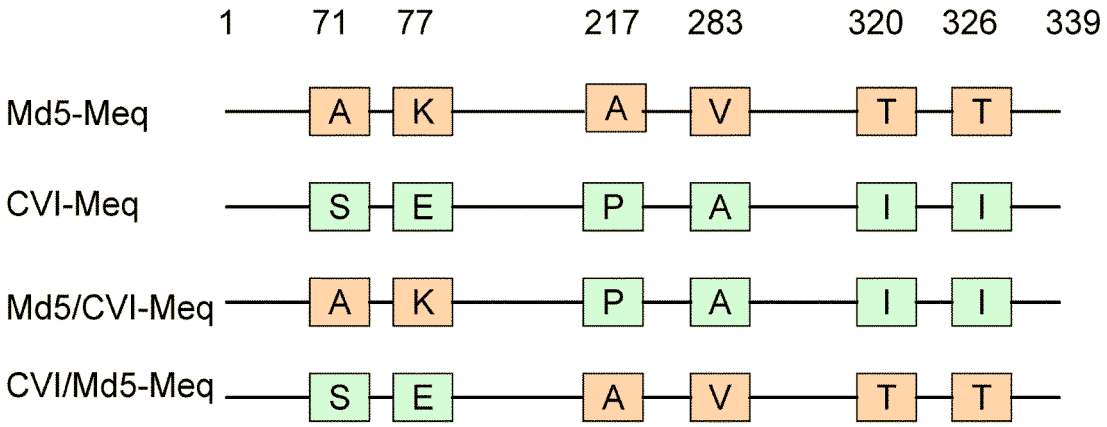

Fig. 15. Schematic representation of Meq proteins. When compared to Md5-Meq, CVI-Meq contains 2 amino acid differences in the DNA binding domain and 4 amino acids differences in the transactivation domain. Chimeric Meq proteins are constructed by exchanging the DNA binding and transactivation domains between Md5-Meq and CVI-Meq.

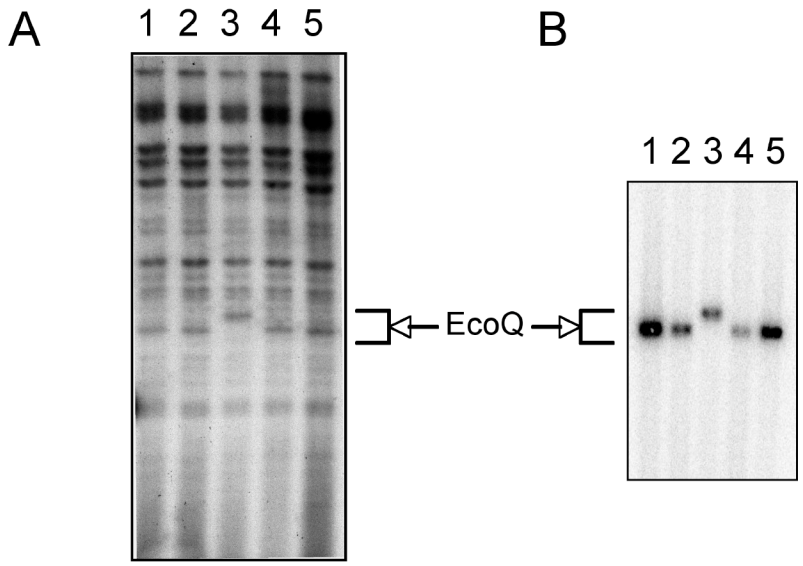

Fig. 16. Southern blot analysis of viral genomes. DNA isolated from DEF infected with recombinant viruses was digested with *EcoR*I and transferred to nylon membranes. The membranes were probed with either radiolabelled viral genome (A) or radiolabelled EcoQ fragment DNA (B). Lanes: (1) rMd5, (2) rMd5-CVI-Meq, (3) rMd5-CVI-LMeq, (4) rMd5-Md5/CVI-Meq, and (5) rMd5-CVI/Md5-Meq.

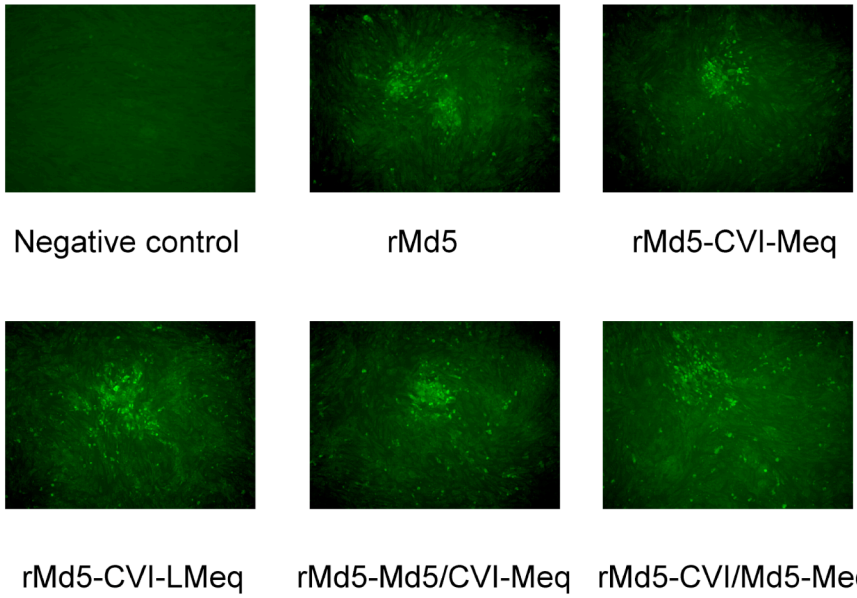

Fig. 17. Expression of Meq. DEF infected with all five recombinant viruses were fixed with ice cold ethanol:acetone solution and tested with polyclonal anti-Meq serum. Except for negative control, Meq expression in nucleus is observed with all five viruses.

the 180 bp insertion in the *meq* gene, rMd5-CVI-LMeq showed no differences in the genome arrangement. Expression of Meq by these viruses was examined in DEFs infected with all five recombinant viruses using rabbit anti-Meq polyclonal antibodies, and was predominantly found in the nucleus (Fig. 17). This shows that chimeric Meq proteins are expressed from rMd5-Md5/CVI-Meq and rMd5-CVI/Md5-Meq viruses and there is no change in their cellular localization properties.

3.3.2. In vitro and in vivo growth properties of rMd5-Md5/CVI Meq and rMd5-CVI/Md5 Meq

Meq has been shown to play no role in the replication of MDV *in vitro* (Lupiani et al., 2004); however, it affects viral replication at or after 2 weeks post infection in

chickens (Brown et al., 2006; Lupiani et al., 2004). To investigate whether chimeric Meq proteins, Md5/CVI-Meq and CVI/Md5-Meq, have any effect on the viral replication, DEFs grown to confluency in 35 mm dishes were infected with 100 PFU of rMd5-Md5/CVI-Meq and rMd5-CVI/Md5-Meq. As controls, rMd5, rMd5-CVI-Meq, and rMd5-CVI-LMeq viruses were also included. As expected, the in vitro growth properties of rMd5-Md5/CVI-Meq and rMd5-CVI/Md5-Meq were comparable to those of rMd5, rMd5-CVI-Meq, and rMd5-CVI-LMeq at all the time points studied (days 2, 3, 4, and 5) (Fig. 18) suggesting that, like parental Md5- and CVI-Meq proteins, the chimeric proteins, have no effect on viral replication *in vitro*.

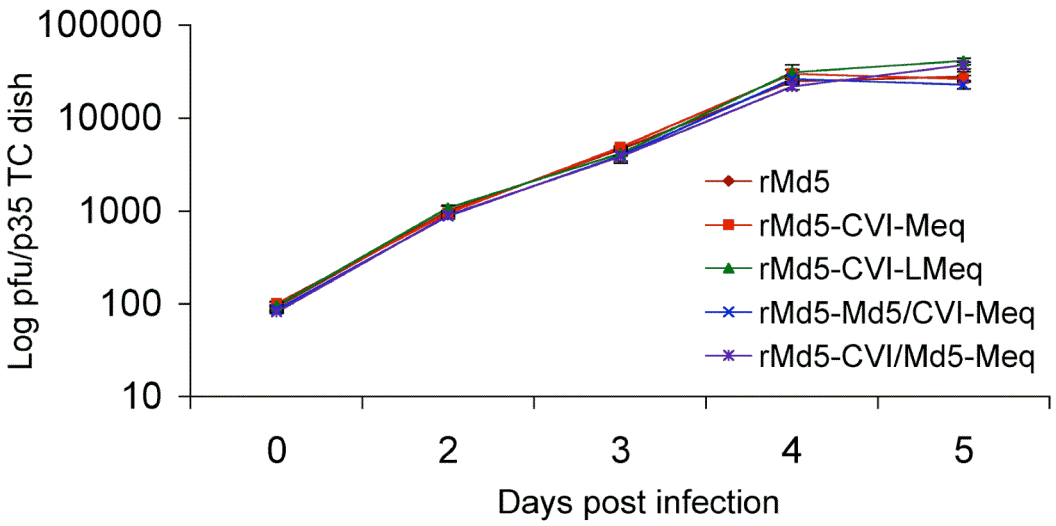

Fig. 18. In vitro growth assay. DEFs were infected with 100 PFU of the indicated viruses. Cells were harvested at days 2, 3, 4, and 5 post infection and virus titers were determined on fresh DEF. The experiment was done twice in duplicates.

