ROLE OF THE LEUCINE ZIPPER OF MAREK'S DISEASE VIRUS ONCOPROTEIN MEQ IN PATHOGENESIS

A Dissertation

by

PAULETTE F. SUCHODOLSKI

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: Veterinary Microbiology

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ABSTRACT

Role of the Leucine Zipper of Marek's Disease Virus Oncoprotein Meq in Pathogenesis.

(May 2009)

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Marek's disease virus (MDV), the etiologic agent of Marek's disease, is a potent oncogenic herpesvirus. MDV is highly contagious and elicits a rapid onset of malignant T-cell lymphomas in chickens within several weeks after infection. The MDV genome encodes an oncoprotein, Meq, which shares resemblance with the Jun/Fos family of bZIP transcription factors. Similar to c-Jun, the leucine zipper region of Meq allows the formation of homo- and heterodimers. Meq homo- and heterodimers have different DNA binding affinities and transcriptional activity; therefore, they may differentially regulate transcription of viral and cellular genes. Previous in vitro data has suggested that Meq homodimers may be involved in regulating viral latency/reactivation, while Meq/c-Jun heterodimers are involved in transformation. Therefore, this research investigates the role of Meq homodimers and Meq-Jun heterodimers in the pathogenicity of MDV, by generating chimeric *meq* genes, which contain the leucine zipper region of the yeast transcription factor GCN4 (*meqGCN*) or leucine zipper region of c-Fos (*meqFos*). Thus producing Meq proteins that exclusively homodimerize (MeqGCN) or heterodimerize with Jun family members (MeqFos). Recombinant viruses (rMd5-MeqGCN and rMd5-

MeqFos) containing the chimeric genes *meqGCN* or *meqFos*, respectively, in place of parental *meq* were generated with overlapping cosmid clones of Md5, a very virulent MDV strain. The chimeric genes were evaluated in vitro and retained DNA binding and transactivation/repressive functions, however, selected cells expressing MeqGCN and MeqFos had reduced colony formation in soft agar. Both the rMd5-MeqGCN and rMd5-MeqFos viruses replicated in vitro and in vivo, but rMd5-MeqGCN was unable to transform T-cells in infected chickens, while rMd5-MeqFos induced preneoplastic nerve lesions in 50% of infected birds. However, a third virus rMd5-MeqFos/GCN, which contains one copy of each *meqGCN* and *meqFos,* induced preneoplastic nerve lesions in 60% of infected chickens and neoplastic lesions in 20% of infected chickens. These data provide the first in vivo evidence that both Meq homodimers and heterodimers are necessary for MDV induced transformation.

DEDICATION

To Jan and my family for their continued support and confidence

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CHAPTER I

INTRODUCTION

Marek's disease

Marek's disease (MD) is a highly contagious and oncogenic cell-associated herpesvirus, of the genus Mardivirus that causes lymphoproliferative and neuropathic disease in chickens. MD was first described in 1907 and is named in honour of the Hungarian veterinarian Jozsef Marek, who first described the disease as fowl paralysis (polyneuritis). Early descriptions of MD indicated the disease only affected the nervous system. However, in the 1920's, significant proportions of affected chickens also had visceral lymphomas, which were cytologically similar to the lymphoid infiltrations in nervous tissue. These occurrences of visceral tumors in MD lead to confusion with lymphoid leukosis (6). Leukosis describes proliferative diseases of the haemopoietic system caused be avian leukosis virus (ALV), and lymphoid leukosis is the most common form (59). Since distinguishing between MD tumors and lymphoid leukosis relied on pathological examination confusion on distinguishing these two etiologies continued and as a result the view that all lymphoid tumors were an indication of a single disease was common. It was not until 1960 that the World Veterinary Poultry Association called for a conference to classify Avian Leukosis Complex and fowl paralysis. At the end of the conference it was recommended that the two diseases be

This dissertation follows the style of the Journal of Virology.

distinguished and that “fowl paralysis” be termed MD (6).

During the 1950’s explosive outbreaks of “visceral lymphomatosis” in addition to neural involvement became prevalent in the USA and Europe, this new form of MD was termed acute MD, young chickens were affected with mortality of 20-30% on average but as high as 60% (6). In addition to higher mortality, acute MDV strains have a higher incidence of visceral tumors compared to classical strains (89). By the 1960’s acute MD was widespread and predominate in countries with a developed poultry industry. Vaccination was introduced in the 1970’s and controlled MD well, however in the late 1970s and again in the 1990’s new vaccine strategies were implemented to circumvent vaccine breaks (6). MD is an economically important disease of poultry and is controlled by vaccination to prevent economic losses by highly contagious and potently transforming MDV. In 2007 it was estimated that over 10 billion chickens in the United States alone were vaccinated against MD (69).

Three serotypes of MDV have been identified based on virus neutralization and agar gel precipitation analysis and are currently classified as *Gallid herpesvirus 2* (GaHV-2) or Marek’s disease virus serotype 1 (MDV-1), *Gallid herpesvirus 3* (GaHV-3) or Marek’s disease virus serotype 2 and the *Meleagrid herpesvirus 1* (MeHV-1) or turkey herpesvirus, only MDV-1 is oncogenic. In addition, a number of different pathotypes exist within MDV-1 ranging from mild to very virulent plus (12, 88). Vaccination with non-oncogenic turkey herpesvirus, HVT, was introduced in the 1970’s and provided excellent protection, however, due to interference with maternal antibodies and increased virulence from field strains, a bivalent vaccine was introduced in the mid

1980's. The bivalent vaccine combined HVT and the non-oncogenic SB-1, MDV serotype-2 and provided better protection than either component alone. Still, vaccine breaks continued and the emergence of more virulent field strains emerged, and in the 1990's the Rispens vaccine was introduced. Rispens is a tissue culture attenuated MDV-1 virus strain called CVI988 (3). The Rispens strain is the most widely used and the most effective MDV vaccine available today. A number of different pathotypes exist within MDV-1 ranging from mild to very virulent plus (12, 88). It is believed that the continued emergence of virulent pathotypes in MDV is a consequence of vaccine pressure (69). Development of new vaccines with increased efficacy continues to be explored in anticipation of the emergence of more virulent strains. The most current potential vaccine is a recombinant MDV-1 strain in which the putative oncogene Meq has been deleted from the genome and results in higher efficacy than the current Rispens vaccine (39).

Pathogenesis of Marek's disease infection

There are three main phases of MDV infection: cytolytic, latent and transforming infection. Lesions associated with MDV include inflammatory lesions during cytolytic infection, and proliferative lesions, which are composed of transformed T-cells. Transformed T-cells can be found in nerves or visceral organs (7, 12). It is the infiltration and proliferation of T-cells in the nerves that causes paralysis and the classical disease. Proliferative lesions in the visceral organs in addition to the nerves are associated with acute MD and increased virulence (89).

Cytolytic infection

Initial infection occurs through the respiratory tract by inhalation of dander containing infectious virus. It is presumed that phagocytic cells pick up virus in the lung and then distribute it to bursa of Fabricius, spleen and thymus. This begins the early cytolytic phase of MDV infection in which virus is produced and released resulting in cell death and necrosis and is evident 3-6 days PI. B-cells are the primary target cell during cytolytic infection, this has been demonstrated by in vitro infection of splenocytes and embryonal bursectomy, which results in impaired cytolytic infection. But, lytic infection has been detected in activated CD4+ and CD8+ T-cells (12). Interestingly, MDV infection of macrophages from infected chickens has been demonstrated by viral antigen detection using flow cytometric analysis and confocal microscopy, thus supporting a role for macrophages as the carrier cells during initial infection. However, since the presence of viral particles was not detected, further work is needed to evaluate if macrophages can maintain productive infection (4). In addition to lymphocytes, epithelial cells are highly susceptible to cytolytic infection and infection of non-lymphoid cells have been found in multiple organs including, kidney, adrenal gland, proventriculus, lung, liver, and crop to list a few (12). A consequence of cytolytic infection is atrophy of thymus and bursa of Fabricius, which is more evident with more virulent pathotypes (14, 70). Cytolytic infection plays an important role in the spread of MDV infection, activation of the immune system and therefore number of target cells available for MDV transformation (12). This has been demonstrated in mutant recombinant viruses that had impaired cytolytic infection and reduced tumor induction

(18, 25, 32, 57). The feather follicle epithelium is the only site of fully productive infection in which infectious cell free virus is produced. It has been suggested that virulence and transmission are interrelated traits, however this has not yet been quantitatively assessed (69).