To investigate the role of chimeric Meq proteins (Md5/CVI-Meq and CVI/Md5-Meq) in virus replication, at 6 dpi, two chickens from each group were euthanized and lymphoid organs (spleen, thymus, and bursa of Fabricius) were collected, routinely processed for immunohistochemistry and stained with pp38, an MDV early protein, monoclonal antibody. As shown in Fig. 19 all five recombinant viruses replicated to similar levels, as no difference in pp38 antigen expression was observed in all three organs examined, suggesting that early cytolytic infection in chickens was not affected by chimeric Meq proteins.

3.3.3. Reactivation properties of rMd5-Md5/CVI Meq and rMd5-CVI/Md5 Meq

In order to determine if chimeric Meq proteins affects the ability of viruses to reactivate from peripheral blood lymphocytes, buffy coats were isolated from three chickens in each experimental group at 35 dpi. For reactivation, $1x10^6$ lymphocytes were added to confluent monolayers of DEFs seeded in 35 mm culture dishes and plaques were counted 7 days later. As shown in Table 1, maximum number of plaques was obtained for rMd5, followed by rMd5- Md5/CVI-Meq; intermediate number of plaques was obtained with rMd5-CVI/Md5-Meq and, as expected, the number of plaques from the rMd5-CVI- Meq, and rMd5-CVI-LMeq was significantly lower.

3.3.4. Transformation properties of rMd5-Md5/CVI-Meq and rMd5-CVI/Md5-Meq

To examine the ability of chimeric Meq proteins to induce tumors, inoculated chickens were observed for up to 8 weeks, and those that developed MD during the experiment or those that remained at termination were euthanized and necropsied (Table 2).

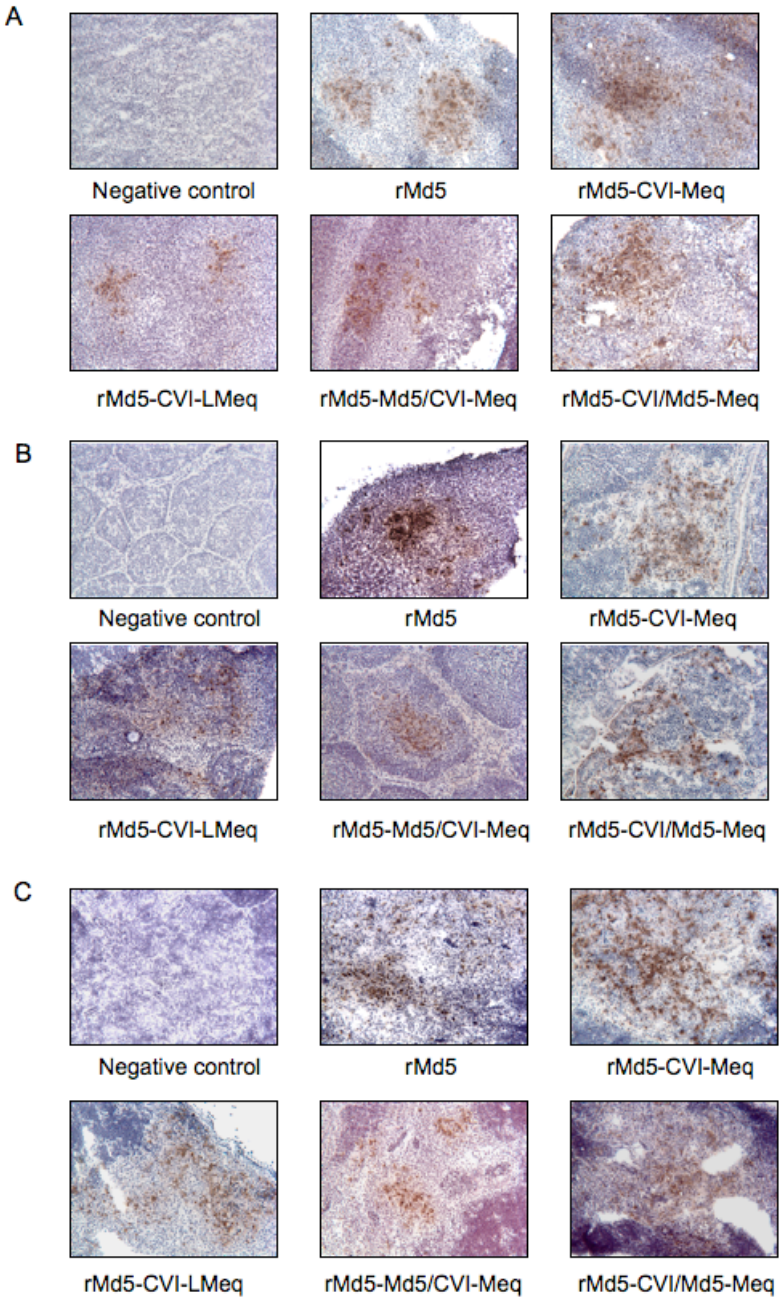

Fig. 19. In vivo replication of viruses in the lymphoid organs. Specific pathogen free chickens were infected with 5000 PFU of indicated viruses. The spleen (A), bursa (B), and thymus (C) were collected randomly from two chickens per group and routinely processed for immunohistochemistry. For staining, anti-pp38 antibody was used as primary antibody.

Table 1. Reactivation assay.

Virus	No of plaques per one million lymphocytes ± SD
Control	0
Rispens	11 ± 4
rMd5	117 ± 21
rMd5-CVI Meq	6 ± 5
rMd5-CVI-LMeq	27 ± 9
rMd5-Md5/CVI Meq	99 ± 27
rMd5-CVI/Md5 Meq	11± 4

Table 2. Incidence of Marek's disease.

Group	Experiment 1		Experiment 2	
	No of chickens with MD/Total chickens (%)	Mean death time (days)	No of chickens with MD/Total chickens (%)	Mean death time (days)
Control	0/10 (0)	NA	0/10 (0)	NA
Rispens	0/8 (0)	NA	ND	NA
rMd5	15/15 (100)	38.7	13/13 (100)	35.6
rMd5-CVI Meq	1/15 (6)	55.0	ND	NA
rMd5-CVI-LMeq	3/15 (20)	52.3	ND	NA
rMd5-Md5/CVI-Meq	15/15 (100)	34.6	15/15 (100)	31.5
rMd5-CVI/Md5-Meq	7/15 (46)	45.0	3/13 (23)	35.6
rMd5-Md5/CVI-Meq #1	ND	NA	12/12 (100)	35.0
rMd5-Md5/CVI-Meq #2	ND	NA	15/15 (100)	32.1
rMd5-CVI/Md5-Meq #1	ND	NA	4/12 (33)	49.3
rMd5-CVI/Md5-Meq #2	ND	NA	6/14 (42)	38.1
rMd5-Md5/CVI-Meq-R	ND	NA	13/13 (100)	34.1
rMd5-CVI/Md5-Meq-R	ND	NA	14/15 (93)	39.6

Note: ND- not done; NA- not applicable

Chickens from the uninoculated and CVI988/Rispens inoculated control groups did not die or develop MD during the duration of the experiment (Fig. 20). As expected, chickens inoculated with rMd5-CVI-Meq and rMd5-CVI-LMeq showed 7 and 20% MD, respectively (Fig. 20). In stark contrast, 100% of the chickens inoculated with rMd5-Md5/CVI-Meq and rMd5, had MD before the completion of the experiment (Fig. 20). On the other hand, 46% of chickens that were inoculated with rMd5-CVI/Md5-Meq had MD (Fig. 20). To confirm that the differences observed in the phenotype were due to the chimeric Meq proteins and not to unwanted mutations in other regions of the MDV genome, two additional clones (#1 and #2) of each of the chimeric viruses were generated in two additional independent transfections and the viruses were tested for pathogenesis in chickens as indicated above (Figs. 21-22).

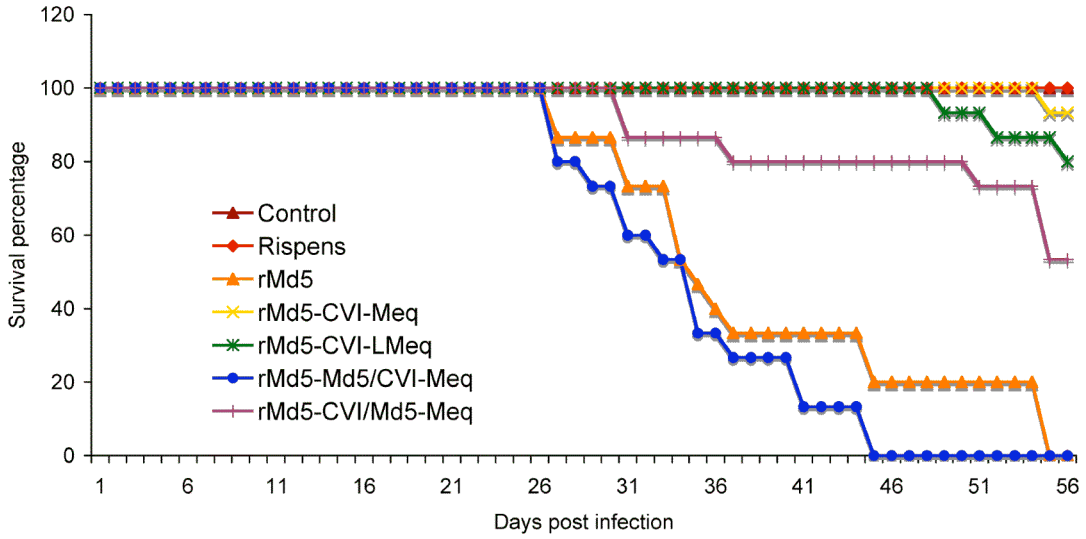

Fig. 20. Survival curve (experiment 1). SPF chickens were infected with 5000 PFU of indicated viruses and were maintained in isolation units for a period of 8 weeks. The mortality pattern for each virus was noted and survival percentage for each virus is indicated in the X-axis.

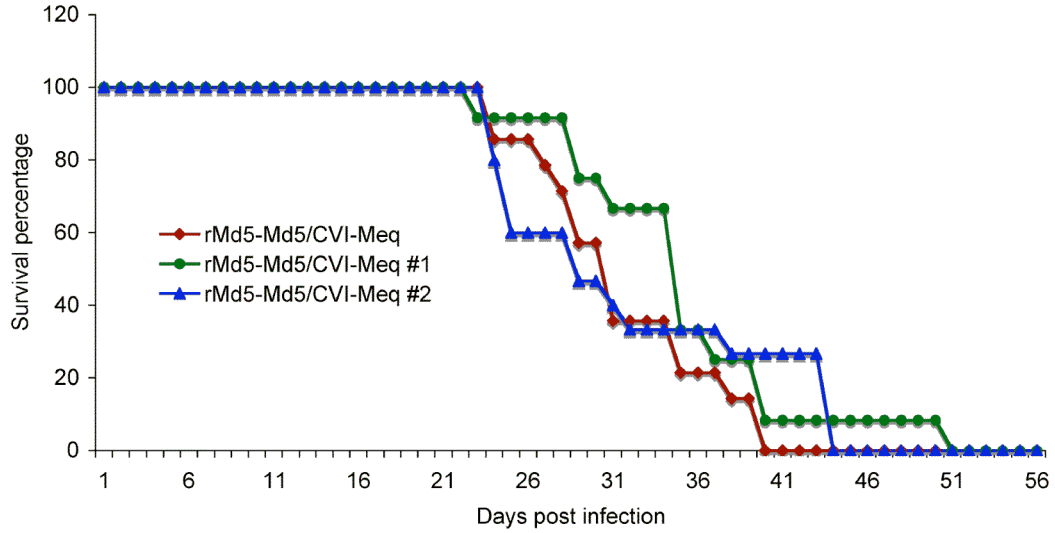

Fig. 21. Survival curve for rMd5-Md5/CVI-Meq (experiment 2). Specific pathogen free chickens were infected with 5000 PFU of rMd5-Md5/CVI-Meq, rMd5-Md5/CVI-Meq #1, and rMd5-Md5/CVI-Meq #2 viruses. Infected chickens were maintained in isolation units for a period of 8 weeks. The mortality pattern for each virus was noted and survival percentage for each virus is indicated in the X-axis.

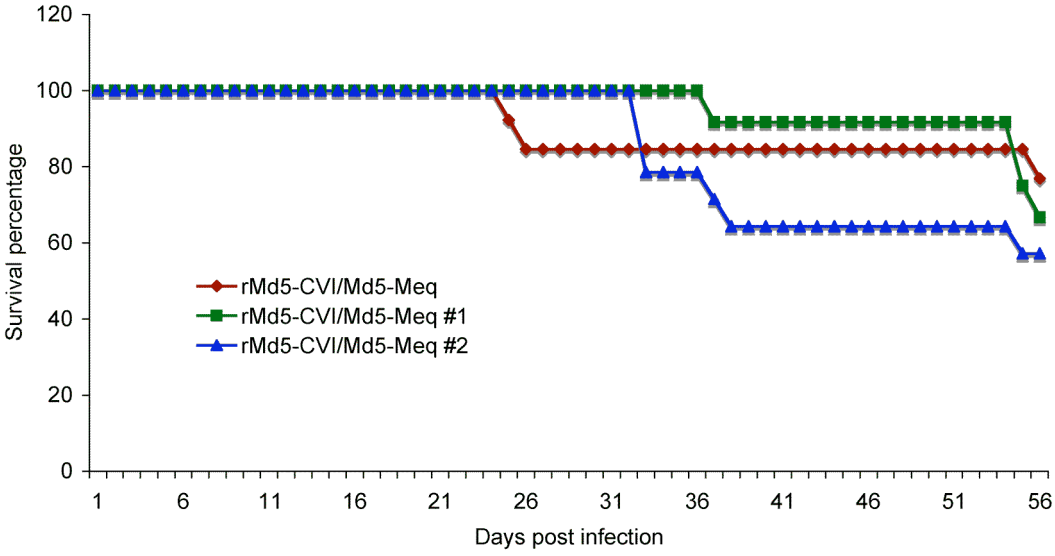

Fig. 22. Survival curve for rMd5-CVI/Md5-Meq (experiment 2). Specific pathogen free chickens were infected with 5000 PFU of rMd5-CVI/Md5-Meq, rMd5-CVI/Md5-Meq #1, and rMd5-CVI/Md5-Meq #2 viruses. Infected chickens were maintained in isolation units for a period of 8 weeks. The mortality pattern for each virus was noted and survival percentage for each virus is indicated in the X-axis.

Inoculation of chickens with rMd5-Md5/CVI-Meq #1 and rMd5-Md5/CVI-Meq #2 resulted in 100% MD mortality (Fig. 21) while the MD percentage for chickens that were inoculated with rMd5-CVI/Md5-Meq #1 and rMd5-CVI/Md5-Meq #2 were 33 and 42%, respectively, which is comparable to that of rMd5-CVI/Md5-Meq group (23% MD) (Fig. 22).

The transformation phenotype of rMd5-Md5/CVI-Meq was quite different from rMd5 and rMd5-CVI/Md5-Meq (Table 3). In addition to lesions in the peripheral nerves (Figs. 23 & 24), rMd5-Md5/CVI-Meq induced tumors at high levels of tumors in the heart (25-60%), spleen (20-50%), kidney (35-60%), and gonads (6-25%) and the tumors were of an unusual large size (Figs. 25-27). Interestingly, in experiment 1, one of the chickens infected with rMd5-Md5/CVI-Meq had tumors in the liver, intestines, pancreas, and proventriculus besides the kidney, spleen, and heart. On the other hand, rMd5 caused visceral tumors at a low frequency in heart (15-33%), spleen (6-23%), kidney (0-6%) and gonads (0-6%) while rMd5-CVI/Md5-Meq rarely induced tumors in the heart (0-8%) and spleen (0-7%). Further, rMd5-CVI/Md5-Meq caused no tumors in the liver, gonads, and kidney.

Table 3. Frequency of gross visceral tumors.