Latent infection

A feature of all herpesviruses is the ability to enter a latent state in their natural hosts. During latency the viral genome is maintained and only a small set of proteins or viral RNA are expressed, and infectious virus is not produced (78). Immune responses to herpesvirus infection play an important role in the switch from cytolytic infection to latency. Approximately 7-8 days PI, cytolytic infection switches to a non-productive latent infection during which viral proteins are not expressed. Immunocompetence influences the switch to latency and is required for maintenance of latency.

Immunosuppressive treatments of infected chickens leads to prolonged cytolytic infection and recrudescence of cytolytic infection after latency has been established (9). Although not defined, extrinsic factors influence the switch to latency (12). Recombinant IFN gamma combined with lipopolysaccharide (LPS), and nitric oxide treatment reduce MDV and HVT replication in vitro. Cytotoxic T-cell (CTL) responses against MDV lytic antigens, ICP4, pp38, gB have been demonstrated which may also drive the switch to latency for the virus to "escape" immune surveillance (71). In contrast to cytolytic infection, activated CD4 + T-cells are the predominate cell type infected during latency. The difference in cell type infected during latency and cytolytic infection may be a

consequence that B-cells are shorter lived or that T-cells are intrinsically less able to support productive infection (12).

Transforming infection

Activated CD4+ T-cells are the target cell for MDV transformation. MATSA (Marek's disease tumor associated surface antigen) originally described in MD tumor cells as an MDV specific marker was later found to be a marker of activated T-cells, and another MD tumor associated marker AV37 is also likely linked with an activated phenotype. The important role of immune activation in transformation has been demonstrated in a study by Calnek et al. (1989), in which alloantigens along with MDV were injected in wing webs and enhanced tumor development was observed. Current knowledge of lymphoma development in MDV entails a series of sequential events that lead to MDV transformation, the first being the initial cytolytic infection, followed by a period of latency, and eventually transformation. The necrotizing infection and inflammatory response that develops during cytolytic infection plays an important role in stimulating the activation of T-cells and recruitment of target cells and the switch to latent infection Lymphomas may be found in any visceral organ, peripheral nerves, eye and skin as early as 12-14 PI with highly virulent pathotypes (12). Like latently infected cells, transformed cells do not produce infectious virus. In addition, few known viral antigens are expressed.

MDV genome and genes associated with pathogenesis

The180 kb linear double-stranded DNA genome of MDV is surrounded by a 100 nm icosahedral protein capsid core. The tegument, is a proteinaceous structure that varies in thickness and surrounds the capsid core, a lipid envelope in turn surrounds the tegument and core. Enveloped virus varies in size from 120 to over 300 nm in diameter depending on the thickness of the tegument and state of the lipid envelope. Although, MDV shares biological characteristics with gammaherpesviruses such as its tropism and its ability to transform lymphocytes, it is classified as an alphaherpesvirus based on viral genome organization and sequence homology (67). The genome structure consists of unique long (U_L) and short (U_S) regions flanked by terminal and inverted repeat long (TR_L, IR_L) and terminal and inverted repeat short (TR_S, IR_S).

Search for MDV oncogene(s)

The highly oncogenic nature of MDV suggests the viral genome encodes oncogene(s). Initial investigations in the search for candidate viral oncogenes focused on transcriptionally active regions of the MDV genome, genes expressed in transformed cells, and identification of genes unique to MDV. In MDV transformed cells transcription is limited to the repeat regions whereas in lytically infected cells transcription throughout the viral genome is detected. Probes from $BamH_1$ and partial EcoQ libraries were used to screen genes expressed in MDV lymphoblastoid cells. Transcription was found to be limited to repeat regions with the highest transcription from Bam I_2 and EcoQ regions with most transcripts originating from the EcoQ region.

Therefore, the EcoQ region was sequenced and a predicted protein coding sequence that shared homology with the bZIP factors, c-Jun and c-Fos, was found and named Meq. The *meq* gene is named after the EcoQ fragment where it is located, "Marek's EcoQ", and two copies are found in the viral genome within the terminal repeat long (TR_L) and internal repeat long (IR_L) regions (60, 66, 67, 80). Antibodies raised against the leucine zipper region of Meq were successful in detecting 40 and 60 kda proteins from the MDV transformed cell line RP4 (33). Since c-Jun and c-Fos are proto-oncogenes (73), the shared homology of Meq with these factors and detection of Meq in tumor cells lines, suggested a role for Meq in MDV transformation.

While MDV pp38 and ICP4 genes are also located in transcriptional active regions of MDV transformed cells, only Meq is consistently expressed in tumor cells and unique to the oncogenic MDV-1 genome (33). Although, pp38 expression has been identified in MDV transformed cells, because pp38 is a lytic associated protein, it is commonly argued this represents a subset of cells undergoing reactivation. In addition, only anti-sense transcripts to ICP4 have been identified in transformed cells (43, 44).

Meq

After the identification of Meq several studies soon followed to characterize this unique MDV putative oncogene. Meq is a bZIP protein with a basic DNA binding domain at the N+ terminus, leucine zipper, and proline rich C+ terminal transactivation domain (33). Meq is a nuclear phosphoprotein and potentially phosphorylated by a number of cellular kinases, including PKA, PKC and MAPK (49). Nuclear and nucleolar

localization signals are located in the basic region, and referred to as BR1 and BR2, respectively (46). A proline/glycine rich region is located adjacent to the basic region that contains a C-terminal binding protein (CtBP) site (8). In addition Meq contains putative p53 and retinoblastoma (Rb) binding sites (45). The proline rich domain of Meq is a potent transactivator with the last 33 amino acids essential for transactivation (62).

The DNA binding and transactivation/repression properties of Meq have been investigated to better understand Meq's potential role as a transcriptional regulator. The cyclic amplification of selected targets (CASTing) technique using bacterial expressed Meq amino acids 1-129 and c-Jun proteins were utilized to determine the optimal DNA binding sites of Meq/c-Jun and Meq/Meq dimers. Meq/c-Jun heterodimers were found to bind tetradecanoylphorbol acetate response element (TRE; TGAGTCA) and cyclic AMP response element (CRE; TGACGTCA) core containing sequences RTGAC(G)TCAY where R is a purine and Y is a pyrimidine referred to as Meq response element (MERE1). Meq/Meq homodimers bound an extended MERE I sequence GAGTGATGAC(G)TCATC and consensus sequence RACACACAY (MERE II). However, MERE II sequences are necessary, but not sufficient, for Meq binding, and additional flanking sequences are required for binding by Meq. EMSA analysis further demonstrated Meq/c-Jun heterodimers bind TRE and CRE sequences but Meq homodimers only bound MERE II like sequences (61). The *meq* promoter contains a CRE/TRE like sequence (TGACGT) and therefore potential Meq binding site. Luciferase assays confirmed that Meq transactivates the *meq* promoter, and transfections together with c-Jun resulted in increased activation (47, 61). The MDV pp38/14

bidirectional promoter contains a sequence (ACACA) that resembles MERE II at the MDV Origin of replication, with which Meq homodimers were shown to bind by EMSA (42, 61). However, unlike the *meq* promoter, Meq repressed both pp14 and pp38 promoters in a dose dependent manner (42). A modified ChIP assay was performed to evaluate Meq binding sites in the MDV genome. Cross-linked sonicated chromatin was recovered from MSB1 cells, an MDV transformed cell line, followed by immuoprecipitation with Meq or c-Jun specific antibody. The recovered DNA fragments were labeled with radioactive P^{32} and used to probe digested cosmid DNA spanning the MDV genome. The results indicated numerous low affinity Meq binding sites were present throughout the MDV genome, with areas of higher affinity. Based on restriction enzyme maps, these areas of high Meq binding corresponded with the *meq* and *ICP4* promoters as well as the MDV Ori. PCR analysis from ChIP samples confirmed Meq was recruited to each of these promoters and the MDV gB promoter as well. In addition, PCR analysis from ChIP samples immunoprecipitated with c-Jun antibody showed that c-Jun was recruited to the Meq and ICP4 promoters. Gel shift assays, coimmunoprecipitation and ChIP assays have confirmed dimerization of Meq and c-Jun in vitro and in MDV transformed cells (42). Considering the oncogenic potential of c-Jun, a role between c-Jun and Meq in MDV transformation is appealing.

Expression of Meq in vitro supports its oncogenic potential. Meq has been shown to promote anchorage independent growth in rat-2, and DF-1 cell lines (41, 47, 48). Expression of Meq also promotes resistance to apoptosis under serum deprivation and treatments with ceramide, TNFα, and UV (48). Meq is essential for T-cell

transformation in vivo, as deletion of Meq from the MDV genome and mutations of the CtBP site have been shown to result in loss of transformation (8). However, the molecular mechanisms of Meq in MDV transformation remain to be defined, recent studies indicate Meq/c-Jun partnership may play a critical role in MDV transformation. Expression of Meq in DF-1 cell line was shown to activate genes associated with the v-Jun pathway, such as HB-EGF, Bcl-2, JTAP-1, and JAC. In addition silencing of c-Jun by siRNA resulted in reduction in colony formation induced by Meq expression (41).

Other MDV genes associated with pathogenesis

A number of genes unique to MDV are encoded within the repeat long region or at its junction with the unique long region, and have been associated with pathogenesis. These genes include *pp38*, *v-IL8*, and *LORF11* genes. Disruption of these genes from oncogenic MDV has been shown to result in reduced tumor incidence. This is likely a consequence of impaired lytic replication in chickens rather than a direct role in transformation (17, 25, 40, 57). The *pp38* gene encodes for an MDV lytic antigen that is evident during cytolytic infection of lymphoid tissues. Although, pp38 may be detected in subsets of cells from MDV transformed cells this may represent virus undergoing reactivation. The MDV virokine, v-IL8, shares significant homology with cellular CXC chemokine IL-8. And bacterial expressed v-IL8 functions as a chemoattractant for chicken peripheral blood mononuclear cells. A reduction in tumor incidence in vivo is evident with v-IL8 deletion mutants, however cytolytic infection is also impaired, therefore v-IL8 deletion mutants may indirectly impair tumor formation by reducing

transformable target cells (18, 57). LORF-11 is an MDV unique gene of unknown function present in oncogenic and non-oncogenic MDV serotypes. Deletion of LORF-11 from the MDV genome resulted in a virus that was able to replicate in vitro but was replication defective in vivo. Antigen detection in lymphoid organs was not detectable at 6 days PI. Therefore, although no tumor incidence was observed in chickens infected with a rMd5ΔLORF-11 this is likely a consequence of absent viral replication (40).

However, disruption of viral telomerase RNA (*vTR)* or *meq* genes from the MDV genome does not disrupt early cytolytic infection, but greatly reduces or abolishes tumor incidence, respectively (50, 79). The MDV-1 genome encodes a functional *vTR* which shares 88% homology with chicken telomerase (*chTR*) and is not found in the non-oncogenic MDV-2 or MeHV-1 genomes (23). In vitro expression of *vTR* in cells significantly enhanced proliferation compared to vector control cells and more importantly deletion of *vTR* from the MDV genome resulted in a 60% reduction of tumor incidence in infected chickens (79).

bZIP- proteins

Basic leucine zipper (bZIP) proteins are a group of transcription factors that consist of a basic DNA binding region adjacent to a leucine zipper region and have been described in plants, insects, and mammals. The sequences of bZIP proteins widely vary but the leucine zipper and basic domain are the common features of this group. The location of the bZIP domain also varies and can be located at either the N+ or C+ terminus. The DNA binding domain is the most conserved region among bZIP proteins

and contain the consensuses sequence, N _ _ A _ _ (C/S) R, ending just before the start of the leucine zipper. The leucine zipper region is less conserved but all consist of a periodic repeat of a leucine every 7th amino acid, however in some proteins one or more of the leucine residues may be replaced by an alternative amino acid. The number of leucine repeats also varies among bZIP proteins and three up to six repeats have been described. Although the sequences of bZIP proteins vary greatly, a high degree of homology across species for a given bZIP exists (19). The importance of bZIP proteins across species is evident, as these factors have been described in a broad range of cellular functions. In mammals, bZIP factors have a critical role in cell proliferation and development of liver, bone and heart during embryogenesis. They are also involved in metabolism, and stress response in mammals (19, 21). In plants bZIP proteins are involved in regulating seed development and flower maturation, and sexual maturation in yeast (19).

The main function of the leucine zipper is to bring the basic regions of each monomer together to allow for specific DNA binding. Characterization of the function of the leucine zipper in dimerization has been carried out with the bZIP proteins c-Jun, c-Fos, and GCN4. Experiments were performed by generating chimeric bZIP proteins by swapping the leucine zipper region and testing dimer formation by EMSA anaylsis (37, 72). The results conclude that the leucine zipper region is interchangeable and dictates whether a bZIP protein will form homodimers or heterodimers. It was demonstrated that the leucine zipper region of c-Jun allows for the formation of both homodimers and heterodimers while the leucine zipper regions of GCN4 and c-Fos exclusively confers

homodimerization and heterodimerization, respectively (37, 72). The seven amino acids of each repeat are denoted as *a*-*g* and the residues in the opposite dimer *a′*-*g′*. Leucine residues are found at position *d*. Position *a* contains hydrophobic residues a characteristic of coil-coil structures, and most charged residues occur at positions *e* and *g*. The charges of the amino acids at the e and g position have been shown to be important determinants of homodimerization and heterodimerization. For example, proteins where the *e* and *g* residues within one heptad are of opposing charge can form both homo- and heterodimers, whereas, *e* and *g* residues of the same charge will form heterodimers with a protein with e and g residues of opposite charge (30).

DNA binding sites recognized by bZIP dimers show dyad symmetry, or two "half sites" which are contacted by each of DNA binding domain in the dimer. Activating protein-1 (AP-1) transcription factors are a group of well-described bZIP proteins that are characterized by their ability to bind and regulate sequence specific gene elements, AP-1, which are found in many genes associated with cell proliferation (73). The c-Jun, c-Fos, and CREB/ATF families are part of the AP-1 complex. The consensus sequences recognized by AP-1 factors are TPA-response element (TRE) TGAGTCA and cyclic AMP response elements (CRE) TGACGTCA. However, it is important to keep in mind since that since optimal binding site are evaluated in vitro, target sequences in natural promoter or enhancer settings may deviate from the optimal recognition sequence (16). It is well established that dimerization impacts the sequences that bZIP proteins bind. For example, as homodimers the Fos/Jun families preferentially bind TRE sequences while the CREB/ATF family preferential bind CRE sequences. In

addition, as heterodimers DNA binding preferences and affinity may change. For instance, c-Jun/c-Fos heterodimers recognize TRE sites, while c-Jun/ATF2 dimers preferentially bind CRE sequences and even though Jun/Jun homodimers bind TRE sites, c-Jun/c-Fos heterodimers bind TRE sites with greater affinity (26, 81). While it is generally accepted that most bZIP proteins dimerize before DNA binding, some bZIP proteins including c-Fos and c-Jun can bind DNA as monomers and then dimerize. In addition, specificity of DNA binding is dependent on the basic region and the spacer region located between the basic region and leucine zipper (82). For example, domain swapping experiments have shown that although the leucine zipper region dictates dimerization therefore impacting DNA binding preference, DNA binding is still influenced by the basic regions. Chimeric proteins containing c-Fos or c-Jun basic and leucine zipper domains can form stable heterodimers and bind AP-1 probes designed from the Jun promoter. However, chimeric proteins with the CREB basic region and c-Jun or c-Fos leucine zipper domains have reduced DNA binding even though dimerization through the c-Fos and c-Jun leucine zipper region is intact (63).