Virus	Heart (%)	Spleen (%)	Liver (%)	Gonads (%)	Kidney (%)
Control (Expt #1)	0/8 (0)	0/8 (0)	0/8 (0)	0/8 (0)	0/8 (0)
Control (Expt #2)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)
Rispens (Expt #1)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)
rMd5-CVI-Meq	0/15 (0)	0/15 (0)	0/15 (0)	0/15 (0)	0/15 (0)
rMd5-CVI-LMeq	0/15 (0)	0/15 (0)	0/15 (0)	0/15 (0)	0/15 (0)
rMd5 (Expt #1)	5/15 (33)	1/15 (6)	1/15 (6)	1/15 (6)	1/15 (6)
rMd5 (Expt #2)	2/13 (15)	3/13 (23)	0/13 (0)	0/13 (0)	0/13 (0)
rMd5-Md5/CVI-Meq (Expt #1)	5/15 (33)	7/15 (46)	4/15 (26)	1/15 (6)	9/15 (60)
rMd5-Md5/CVI-Meq (Expt #2)	7/14 (50)	6/14 (42)	1/14 (7)	3/14 (21)	5/14 (35)
rMd5-Md5/CVI-Meq #1 (Expt #2)	3/12 (25)	6/12 (50)	0/12 (0)	3/12 (25)	6/12 (50)
rMd5-Md5/CVI-Meq #2 (Expt #2)	9/15 (60)	3/15 (20)	0/15 (0)	2/15 (13)	6/15 (40)
rMd5-CVI/Md5-Meq (Expt #1)	0/15 (0)	0/15 (0)	0/15 (0)	0/15 (0)	0/15 (0)
rMd5-CVI/Md5-Meq (Expt #2)	0/13 (0)	0/13 (0)	0/13 (0)	0/13 (0)	0/13 (0)
rMd5-CVI/Md5-Meq #1 (Expt #2)	1/12 (8)	0/12 (0)	0/12 (0)	0/12 (0)	0/12 (0)
rMd5-CVI/Md5-Meq #2 (Expt #2)	0/14 (0)	1/14 (7)	0/14 (0)	0/14 (0)	0/14 (0)
rMd5-Md5/CVI-Meq-R (Expt #2)	3/13 (23)	10/13 (76)	0/13 (0)	7/13 (53)	8/13 (61)
rMd5-CVI/Md5-Meq-R (Expt #2)	3/15 (20)	3/15 (20)	0/15 (0)	0/15 (0)	0/15 (0)

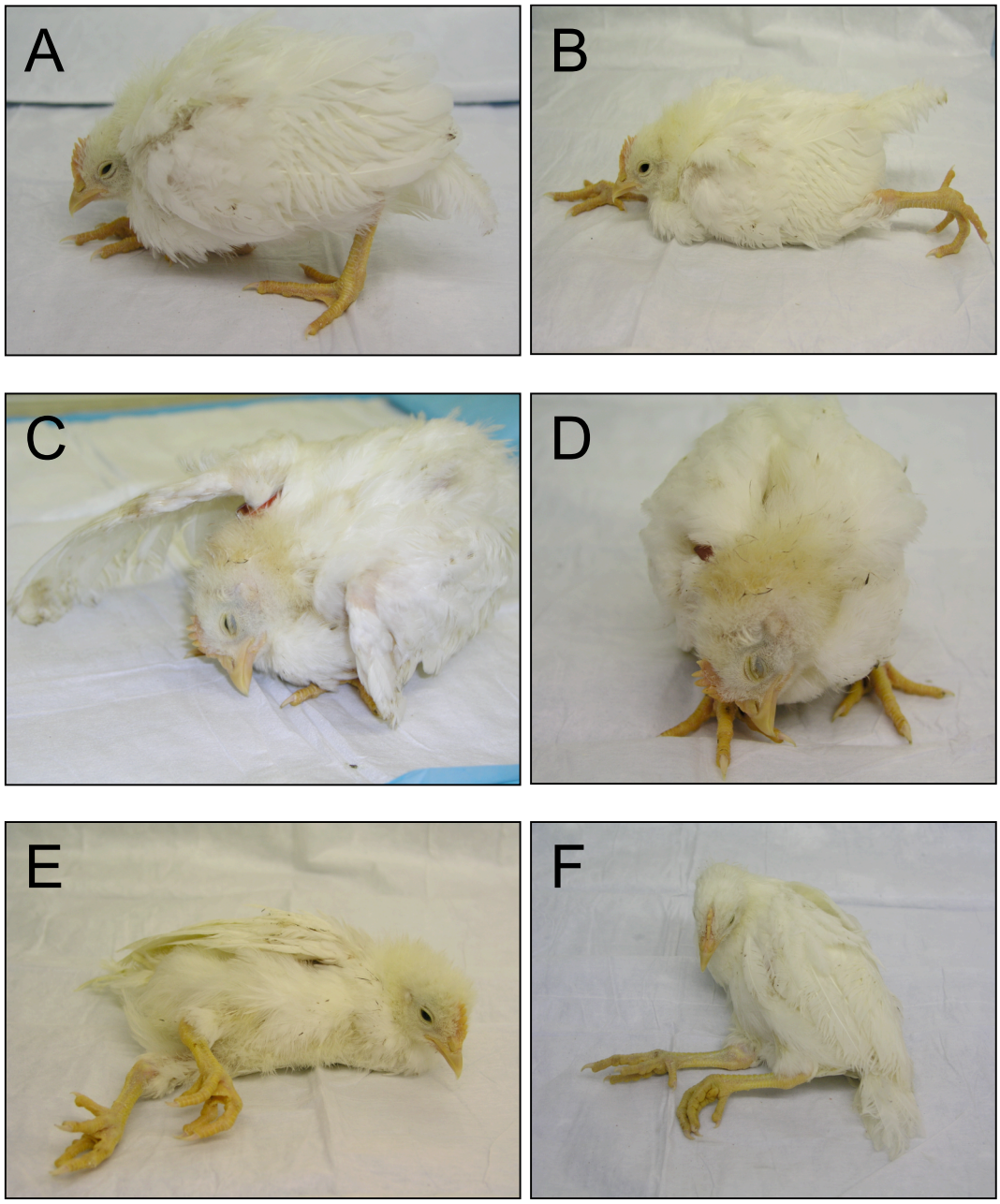

Fig. 23. Classical MD symptoms in chickens. Chickens infected with rMd5-Md5/CVI-Meq showed unilateral (A) or bilateral leg paralysis (B) or wing paralysis (C) or torticollosis (D). Similar neurological signs were also observed in rMd5 (E) and in rMd5-CVI/Md5-Meq (F) infected chickens.

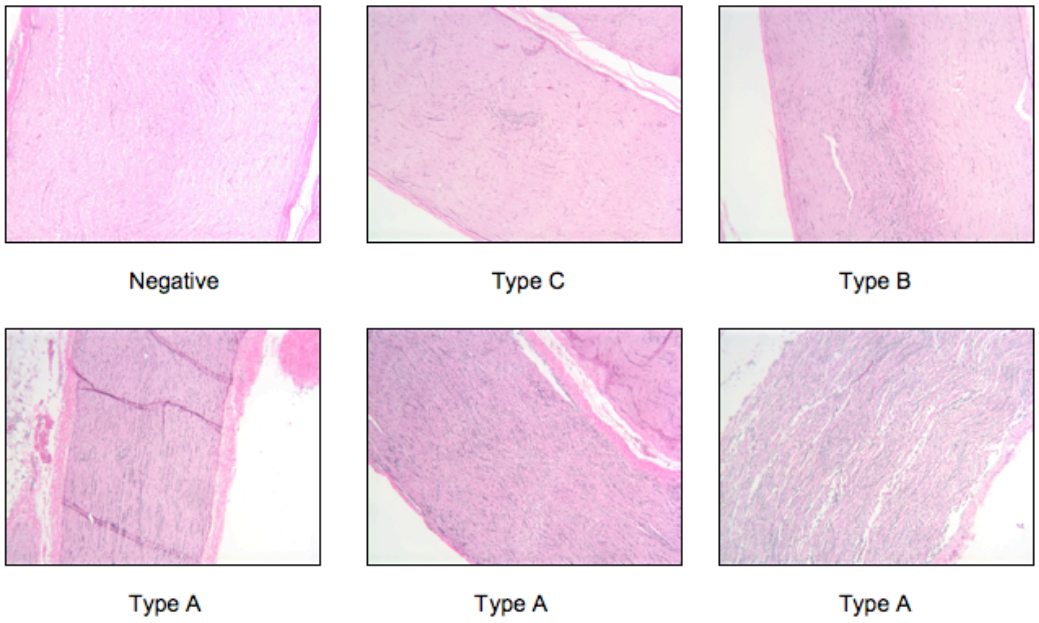

Fig. 24. Histology of nerve lesions. Nerves from control birds show neither lymphocyte nor lymphoid infiltration (negative). Type C lesion is characterized by minimal infiltration of small lymphocytes. Type B lesion has moderate infiltration of lymphoid cells along with edema. Type A lesion is severe and is characterized by massive infiltration of lymphoid cells. Type A lesions were observed in rMd5 and rMd5-Md5/CVI-Meq infected chickens. Few birds infected with rMd5-CVI/Md5-Meq had type A lesions and the rest had type C lesions. Lesions in rMd5-CVI-Meq or rMd5-CVI-LMeq infected birds were of type C.

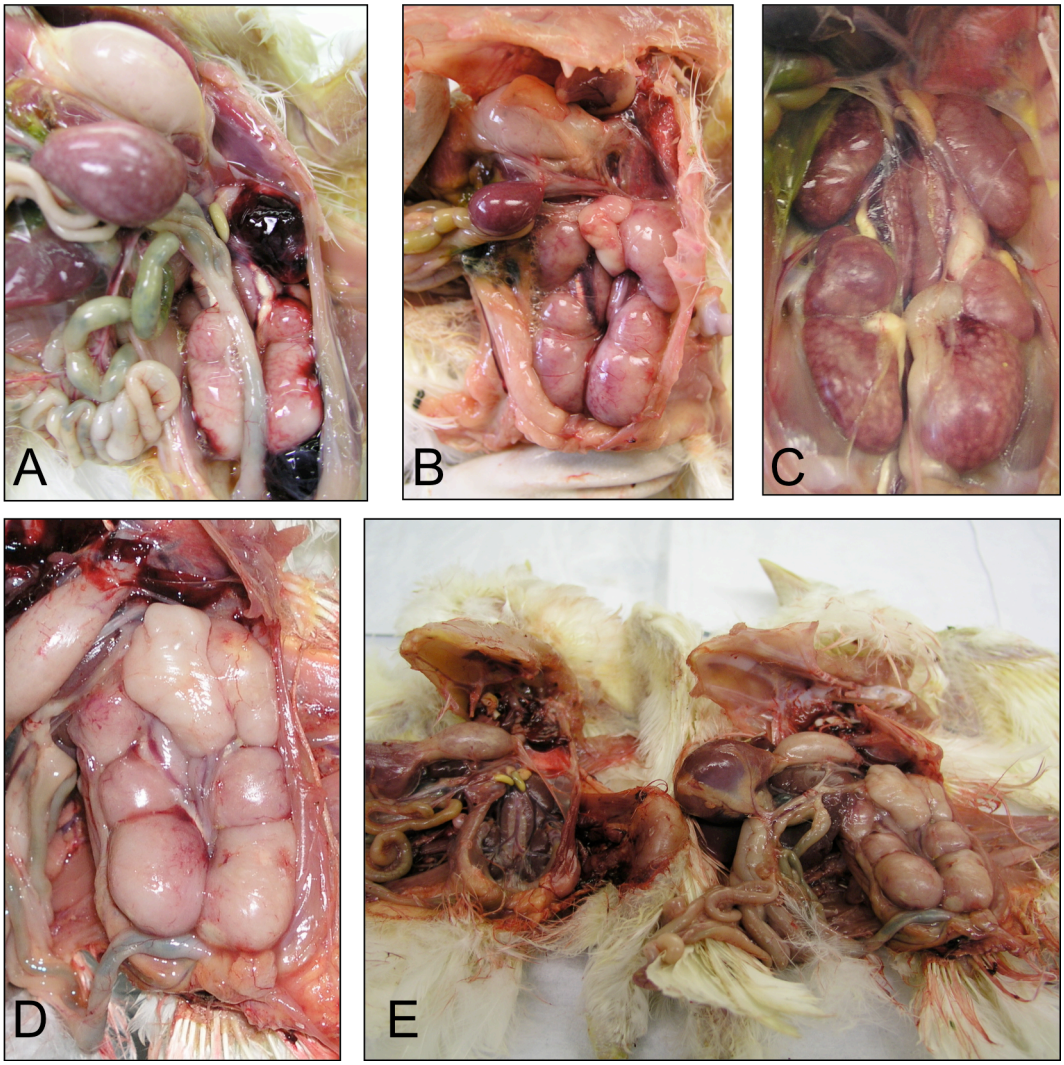

Fig. 25. Kidney tumors in rMd5-Md5/CVI-Meq infected chickens (A-E). Tumors are bigger in size and generally involve all the lobes. Tumors of infected chicken are compared with a control bird on the left in E.

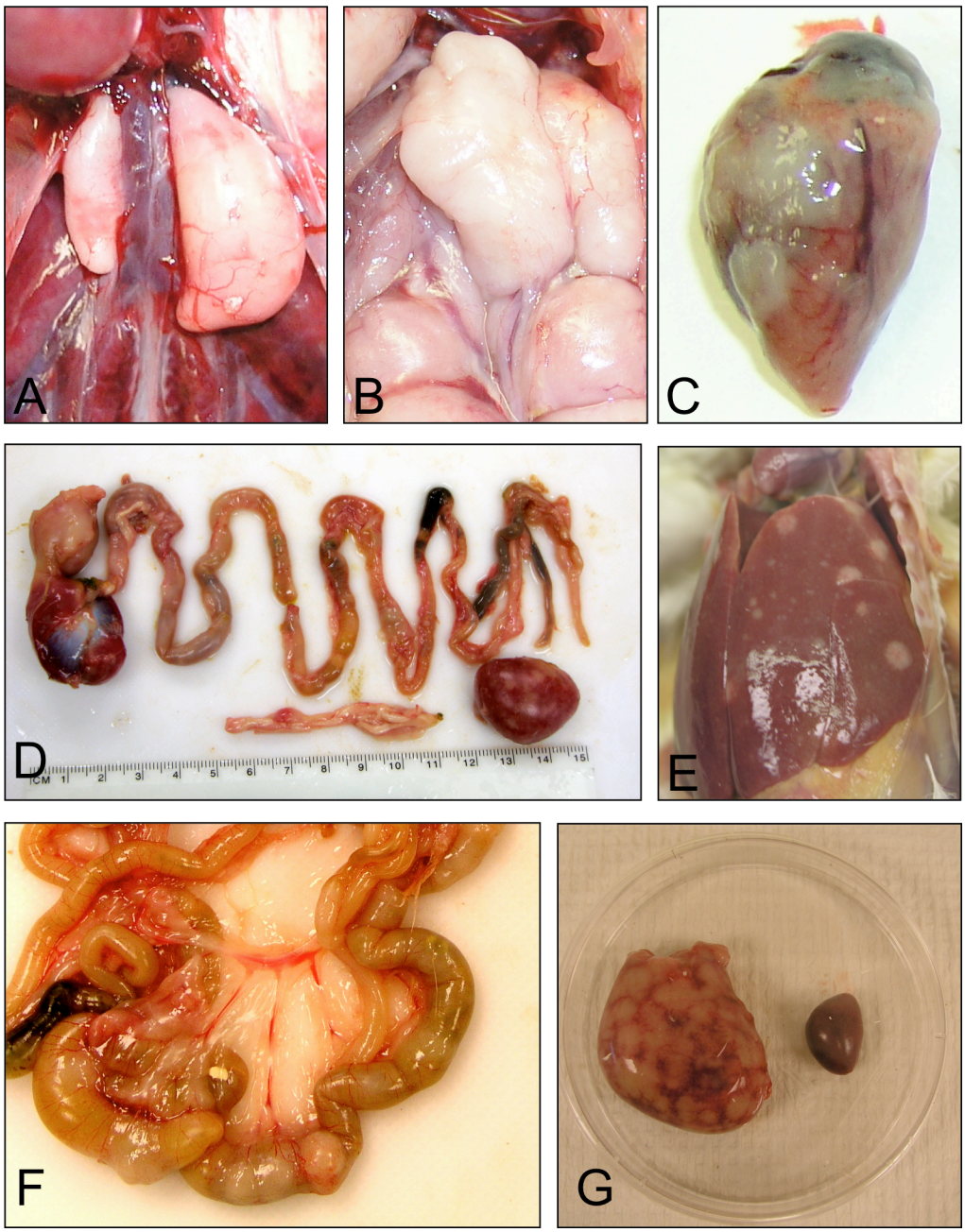

Fig. 26. Visceral tumors in rMd5-Md5/CVI-Meq infected chickens. A. Tumors in the testicles. B. Tumors in the ovary. C. Multiple tumors in the heart. D. Tumors in the proventriculus, entire intestines, pancrease, and spleen. E. Tumors in the liver in multiple areas. F. Tumors in the intestine. G. Tumors in the spleen. Compare the size with the uninfected spleen.