In addition to the bZIP domain, nuclear localization signals, and activation and repression domains are also typically found in bZIP proteins and contribute to their role as transcriptional regulators. Nuclear localizations signals are generally found in the basic domain of bZIP proteins and ensure transfer to the nucleus. Activation and repression domains contribute to the regulatory activity of bZIP proteins, which are in turn regulated by phosphorylation. In addition AP-1 members regulate each other, for instance Jun promotes cell proliferation while JunD and JunB are negative regulators of

cell proliferation (21). The ability of cross dimerization between bZIP members results in a cell specific competition for dimerization partners and DNA binding sites that results in a complex system of transcriptional regulation (82). In addition, the activity of bZIP proteins are further regulated by phosphorylation, that depends on the activated kinases present in a particular cell. Therefore, overexpression of AP-1 members, mutations or deletions in regulatory domains such as in v-Jun lead to deregulated transcription and transformation.

c-Jun

The viral counterpart of c-Jun, v-Jun, was first cloned from the avian sarcoma virus 17 in 1987 and Jun was later identified as the cellular homologue of v-Jun and a component of AP-1. In fact, the name "jun" is derived from the Japanese word ju-nana, meaning 17 (85). The connection of c-Jun and AP-1 came about after the homology between c-Jun and GCN4 was recognized. AP-1 proteins were initially recognized as a TPA inducible transcription factor(s) that bound specific elements in the metallothionein gene and 72 base pair repeat in the SV40 enhancer region, because it was known that GCN4 also bound AP-1 sequences, Jun was discovered to be a component of AP-1 (84).

A 27 amino acid (delta) deletion and two to three amino acid substitutions (depending on the clone) are found in v-Jun compared to c-Jun. The delta deletion results in deletion of the Jun-N-terminal Kinase (JNK) binding site found in Jun. JNK phosphorylation of Jun results in transcriptional activation by weakening the interaction with the histone deacetylase (HDAC-3) inhibitor complex. Even though v-Jun is not

activated by JNK, it is a strong transactivator because it is relieved of inhibition from the HDAC-3 inhibitor complex. Another mechanism in which v-Jun escapes negative regulation is due to a phosphorytaion mutation at position 243. In c-Jun serine 243 is phosphorylated by ERK, which in turn promotes phosphorylation of threonine 239 by GSK-3. Phosphorylated threonine 239 provides an attachment site for Fbw7 ubiquitin ligase and subsequent proteasomal degradation of c-Jun (86).

A number of studies have been performed in order to understand the transformation pathway of c-Jun and delineate the function of c-Jun homodimers and heterodimers. To study Jun homodimers in transformation chimeric c-Jun proteins with the leucine zipper of EB1 or GCN4 were utilized. Chimeric c-Jun homodimers proteins were able to transform CEF as assessed by proliferation in reduced serum and anchorage independent growth, thus supporting that Jun homodimers promote transformation. However, chimeric c-Jun proteins did have reduced colony formation compared to wild type morphologically had a more condensed than elliptical appearance (15). A similar study using chimeric c-Jun homodimers was performed but with v-Jun. The chimeric v-Jun proteins also induced anchorage independent growth; with reduced numbers compared to v-Jun. Interestingly in vivo the v-Jun-GCN4 construct was similar to v-Jun while v-Jun-EB1 was more oncogenic (34). However, it should be noted that the described chimeric c-Jun proteins are more stable than wild type c-Jun/c-Jun homodimers and can bind AP-1 sites with affinity comparable to c-Jun/c-Fos dimers. Van Dam investigated the role of c-Jun/c-Fos and c-Jun/ATF heterodimers by generating point mutations in the leucine zipper region that resulted in Jun proteins that showed a

preferred dimerization with c-Fos or ATF2. Using these constructs he demonstrated that c-Jun/c-Fos dimers promoted anchorage independent growth while c-Jun/ATF2 dimers promoted growth factor independence (81). Together, these studies show that homodimerization and cross dimerization contribute to transformation.

Unique viral bZIP proteins

Only two other bZIP proteins encoded by herpesviruses have been described, Zta, (also known as ZEBRA, or EB-1) from Epstein barr virus (EBV) and K-bZIP (also known as (RAP, or K8α) from Kaposi sarcoma associated virus (KSHV). However, both Zta and K-bZIP differ from typical bZIP proteins in that the dimerization domain of Zta is weak compared to other bZIP proteins, and K-bZIP does not contain a basic DNA binding domain. Zta and K-bZIP are expressed during lytic infection but are silent during latency. Expression of Zta is sufficient to disrupt latent infection, however this does not apply to K-bZIP. Zta functions as transcription factor and activated early viral gene transcription and can repress transcription of viral late genes. In addition to regulation of viral gene transcription Zta also binds to the origin of lytic replication (ori_{LYT}) and is necessary for lytic replication by recruiting and interacting with viral proteins essential for viral replication. In contrast K-bZIP does not activate transcription of viral early genes but is necessary for viral replication (75).

Cellular proteins that have been described to interact with Zta and K-bZIP include p53, CREB-binding protein (CBP), C/EBPα, and TATA binding protein (TBP). The bZIP region of both Zta and K-bZIP interact with the C-terminal region of p53 and

can interfere with p53 dependent transcription (58, 75). However, activation and enhancement of p53 by Zta in some cell lines has also been described and leads to cell cycle arrest. Interactions of Zta and with the co-activator CBP enhances transcriptional activity of Zta and can relieve K-bZIP mediated repression. The C-terminal domains of both Zta and K-bZIP interact with the bZIP transcription factor C/EBPα leading to enhanced expression of cyclin-dependent kinase inhibitor, $p21^{CIP1}$, which also promotes cell cycle arrest (5, 75). Lastly, Zta has also been shown the interact directly with TBP and stabilizes the association of TFII with the TATA element and therefore promotes transcription from genes with Zta response elements (ZRE) but it is unclear if Zta promotes transcription from ZRE less promoters (75).

EBV also encodes an atypical bZIP protein EBNA3C that contributes to EBV transformation. Although, EBNA3c contains a bZIP domain both the basic and leucine zipper domains do not share homology with previously described bZIP proteins. In addition EBNA3c specific DNA binding sequences have not been defined and it is unclear if EBNA3c can bind DNA directly, but EBNA3c interacts with the transcription factor, RBP-Jκ α, which likely directs DNA binding (87). Still, EBNA3c is a potent repressor and interacts with cellular co-repressors, HDAC 1, and CtBP. The transforming properties of EBNA3c are linked to disruption of cell cycle checkpoints and cell cycle progression. EBNA3c is able to disrupt the cell cycle checkpoints through it's interaction with Rb, CtBP, and cyclin A (87). In addition EBNA3c has been shown to cooperate with H-Ras in transformation of Rat-2 fibroblasts, and is necessary for the growth and maintenance of lymphoblastoid cells (51).