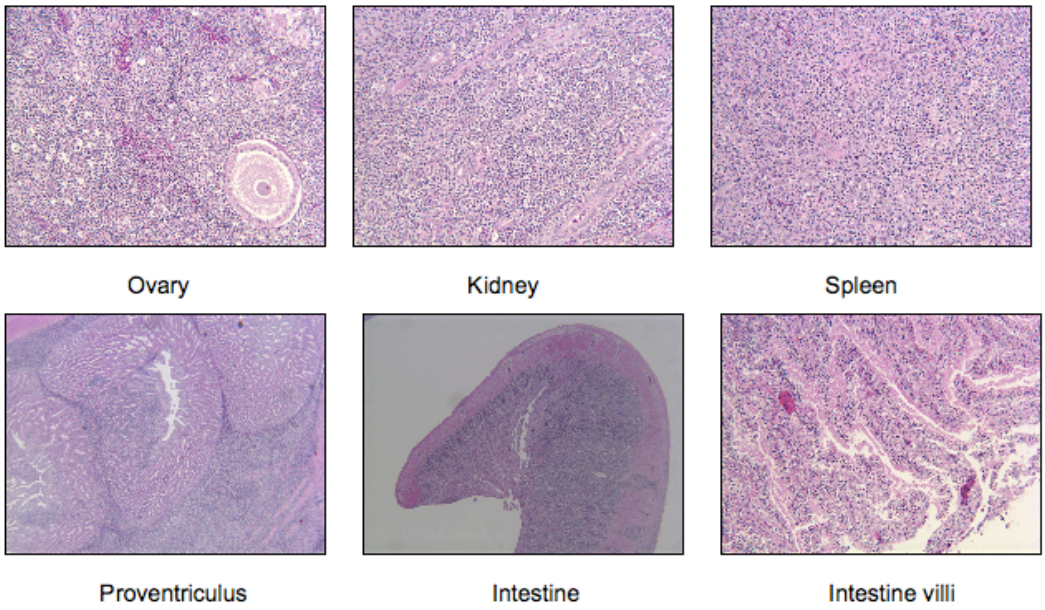

Fig. 27. Histological examination of visceral tumors. Massive infiltration of lymphoid cells in the ovary, kidney, spleen, proventriculus, and intestines were observed in chickens infected with rMd5-Md5/CVI-Meq.

These data collectively suggest: (1) Md5/CVI-Meq is more oncogenic than CVI/Md5-Meq; (2) Md5/CVI-Meq is more or at least as oncogenic as Md5-Meq (3) Md5/CVI-Meq retains the pathogenicity of Md5-Meq albeit containing the TAD of CVI988, thus indicating that DBD plays an important role in transformation; (4) When compared to CVI-Meq, CVI/Md5-Meq, which contains the TAD of Md5, is more virulent, thus suggesting that the TAD also plays a role in transformation; and (5) combination of DBD and TAD determines the transformation potential of Meq.

3.3.5. Transmission ability of rMd5-Md5/CVI Meq and rMd5-CVI/Md5 Meq

Fully productive replication of MDV occurs only in feather follicular epithelial cells (FFE), where cell free virus is produced and released serving as a source of infection for other susceptible chickens. We therefore investigated whether rMd5-Md5/CVI-Meq, and rMd5-CVI/Md5-Meq viruses replicate in FFE, an indication of transmissibility. To address this, skin from two randomly selected chickens from groups of chickens that were infected with rMd5, rMd5-CVI-Meq, rMd5-CVI-LMeq, rMd5-Md5/CVI-Meq or rMd5-CVI/Md5-Meq were collected at 8 weeks post inoculation, processed, and stained with anti-pp38 antibody. As shown in Fig. 28, positive pp38 staining was observed in all five recombinant viruses.

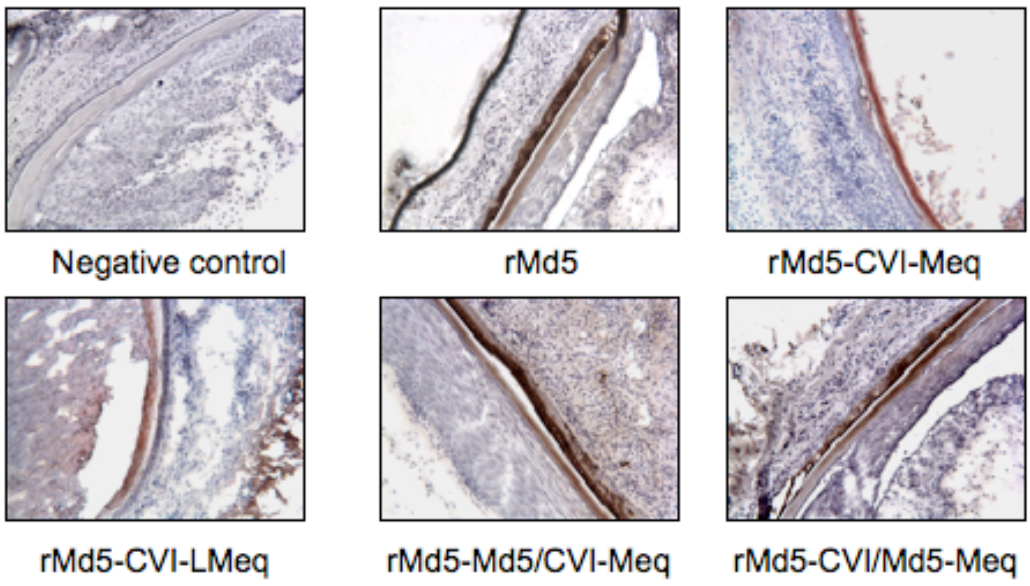

Fig. 28. Viral replication in feather follicular epithelium (FFE). Feather follicles were collected from few chickens in each group at the end of the experiment and stained with H19 pp38 monoclonal antibody.

In addition, inoculation of DEFs with buffy coats collected from contact chickens resulted in the formation of plaques, which were visualized by IFA (Fig. 29). Furthermore, buffy coat DNA from contact birds was analyzed for meq gene by PCR (data not shown). All five recombinant viruses were positive for Meq PCR. All the above results indicate that rMd5-Md5/CVI-Meq, and rMd5-CVI/Md5-Meq viruses can transmit horizontally among chickens.

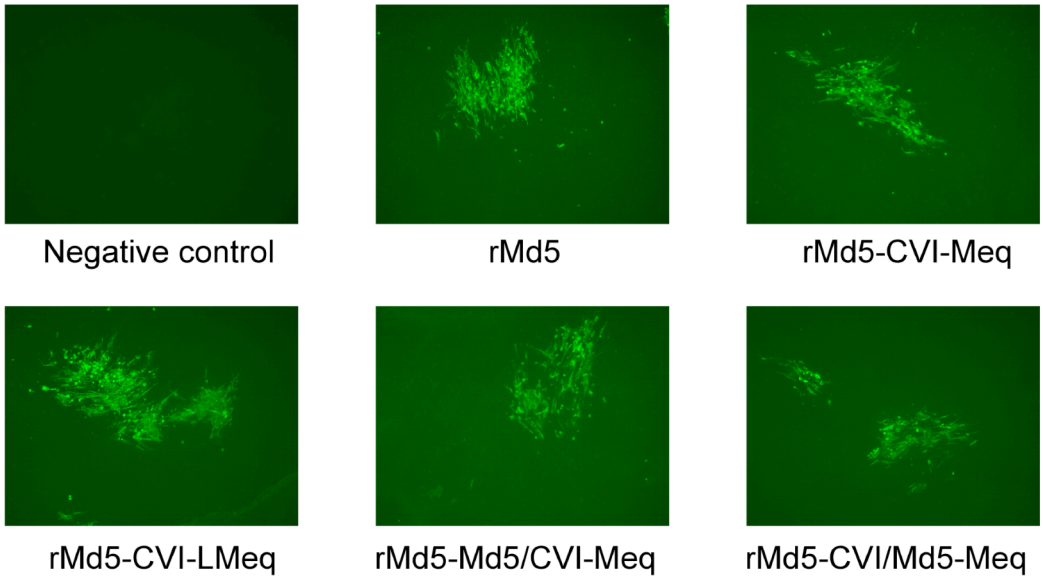

Fig. 29. Virus isolation in contact chickens. Lymphocytes were collected from contact chickens from each group and used to infect fresh DEF. Cells were stained 7 days post inoculation with H19 pp38 monoclonal antibody.

3.4. Discussion

MDV is regarded as one of the most potent oncogenic herpesviruses since it induces lethal T lymphomas in chickens as rapidly as 3 weeks, suggesting a direct role

for one or more viral proteins in the transformation process (Calnek, 2001). Search for such proteins has led to the discovery of Meq, a bZIP protein, which is highly expressed in both tumors and MDV-transformed cell lines (Jones et al., 1992). Based on *in vitro* transformation assays, it was suggested that Meq has the potential to function as an oncoprotein (Levy et al., 2005; Liu et al., 1999a; Liu et al., 1998; Qian et al., 1995). Subsequent experiments in chickens with a Meq knockout virus convincingly proved that Meq is the major oncogenic determinant of MDV (Brown et al., 2006; Lupiani et al., 2004). Following these findings, three studies that focused on the mechanisms of Meq mediated transformation, critical for the design of new MDV vaccines, have been published. First, Meq has been suggested to transform via the v-Jun pathway since it activates genes such as JTAP-1, JAC, and HB-EGF (Levy et al., 2005). Second, like EBNA 3A and 3C of Epstein-Barr virus, Meq has also been shown to interact, through a motif located in its DNA binding domain, with C-terminal binding protein (CtBP) and this interaction has been demonstrated to be critical for transformation (Brown et al., 2006). Finally, a recent work in our lab has indicated that homodimerization of Meq is insufficient for inducing tumors in chickens (Suchodolski et al., 2009).