Human T-cell lymphotropic virus (HTLV-1) (HTLV-1) encodes the bZIP protein HBZ which interacts with CREB, c-Jun, JunB and JunD. Unlike v-Fos, and v-Jun, HBZ is a unique viral protein and not a viral homologue of a cellular factor. HBZ together with CREB mediates transcriptional repression of the viral oncoprotein Tax. It is postulated that down regulation of Tax may be a mechanism to down regulate immune responses targeting Tax. In addition, HBZ attenuates AP-1 transcription by disrupting DNA binding through it's interaction with JunB and c-Jun. Although HBZ is not necessary for transformation of T-cells in culture, HBZ contributes to T-lymphocyte proliferation in vivo, however the molecular mechanism remain to be defined (2).

CHAPTER II

HOMODIMERIZATION OF MAREK'S DISEASE VIRUS ENCODED MEQ PROTEIN IS NOT SUFFICIENT FOR TRANSFORMATION OF LYMPHOCYTES IN CHICKEN*

Introduction

Marek's disease virus (MDV), the etiologic agent of Marek's disease, is a potent oncogenic herpesvirus which elicits a rapid onset of malignant T-cell lymphomas in chickens within several weeks of infection, resulting in mortality. MDV is classified as an alphaherpesvirus based on viral genome organization and sequence but shares biological characteristics with gammaherpesviruses such as its tropism and its ability to transform lymphocytes (67). Of the previously described serotypes of MDV, now classified as *Gallid herpesvirus 2* (GaHV-2) or Marek's disease virus serotype 1 (MDV-1), *Gallid herpesvirus 3* (GaHV-3) or Marek's disease virus serotype 2 and the Meleagrid herpesvirus 1 (MeHV-1) or turkey herpesvirus, only MDV-1 is oncogenic. In addition, a number of different pathotypes exist within MDV-1 ranging from mild to very virulent plus (12, 88).

The search for candidate viral oncogenes in the MDV genome led to the discovery of *meq*, which is abundantly expressed in MDV transformed cells, and is

* Reprinted with permission from "Homodimerization of Marek's Disease Virus-Encoded Meq Protein Is Not Sufficient for Transformation of Lymphocytes in Chickens" by Paulette F. Suchodolski, Yoshihiro Izumiya, Blanca Lupiani, Dharani K. Ajithdoss, Oren Gilad, Lucy F. Lee, Hsing-Jien Kung, and Sanjay M. Reddy, 2009. *Journal of Virology*,Vol. 83 , 859-869, Copyright [2009] by American Society for Microbiology.

encoded only by the genome of MDV-1. The *meq* gene is named after the EcoQ fragment where it is located, "Marek's EcoQ", and two copies are found in the viral genome within the terminal repeat long (TR_L) and internal repeat long (IR_L) regions (60, 66, 67, 80). Meq, a 339 amino acid nuclear phosphoprotein, is a bZIP (basic-region leucine zipper) protein, which shares significant homology, in the bZIP domain, with the protooncogene c-Jun a transcription factor of the AP-1 (activating protein) complex (33, 46, 49). AP-1 transcription factors are a group of well-described proteins that are characterized by their ability to bind and regulate sequence specific gene elements, AP-1 sites, which are found in many genes associated with cell proliferation (73). Transformation by deregulated expression of c-Jun or its viral counterpart v-Jun, is well documented and therefore the shared homology between Meq and Jun is intriguing (84).

In vitro data support the oncogenic nature of Meq, which can promote anchorage-independent growth, cell-cycle progression, and anti-apoptosis (48, 49). Recently in vitro expression of Meq was shown to upregulate genes similar to those upregulated by v-Jun suggesting that Meq transforms *via* a v-Jun transforming pathway (41). In addition, knockdown of *c-jun* diminishes Meq's transforming ability in vitro, strongly suggesting that Meq-Jun partnership plays a key role in Meq's oncogenic potential. Yet, the most convincing evidence for Meq's oncogenic property was the characterization of a Meq null recombinant MDV virus (rMd5ΔMeq) which replicated in chickens and did not induce tumors (50). Significantly, the Meq null virus also provided better protection than currently available vaccines in chickens, upon challenging with the most virulent strains of MDV (39) pointing to a potential strategy for further vaccine

improvement, where more subtle mutation(s) of Meq are engineered, to abolish its transforming ability, while retaining its ability of establishing infection in vivo and associated antigenicity. To this end, it is important to further dissect the transforming and replication functions of Meq, which at present, remain largely unexplored.

Like c-Jun, the leucine zipper region of Meq allows the formation of homodimers and heterodimers (42). It has been shown that dimerization partners of bZIP proteins are important determinants of DNA binding specificity and therefore transcriptional regulation. For example, different Jun dimers have been shown to play distinct roles in transformation, i.e. anchorage or serum independent growth (84). Again, similarly to c-Jun, the DNA binding properties of Meq depend on its dimerization partner. Previous characterization of the in vitro DNA binding properties of Meq revealed Meq/c-Jun heterodimers bind AP-1 sequences, while Meq homodimers in addition to AP-1 sequences also bind sequences found at the MDV origin of replication (Ori) (42, 61). Transcriptional analysis of Meq on the AP-1 containing *meq* promoter, and the MDV *pp14/38* bidirectional promoter which contains the MDV Ori, revealed that Meq transactivates the *meq* promoter but represses the bidirectional *pp14/38* promoter. Because Meq has been shown to bind the MDV Ori, it is possible that Meq homodimers repress the *pp38/14* promoter by binding this sequence element. In addition to regulating pp38 expression, an MDV protein highly expressed during lytic infection, Meq was also shown to bind the ICP4 promoter by ChIP analyses. Therefore Meq may also potentially regulate the expression of an additional lytic protein ICP4 (42, 61). This, together with luciferase reporter data, suggests that Meq heterodimers activate AP-1 containing

promoters, therefore potentially activating genes associated with cell proliferation, while Meq homodimers may repress genes associated with lytic infection and consequently may be involved in regulating lytic or latent infection. Collectively, these data point to a role of Meq heterodimers in transformation and Meq homodimers in regulation of viral replication. In order to delineate the functions of Meq and address the role of Meq homodimers in MDV pathogenesis, a recombinant Meq mutant virus (rMd5-MeqGCN) expressing a chimeric *meq* gene (*MeqGCN*) was constructed by substituting the parental Meq leucine zipper with that of the leucine zipper of the yeast protein GCN4. The leucine zipper region of GCN4 allows for the formation of only homodimers (37), thus conferring homodimerization of MeqGCN. The homodimerization and DNA binding and transactivation/repression properties of MeqGCN were tested in vitro. Recombinant virus expressing MeqGCN was studied in vitro *and* in vivo and compared to parental rMd5 and rMd5ΔMeq viruses. In vitro rMd5-MeqGCN replicated similarly to parental rMd5; however, infection of chickens with rMd5-MeqGCN did not result in tumor formation. We therefore present the first in vivo evidence that Meq homodimers are not sufficient in MDV transformation. Our data also showed that subtle mutations of Meq are effective in generating a non-virulent recombinant MDV, paving the way to the development of improved recombinant vaccine based on Meq mutations.

Materials and methods

Cells and viruses

Primary duck embryonic fibroblasts (DEF) were used for virus propagation, virus

reactivation assay, growth curves and DNA transfections. Recombinant viruses were generated from cosmids derived from a very virulent MDV strain, Md5 (64). The previously described rMd5ΔMeq was also used in this study (50). Chicken embryonic fibroblasts (CEF) were inoculated with rMd5-MeqGCN virus to obtain viral DNA used for transfection to recover a revertant virus (rMd5-MeqGCNR). The DF-1 cell line was used for luciferase assays and co-immunopreciptation (Co-IP) experiments (35). DEF and CEF were maintained in Leibowitz-McCoy (LM) media supplemented with 5% bovine calf serum (BCS) and penicillin-streptomycin at 37°C. The DF-1 cell line was maintained in LM media supplemented with 4% fetal bovine serum (FBS) and penicillin-streptomycin at 37°C.

Cosmids

Previously described cosmids SN5, P89, SN16, A6 and B40, encompassing the entire genome of the very virulent strain Md5 (64) were used to generate a recombinant Md5 (rMd5) virus and a recombinant Md5 with a chimeric Meq gene containing the leucine zipper from GCN4 (Fig. 1, 2).

To generate mutations in the *meq* gene, a 741 bp DNA fragment referred to from here forward as *meq-Kpn*I, which contains the coding sequence for *meq* nucleotides 1-385 was mutated by a series of subcloning steps and overlapping PCR. The *meq-Kpn*I fragment is located within the EcoQ fragment of the Md5 genome (Fig. 1).

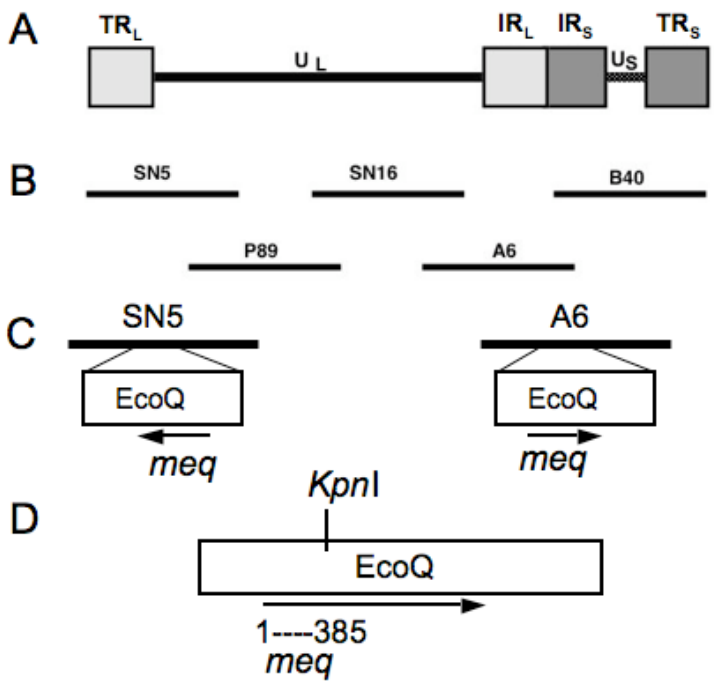

Fig. 1. Cosmid clones used to recover recombinant viruses. (A) Organization of the serotype 1 MDV genome. (B) Schematic representation of the overlapping cosmid clones used to reconstitute recombinant viruses, rMd5 and rMeqGCN, derived from a very virulent (vv) strain of MDV (Md5). (C) Location of EcoQ fragment and Meq gene in cosmids SN5 and A6. (D) Location of *Kpn*1 site described in materials and methods, located within the EcoQ fragment at nucleotide 385 of the *meq* gene.

First, the EcoQ fragment was released from previously described cosmids A6 and SN5 using recA assisted restriction endonuclease (RARE) method (22). Briefly, 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') were used to protect the flanking *Eco*RI sites of the EcoQ fragment during the *Eco*RI methylation reaction. The unmethylated *Eco*RI sites were digested with *Eco*RI releasing. the EcoQ fragment, which was subsequently cloned

into pCR2.1 (Invitrogen, Carlsbad, CA). The digested A6 and SN5 cosmids were self-ligated generating A6ΔEcoQ and SN5ΔEcoQ and HB101 competent bacteria transduced using Gigapack III (Stratagene, La Jolla, CA) according to manufacturer's recommendations. Next, the *meq*-*Kpn*I fragment was released from pCR2.1-EcoQ by digestion with *Kpn*I, and the fragment gel purified, using the QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA) and cloned into *Kpn*I digested pCR2.1 vector. Overlapping PCR, in a combination of three PCR reactions, was performed to replace the leucine zipper region of *meq* with the leucine zipper region of *GCN4*. Two primary PCR reactions were performed to generate the 5' and 3' ends of *meq*-*Kpn*I fragment. The 5' reaction was performed using primers M13R (5'- CAG GAA ACA GCT ATG AC -3') and SR1192-*GCN4* Leucine Zipper Reverse (5'- CTC ATT TTC CAA GTG ATA ATT TTT CGA AAG CAA TTC TTC AAC CTT GTC TTC AAG TTT GTC TAC ATA GTC CGT CTG CTT CCT -3'). The 3' end PCR reaction was performed using primers SR1190 (5'- GAC CGA GAT AGG GTT GAG TG -3') and SR1193-*GCN4* Leucine Zipper Forward (5'- AAA AAT TAT CAC TTG GAA AAT GAG GTT GCC AGA TTA AAG AAA TTA GTT GGC GAA CGC CGT GTA CAG TTG GCT TGT CAT GAG CCA -3'). Both amplicons were gel purified, mixed together and used as templates in a third PCR reaction with primers M13R and SR1190, generating a full *meq*-*Kpn*I fragment containing the *GCN4* leucine zipper in place of the *meq* leucine zipper (*meq*-*GCN4*-*Kpn*I). The *meq*-GCN4-*Kpn*I fragment was subsequently cloned into pCRBlunt (Invitrogen) transformed into *E.coli* Top10 cells and positive clones were identified by restriction digestion and fully sequenced. The *meq*-GCN4-*Kpn*I fragment was then

released by *Kpn*I digestion and cloned into pcr2.1-EcoQΔ*Kpn*I, replacing the parental *meq*-*Kpn*I fragment contained in this region. The newly generated EcoQ fragment (EcoQ-MeqGCN) containing the mutated *meq* leucine zipper (MeqGCN) was then transferred into cosmids A6ΔEcoQ and SN5ΔEcoQ using RARE. The *Eco*RI site of cosmids A6ΔEcoQ and SN5ΔEcoQ was protected during the methylation reaction using recA and primer SR1130 (5'- GAA TCG GAT TTG GAA TAA CCG AAT TCT CGG GAT AAT CTC CCG ATG G -3') then subjected to *Eco*RI restriction digestion to linearize the cosmids and subsequently ligated to the EcoQ-MeqGCN fragment yielding cosmids SN5-MeqGCN and A6-MeqGCN. Following ligation and transduction, clones containing the Eco-MeqGCN fragment in the correct orientation were identified by PCR and selected positive colonies sequenced across the junction regions. The integrity of the SN5-MeqGCN and A6-MeqGCN cosmids was confirmed by evaluating their restriction digestion pattern.

Transfections

Parental and mutant cosmids DNA were digested with *Not*I, to release the viral insert, and purified by phenol chloroform extraction and ethanol precipitation before transfection. To generate rMd5 and rMd5-MeqGCN, 500 ng of cosmids P89, SN16, B40, SN5 and A6 or P89, SN16, B40, SN5-MeqGCN and A6-MeqGCN, respectively, were used to transfect 1.2 X 10^6 DEF in 60 mm dishes by the calcium phosphate procedure. Five days after transfection, cells were trypsinized and seeded onto a 100 mm dish and monitored for cytopathic effects. Viral stocks of recovered viruses were

subsequently made in DEF for further analysis.

Revertant virus

To generate a revertant virus containing the parental *meq* gene, gel purified parental Md5 EcoQ fragment together with proteinase K digested and phenol-chloroform extracted rMd5-MeqGCN genomic DNA, were used to co-transfect CEF by the calcium phosphate procedure. After viral plaques were evident, the cells were overlayed with 0.9 % bactoagar in growth media and individual plaques harvested by trypsinization. Cells from each plaque were divided into two aliquots: one was used to infect DEF and the other for DNA extraction and PCR analysis. Presence of parental *meq* or *GCN4* leucine zipper sequences in individual plaques were detected by PCR using the following primers: SR1118-*meq* start primer (5'- GAT CCC GGG GAG ATG TCT CAG GAG CCA GAG C -3') and leucine zipper specific reverse primers SR3073-*meq* leucine zipper (5'- GTC CTT AGA TCT CGA ATT TCC -3') or SR3074-*GCN4* leucine zipper (5'- CTA ATT TCT TTA ATC TGG CAA C -3'), for parental and GCN4, respectively.