We have previously shown that while Md5-Meq and CVI-Meq proteins are able to equally transform rat and mouse embryonic fibroblasts, they differ in their transactivation activity on the *meq* promoter. Interestingly, chimeric Meq proteins in which the DNA binding and transactivation domains had been swapped between Md5-Meq and CVI-Meq showed intermediate transactivation activity on the *meq* promoter (Ajithdoss et al., 2009). While Md5 causes MD in 100% of infected chickens, a

recombinant Md5 virus expressing the Meq proteins from the attenuated vaccine strain CVI988 only caused MD in 6.6 % of inoculated chickens (Reddy and Lupiani, unpublished results). The main objective of this research was to identify the domains of the Meq protein of a very virulent strain, Md5, involved in transformation. To address this we constructed and studied the growth, transmission, and transformation properties of two chimeric Meq proteins, Md5/CVI-Meq and CVI/Md5-Meq, in the context of other Md5 genes. These chimeric proteins were constructed by swapping domains between Md5-Meq and CVI-Meq. It has been previously shown that the Meq is not essential for viral replication in cell culture (Lupiani et al., 2004). In agreement with this study, our results showed that the two chimeric Meq proteins did not alter the replication of the virus in vitro. In addition, analysis of early cytolytic replication of rMd5-Md5/CVI-Meq and rMd5-CVI/Md5-Meq in lymphoid organs (spleen, bursa, and thymus) indicated that their replication was comparable to that of rMd5. Thus, chimeric Meq proteins do not affect in vivo replication of the virus.

It is well known that MDV is a highly contagious virus since it rapidly spreads among susceptible chickens. Several MDV genes such as U_S2, U_L13, gC, and LORF11 affect the horizontal transmission of the virus (Jarosinski et al., 2007; Lee et al., 2007). However, thus far transmission has not been shown to be affected by Meq (Lupiani et al., 2004). In corroboration with this study, rMd5-Md5/CVI-Meq and rMd5-CVI/Md5-Meq viruses were able to spread to other chickens as indicated by virus replication in both FFE and contact birds. Therefore, it is concluded that chimeric Meq proteins do not affect the transmission of virus.

The hallmark of MDV infection in chickens is the transformation of T lymphocytes. In this regard, both chimeric proteins induced lymphomas in chickens, but some differences were observed in the transformation phenotypes. First, Md5/CVI-Meq caused 100% mortality with mean death time of 33.3 days where as CVI/Md5-Meq on average caused 36 % mortality with mean death time of 42.3 days. Second, Md5/CVI-Meq induced 100 % lesions in peripheral nerves and 53.3 % gross lesions in visceral organs whereas CVI/Md5-Meq induced tumor lesions primarily in the nerves (36 %) and rarely in visceral organs (7.5 %). rMd5 caused 100 % mortality with mean death time of 37.1 days. Lesions were mainly in the nerves (100%) and were less frequently present in visceral organs (26%). Witter (1997) reported that high incidence of visceral lymphomas was associated with very virulent plus MDV strains (Witter, 1997), suggesting that the frequency of gross MD lesions in visceral organs is greatly influenced by the virulence of the virus. Our study showed that rMd5-Md5/CVI-Meq is more virulent than rMd5-CVI/Md5-Meq and even rMd5 as the tumors were more aggresive. The data suggest that Meq is responsible for the difference in transformation phenotypes.

In the present study, Md5-Meq, Md5/CVI-Meq and CVI/Md5-Meq, all in the context of Md5 genes, caused lesions in visceral organs, albeit at varying rates. Md5-Meq caused tumors at low frequency in the heart and spleen whereas CVI/Md5-Meq rarely induced lesions in the heart and spleen. Quite strikingly, Md5/CVI-Meq induced lymphomas not only in the heart, and spleen, but also in the kidney, and gonads at a high rate and also caused lesions at lower levels in the intestines, pancreas, proventriculus, and liver. It is noteworthy that these tumors were generally bigger in size than those

induced by rMd5. Interestingly, the amino acid sequence of Md5/CVI-Meq, except at position 326 (T instead of I) in the transactivation domain, is similar to that of Meq of GA strain, which is known to cause only tumors in the liver, kidney, spleen, and gonads, but no nerve lesions in young chickens (Eidson and Schmittle, 1968). Like rMd5-Md5/CVI-Meq, GA induces larger tumors. The DNA binding domain of Md5-Meq, Md5/CVI-Meq, and GA-Meq is identical whereas Md5/CVI-Meq, and GA-Meq have similar transactivation domain that is different from Md5-Meq. Since Md5/CVI-Meq, and GA-Meq, unlike Md5-Meq, cause tumors in the kidney and gonads at higher rate, this kind of tissue distribution could be attributed to their transactivation domain. However, it is important to note that the transactivation domain alone can not be responsible for this phenotype because CVI-Meq, which cause only nerve lesions in the background Md5 genes have same transactivation domain as Md5/CVI-Meq, and GA-Meq but differ in their DNA binding domain. In other words, phenotypic changes observed with rMd5/CVI-Meq and rCVI/Md5-Meq could not be attributed to either DNA binding or transactivation domain alone. Based on these observations, we suggest that a combination of DNA binding domain and transactivation domain determines the incidence, nature and size of the tumors. It is important in the future to generate single amino acid mutant Meq viruses to understand the role of these six amino acids in the transformation process.

The DNA binding domain of Meq is composed of two regions; (i) a basic amino acids rich region that binds DNA, and (ii) a leucine zipper motif, also known as dimerization domain, which facilitates dimerization. Meq through its basic region binds

specific sequences, known as MERE I, and MERE II, found in various promoters. Prior to binding to the DNA, it may dimerize with itself, forming homodimers or with various bZIP proteins, particularly c-Jun, forming heterodimers. Following binding to the DNA, the C-terminal domain of Meq can either activate or repress transcription depending upon the type of the promoter. These three regions of Meq, either alone or in cooperation could affect the development of tumors. First, the basic region might influence where Meq binds, and in turn will regulate the genes that are critical for transformation. Because of difference in the basic regions, Md5/CVI-Meq and CVI/Md5-Meq might target different promoters. The knowledge about cellular promoters that are targeted by Meq is very limited. Importantly, there could be variation in their DNA binding affinities. Its DNA binding activity is affected by phosphorylation of serine at position 42, which is present in both CVI/Md5-Meq and Md5/CVI-Meq. It is well established in case of Jun that phosphorylation reduces its DNA binding activity (Boyle et al., 1991, Oehler et al., 1993). Interestingly, CVI/Md5-Meq has a potential phosphorylation site at position 71. Whether these factors are responsible for the attenuated phenotype of CVI/Md5-Meq remains to be studied. Secondly, the homodimerization or heterodimerization of Meq or both might be crucial for transformation. The functions of bZIP proteins are largely affected by the interacting proteins. For example, Jun-Fos interaction confers anchorage independent growth while Jun-ATF2 interaction is responsible for serum independence (van Dam et al., 1998). Meq/Meq homodimers and Meq/Jun heterodimers have different functions. We have recently shown that Meq/Meq homodimers cannot cause tumors in chickens (Suchodolski et al., 2009). On the other

hand, heterodimerization of Meq can cause microscopic lesions in the nerves (Suchodolski et al. manuscript under preparation). Because both Md5/CVI-Meq and CVI/Md5-Meq have identical leucine zipper region, it's tempting to speculate that this region is less likely responsible for the transforming phenotype. It is important to note that there is serine at position 71 that can be phosphorylated in CVI/Md5-Meq whereas Md5/CVI-Meq has alanine at the same position. Interestingly, the DNA binding domains of Jun and of Meq are functionally exchangeable, which emphasizes the importance of phosphorylation. We have recently shown that Md5/CVI-Meq and CVI/Md5-Meq proteins, unlike CVI-Meq, transactivate the *meq* promoter, although at a lower level compared to Md5 Meq (Ajithdoss et al., 2009). Although Md5/CVI-Meq and CVI/Md5-Meq proteins have comparable transactivation abilities in cell culture, it could not be correlated directly with their transformation properties. It is not surprising since such lack of direct correlation between transactivation activity and transformation has also been described for Jun (Havarstein et al., 1992). It is conceivable that the phenotypic differences between Md5-Meq and Md5/CVI-Meq are due to differences in the transactivation domain. In conclusion, it is clear that both DNA binding domain and transactivation domain of Meq contribute to the transformation phenotype.