Indirect immunofluorescence assay (IFA) and immunohistochemistry (IHC)

Confluent DEF monolayers were infected with rMd5 or rMd5-MeqGCN, and when viral plaques were apparent, cells were fixed with ice-cold acetone/alcohol (6:4) and expression of Meq evaluated by IFA using rabbit polyclonal anti-Meq antibodies (1:300). Lymphocytes collected for reactivation assays were deposited on a microscope

slide using a cytospin centrifuge, fixed with ice-cold acetone/alcohol (6:4) and expression of pp38 evaluated by IFA using the H19 pp38 specific monoclonal antibody (1:400). For IHC, lymphoid organs (thymus, spleen and bursa of Fabricius), and feather follicles from infected and uninfected chickens were embedded in optimal cutting temperature compound (Tissue-Tek, OCT, Sakura Finetek, Torrance, CA), immediately frozen in liquid nitrogen and stored at -80°C until use. Six- to 8-μm-thick cryostat sections of tissue were prepared and fixed with cold acetone at -20°C for 5 min, and air-dried. Immunostaining was carried out using H19 pp38 monoclonal antibody (1:3000) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions.

MeqGCN homodimerization

For in vitro homodimerization assays, parental *meq* and *MeqGCN* were cloned into the pcDNA3.1 (Invitrogen) eukaryotic expression vector. Both *meq* and *MeqGCN* genes were cloned in frame with HA and Flag tags to aid in protein detection (31). The resulting plasmids were denoted as pHA-MeqGCN, pFlag-MeqGCN, pHA-Meq, and pFlag-Meq.

DF-1 cells were cotransfected with 2 μg of either pFlag-Meq or pFlag-MeqGCN and 2 μg of pHA-Meq-, pHA-MeqGCN, pCMV-cJun or pRCAS-cFos using Fugene 6 (Roche, Mannheim, Germany). Both pCMV-cJun and pRCAS-cFos are generous gift from Dr. Junnlin Liu, M. D. Anderson). Cells were harvested 48 hours after transfection and lysed in RIPA buffer (10 mM Tris-HCl [pH 7.4], 1% NP-40, 0.1% sodium

deoxycholate, 150mM NaCl, 1mM EDTA, 1mM PMSF). Cell lysates (500 µl) were immunoprecipitated with 20 µl of Flag antibody-conjugated agarose beads (Sigma, St. Louis, MO). Meq/c-Jun or Meq/c-Fos heterodimer were immunoprecipitated with 3 µg of either anti-cFos antibody (Santa Cruz, Santa Cruz, CA) or anti-cJun antibody (Santa Cruz) for two hours. The immune complex was then captured by the addition 20 µl of a protein A and protein G sepharose bead mixture and rocked for an additional 2 h at 4°C. Beads were washed four times with RIPA buffer and boiled for 5 min in 20 µl of 2 X sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.6% bromphenol blue) and subjected to SDS-PAGE electrophoresis and transferred to PVDF membranes. After blocking for 1 h at room temperature (RT) with 5 % skim milk in TBST (20 mM Tris-HCl [pH 7.5], 137 mM NaCl, 0.05% Tween 20), the membranes were incubated with primary antibodies for 2 h at RT. The membranes were washed with TBST three times for 10 min each at RT, and incubated with horseradish peroxidase-conjugated antibodies for 1 hour at RT. Membranes were washed three times with TBST and visualized with enhanced chemiluminescence reagent (Amersham-Pharmacia, Piscataway, NJ). Final dilutions of the primary antibodies for immunoblotting were 1:500 for anti-HA tag antibody (Convance, Berkley, CA), 1:4000 for anti-Meq (41), 1 µg/mL for anti-cFos (Santa Cruz, CA), and 1 µg/mL for anti-cJun (Santa Cruz).

Expression of recombinant Meq and MeqGCN proteins

Recombinant parental Meq and MeqGCN proteins were produced using a baculovirus expression system (Invitrogen). Parental *meq* and *meqGCN* genes were cloned into a modified pFAST-BAC vector, which carries a Flag tag. One hundred million Sf9 cells were infected with recombinant baculoviruses, expressing either Flag tagged Meq (Flag-Meq) or Flag tagged MeqGCN (Flag-MeqGCN). Cells were harvested 48 hours after infection and lysed in lysis buffer (50 mM Tris-HCl [pH7.5], 500 mM NaCl, 2% glycerol) supplemented with protease inhibitor cooktails (Roche). Cleared lysates were incubated overnight with 100 µl of Flag antibody-conjugated agarose beads followed by four washes with lysis buffer. Recombinant proteins were then eluted with 3x Flag peptides according to manufacture's protocol. The purity and concentration of protein was measured by SDS-PAGE and Coomassie blue staining using BSA as a standard.

Electromobility shift assay (EMSA)

EMSA analysis was performed with baculovirus expressed recombinant purified Meq proteins. The DNA binding activity of Flag-Meq and Flag-MeqGCN was tested with two different probes, an AP-1 consensus oligo (5'- CGC TTG ATG AGT CAG CCG GAA -3') (Promega, Madison, WI) and a probe derived from the MDV Ori (5'- TGC TCA TTT GCA TAC ACA TCA CGT GAT AGT -3'). Probes were labeled at the 5' ends with [^{32}P] ATP and polynucleotide kinase following manufacturer's instructions (Promega). Purified recombinant Meq, or MeqGCN proteins (160 ng) were incubated for

20 min at 30°C in gel shift buffer (Promega) followed by additional 20 min incubation with approximately 10,000 cpm labeled probe. For competition reactions, purified proteins were first incubated with unlabeled probes for 20 min at 30°C prior to incubation with labeled probes. Reaction products were resolved on nondenaturing 6% polyacrylamide-Tris-glycine gels and visualized using a Fuji film Bas-1800 II phosphor imaging system.

Luciferase assays

The transactivation/repression activity of the MeqGCN protein was evaluated on MDV promoters by luciferase assay. The *meq* promoter region (nucleotides -355 to -1) was cloned into the pGL3 vector (Promega) to form the pGL3-*meq* promoter. The pp38 and pp14 promoters cloned into the pGL3 reporter vector and have been previously described (42). Three tandem copies of the MDV Ori sequence (TGC TCA TTT GCA TAC ACA TCA CGT GAT AGT) found within the pp38/pp14 bidirectional promoter were cloned into pGL2-TATA luciferase vector (a kind gift from Dr. Stephen Safe, Texas A&M University) to generate pGL2-3XOri. The *meq* and *MeqGCN* genes were cloned into the pcDNA 3.1 Zeo vector (Invitrogen) to generate expression vectors, pcDNA-Meq and pcDNA-MeqGCN. DF-1 cells (1×10^5) were seeded in 12-well plates 16 hr prior to transfection and incubated at 37°C under standard conditions. Transfections were performed using siPORT XP-1 (Ambion, Austin, TX), 500 ng of pcDNA (empty vector control), pcDNA-Meq, or pcDNA-MeqGCN and either 250 ng of pGL3-*meq* promoter vector, pGL2-Ori or 500 ng of pp38 and pp14 promoter vectors,

according to manufacturer's recommendations. Cell lysates were harvested 48 hours post-transfection with active lysis buffer (Promega) and luciferase assay performed using a Biotek Clarity luminometer (Biotek, Winooski, VT). The protein concentration in each transfected sample was measured by Bradford assay (Bio-Rad, Hercules, CA) and luciferase activity was normalized to protein concentration. Assays were performed in triplicate and three independent experiments were performed for each reporter vector tested. Transactivation or transrepression activity was expressed as fold difference relative to empty pcDNA vector control. Results of all three experiments were analyzed by one-way ANOVA followed by Tukey HSD test using the SPSS®, Version 14.0 software (SPSS Inc., Chicago, IL, USA). For all analysis, $p \leq 0.05$ was considered statistically significant.