4. SUMMARY

Serotype 1 Marek's disease virus (MDV-1) causes T cell lymphomas in chickens. Attenuated and oncogenic MDV-1 strains code for an oncoprotein knows as Meq. The overall objective of this study was to characterize the Meq proteins of the attenuated non-oncogenic MDV-1 vaccine strain CVI988/Rispens. Two forms of Meq proteins have been detected in CVI988/Rispens vaccinated chickens: CVI-Meq and CVI-L Meq. CVI-Meq and CVI-L Meq differ by six amino acids from Md5-Meq encoded by a very virulent MDV strain that causes lymphomas in chickens. In addition, CVI-LMeq contains a 59 amino acid insertion in its transactivation domain. It is noteworthy that Meq is essential for the development of lymphomas in chickens. We hypothesized that CVI-Meq and CVI-LMeq proteins lack transactivation and transformation activities due to these amino acid changes.

In the current study, Md5-Meq protein bound and activated the *meq* promoter, in a dose-dependent fashion, in a luciferase reporter assay. In stark contrast, CVI-Meq and CVI-LMeq proteins failed to activate their own promoter even though they were able to bind. Interestingly, they activated the promoter in the presence of c-Jun overexpression. When co-transfected with Md5-Meq, CVI-Meq and CVI-LMeq proteins reduced the transactivation of the *meq* promoter in a dose-dependent manner. Thus, CVI-Meq and CVI-LMeq proteins could reduce the expression of Md5-Meq in case of Md5 super infection in CVI988/Rispens vaccinated chickens leading to no transformation possibly because of low levels of Md5-Meq. However, this hypothesis remains to be tested.

Unlike CVI-Meq proteins, chimeric Meq proteins CVI/Md5-Meq, Md5/CVI-Meq, and Md5/CVI-LMeq, which were constructed by exchanging the DNA binding domain (DBD) and transactivation domain (TAD) among Md5- and CVI-Meq proteins, activated the the *meq* promoter, although to a significantly lower level than Md5-Meq protein. Thus, either the DNA binding domain or transactivation domain of Md5-Meq protein can confer transactivation function to CVI-Meq proteins. The above findings suggest: (1) the binding affinity of the DNA domain of CVI-Meq may not be as good as that of Md5-Meq (2) the transactivation ability of the transactivation domain of CVI-Meq and CVI-LMeq is inferior to that of Md5-Meq, and (3) both domains are important for stronger transactivation activity by Md5-Meq. In the future, it is important to analyze the DNA binding affinities of parental and chimeric Meq proteins.

Among the six amino acid residues in Md5-Meq that are different from CVI-Meq, alanine at position 71, in the DNA binding domain, and threonine at position 320, in the transactivation domain, were important for transactivation. This further supports the importance of both domains in transactivation function. Interestingly, CVI-Meq proteins, have serine at position 71,which is a potential phosphorylation site. It will be interesting to study whether this serine is phosphorylated and if so, whether it modulates its DNA binding activity. Conversely, CVI-Meq proteins have an isoleucine at position 320, resulting in a loss of phosphorylation residue when compared to Md5-Meq. Whether amino acid 320 is responsible for its lack of transactivation function in CVI-Meq proteins could be studied by site-directed mutagenesis. Thus far, the

phosphorylation status of Meq is poorly understood. Our study suggests a potential role for phosphorylation in the biology of Meq that should be explored in the future.

Md5-Meq, CVI-Meq and CVI-LMeq proteins suppressed transcription from the pp38/pp14 bidirectional early promoter as well as from the Meq binding sequence in the MDV Ori. These findings suggest that Meq may play a role in the maintenance of latency by suppressing the expression of early proteins pp38 and pp14. We showed, for the first time, that Meq can activate MDV gB, MMP-3, and Bcl-2 promoters. Although all three Meq proteins activated these promoters, activation by Md5-Meq was generally higher.

CVI-Meq and CVI-LMeq proteins transformed NIH3T3 and Rat-2 fibroblast cell lines when overexpressed using a replication defective retrovirus system. These transformed cells formed foci and colonies in soft agar very similar to Md5-Meq transformed cells. Therefore, the in vitro transformation ability of CVI-Meq and CVI-L Meq is not affected by the amino acid differences. Since MDV targets T cells for transformation, it is appropriate in the future to study the transformation properties of CVI-Meq and CVI-LMeq proteins using an in vitro chicken T cell system, which is lacking at present.

To understand the role of Meq DNA binding and transactivation domains in the development of lymphomas, we constructed two recombinant rMd5 viruses: rMd5-Md5/CVI-Meq (DNA binding domain of Md5-Meq and transactivation domain of CVI-Meq) and rMd5-CVI/Md5-Meq (DNA binding domain of CVI-Meq and transactivation domain of Md5-Meq). rMd5, rMd5-CVI-Meq, rMd5-CVI-LMeq, rMd5-Md5/CVI-Meq

and rMd5-CVI/Md5-Meq viruses induced lymphomas in 100 %, 6%, 20%, 100% and 36% of chickens, respectively. No disease was detected with CVI988/Rispens vaccine or control birds. All five recombinant viruses replicated well both in vitro and in vivo and all of them were capable causing infection in contact birds through horizontal transmission.

Parental rMd5 caused 100% nerve lesions and 28% visceral lesions. rMd5-Md5/CVI-Meq induced 100% lesions in nerves and 55% lesions in visceral organs whereas rMd5-CVI/Md5-Meq induced 36% lesions in nerves and 7% lesions in visceral organs. Type A nerve lesions, characterized by diffuse infiltration of lymphoid cells, were predominantly found in rMd5 and rMd5-Md5/CVI-Meq infected chickens. In contrast, rMd5-CVI/Md5-Meq induced commonly type C lesions, characterized by occasional lymphocyte infiltration in the nerves. rMd5 caused visceral lesions in the heart (15-33%) and the spleen (6-23%) and very rarely (6%) in the liver, gonads, and kidney. Interestingly, rMd5-Md5/CVI-Meq caused tumors in visceral organs at a significantly higher rate: heart (25-60%), spleen (20-50%), liver (0-26%), gonads (6-25%) and kidney (35-60%). It is of interest that kidneys with tumors were at least 3 times their normal size,. On the other hand, rMd5-CVI/Md5-Meq induced very rarely lesions in the spleen (0-7%) and heart (0-8%) and no lesions in the kidney or gonads were observed.

Our data collectively suggests that: (1) Md5/CVI-Meq is more oncogenic than CVI/Md5-Meq; (2) Md5/CVI-Meq is more or at least as oncogenic as Md5-Meq; (3) Md5/CVI-Meq retains the pathogenicity of Md5-Meq albeit containing the TAD of

CVI988/Rispens, thus indicating DBD plays an important role in transformation; (4) when compared to CVI-Meq, CVI/Md5-Meq, which contains the TAD of Md5, is more virulent, thus suggesting that the TAD also plays a role in transformation; (5) combination of DBD and TAD determines the transformation potential of Meq; and (6) Meq, is essential for transformation and plays a role in tumor dissemination.

Based on the results presented here, future lines of research could include: (1) study whether the DNA binding domain or the transactivation domain of Meq alone can cause lymphomas in chickens; (2) study the role of individual amino acids, particularly residues at 71 and 320, in transformation by the generation of recombinant viruses; (3) to study the transformation properties of Md5-Meq in the context of CVI988 genes to better understand the role of other MDV genes in the transformation process; (4) to carry out microarray analysis in tumor cell lines derived from different recombinant viruses in order to identify genes that are regulated by Meq, especially those that play a role in tumor metastasis.

In conclusion, CVI-Meq proteins transform fibroblasts and function as weak transcriptional factors in vitro. In addition, our studies proved that the DNA binding domain of CVI-Meq is not as efficient as that of Md5-Meq in inducing lymphomas and the transactivation domain of CVI-Meq may play a role in tumor dissemination.

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VITA

Name: Dharani Kumar Ajithdoss

Address: MS4467 Department of Veterinary Pathobiology
Texas A&M University, College Station, TX 77843

Email Address: dajithdoss@cvm.tamu.edu

Education: 2000 Bachelor of Veterinary Science (B.V.Sc), D.V.M equivalent
Tamil Nadu Veterinary and Animal Sciences University, India
2002 Master of Veterinary Science (M.V.Sc), Microbiology
A.N.G.Ranga Agricultural University, India
2009 Doctor of Philosophy (Ph.D.), Poultry Science
Texas A&M University, College Station, TX

Awards and Honors:

- Richard B. Rimler Memorial Paper Award, The American Association of Avian Pathologists (2008)
- Fellow of Graduate Teaching Academy, Texas A&M University (2008)
- First prize in poster presentation, Student Research Week, Texas A&M University (2008)
- First prize in oral presentation, Student Research Week, Texas A&M University (2007)
- Environmental Health and Safety Award, Student Research Week, Texas A&M University (2007)
- Graduate Student Research and Presentation Grant, Texas A&M University (2005)
- Texas Broiler Council Endowed Graduate Fellowship (2003-09)