Colony formation in soft agar

DF-1 cells were transfected with pcDNA, pcDNA-Meq or pcDNA-MeqGCN using siPORT XP-1 (Ambion). Approximately 48 hours after transfection the transfected DF-1 cells were selected with 300 μg/ml zeocin (Invitrogen) for 4 weeks. Expression of Meq was confirmed by IFA using Meq antibody as described above. Pools of resistant cells ($5 \text{ X } 10^3$) were seeded in 0.33% agarose containing LM media with 150 μg/ml zeocin and 10% FBS overlaid on a 0.5% agarose in a 35-mm plate. After 3 weeks of culture colonies were examined under a light microscope and photographed using 12 X magnification. Three different fields were randomly selected and colonies greater than

100 μm were counted. Two independent experiments were performed and each experiment was performed in triplicate.

Southern blot

DNA from rMd5, rMd5-MeqGCN and rMd5ΔMeq infected DEF was isolated by proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation. Three micrograms of each DNA sample was digested with either *Eco*RI or *Pst*I, separated on a 1% agarose gel, and transferred to nylon membranes. ^{32}P-dCTP-labeled probes representing the complete MDV genome (cosmids SN5, P89, SN16, A6 and B40) or EcoQ fragment (2,456-bp fragment) were generated by random priming using High Prime DNA Labeling Kit (Roche) and were used to hybridize to viral DNA, using standard protocols (68).

In vitro growth kinetics

To compare the growth characteristics of rMd5 and rMd5-MeqGCN, DEF seeded on 35 mm plates were inoculated with approximately 50 plaque forming units (pfu) of each virus. On days 2, 4 and 6 after inoculation, the infected cells were trypsinized, serial dilutions inoculated onto DEF monolayers, seeded on 35 mm plates, and plaques at the different dilutions counted 7 days later.

Pathogenesis experiments

Experiment 1

To study the effect of Meq homodimers on viral replication, 4-week-old SPF chickens (Hy-Vac, Adel, Iowa) were randomly sorted into experimental groups of nine chickens each. One group remained as a non-inoculated control, whereas the other groups were inoculated subcutaneously with 3,000 pfu of rMd5, rMd5-MeqGCN or rMd5ΔMeq. Three chickens were randomly selected at each time point, except at day 6 in which two chickens were selected. On 14 and 21 dpi blood samples were collected in heparin, for reactivation assays (see below), and were subsequently euthanized for tissue sample collection. On 6 and 21 dpi lymphoid organs and feather follicles were collected for IHC. On 14 dpi, lymphoid organs were collected to determine lymphoid organ/body weight ratios. Organ/body weight ratios between groups were analyzed using the Kruskal-Wallis test with significance set at $p<0.05$.

Experiment 2

To study the role of the Meq homodimers on oncogenesis, SPF (Hy-Vac) day old chicks (nine per group) were inoculated subcutaneously with 2,000 pfu of one of the following viruses: rMd5, rMd5-MeqGCN or rMd5ΔMeq and reared in modified Horsfall-Brauer isolation units for 8 weeks. Weekly mortality was recorded and all chickens were necropsied at time of death or at termination of the experiments and evaluated for MDV-specific lesions in the viscera and the nerves. To confirm that the phenotypic changes observed in rMd5-MeqGCN were due to the exclusive

homodimerization of Meq, a revertant virus (rMd5-MeqGCNR) was inoculated into three chickens (3,000 pfu) and evaluated for MDV specific lesions.

Experiment 3

To study the role of Meq homodimers on horizontal transmission, six SPF (Charles River) day old chicks were inoculated subcutaneously with 3,000 pfu with rMd5 or rMd5-MeqGCN, three additional uninoculated chicks were reared with each group and served as contacts to assess horizontal transmission. At 8 weeks post-infection (PI) buffy coats were obtained from heparinized blood by centrifugation at 500 X g. DNA was extracted using Purelink Genomic DNA extraction kit (Invitrogen) and PCR was performed using MDV specific Meq 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') to detect the presence of MDV genome.

Reactivation assay

Buffy coats were collected as described above at 14 and 21 dpi. DEF monolayers seeded in 35 mm plates, were inoculated with 10^6 lymphocytes in duplicate and viral plaques counted 7 days post inoculation.

Results

Mutations in the leucine zipper of Meq are sufficient to confer homodimerization

It has previously been shown that Meq has the ability to form homodimers and heterodimers like the Jun family of bZIP transcription factors with which Meq shares considerable homology (42). It is well established that the specific amino acid residues within the leucine zipper of bZIP proteins determine their dimerization properties. Among these transcription factors, the yeast protein GCN4 is well characterized and the leucine zipper region is known to allow for the exclusive formation of homodimers (37). Like other studies (27, 29, 34), we also utilized the leucine zipper region of GCN4 in place of the parental leucine zipper of Meq, to study the function of Meq homodimers in MDV pathogenesis. The leucine zipper of Meq was successfully "swapped" with the leucine zipper of GCN4 using overlapping PCR, resulting in a Meq homodimer mutant, MeqGCN (Fig. 2).

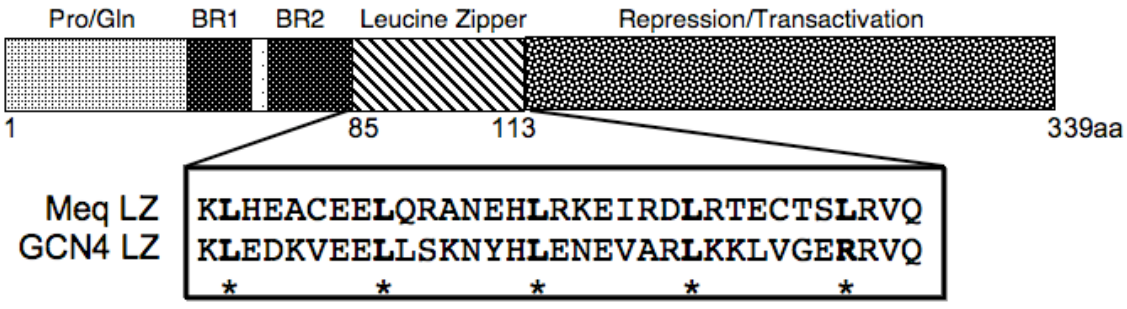

Fig. 2. Schematic representation of the *meq* and GCN4 leucine zippers (LZ). The DNA binding basic regions (BR1 and BR2) and transactivation domains, are depicted. The LZ sequence of parental *meq* and *GCN4* used to replace parental *meq* LZ in rMd5-MeqGCN are shown. Asterisks indicate the conserved leucine sites.

The open reading frames of Meq and MeqGCN, fused to the FLAG and HA epitope tags (HA-Meq, Flag-Meq, HA-MeqGCN, Flag-MeqGCN) were expressed in DF-1 cells. Co-immunoprecipitation experiments were performed with anti-Flag antibody-conjugated beads to test if the leucine zipper mutations in MeqGCN were sufficient to confer homodimerization (Fig. 3). First, Flag-Meq or Flag-MeqGCN was co-transfected with HA-Meq in DF-1 cells (Fig. 3a, left panel). Flag-Meq protein complexes were immunoprecipitated with anti-Flag antibody and evaluated for the presence of HA-proteins by Western blot analysis. Flag-Meq, but not Flag-MeqGCN effectively precipitated HA-Meq. Conversely, Flag-MeqGCN precipitated HA-MeqGCN but not HA-Meq (Fig. 3a right panel). These results demonstrated the strong homodimerization property of MeqGCN.

As a next step, the ability of Meq and MeqGCN to dimerize with c-Jun or c-Fos was examined (Fig. 3b). Meq or MeqGCN were co-expressed with c-Fos or c-Jun, and their ability to heterodimerize with other bZIP family protein assessed. As shown in the left panel of Fig. 3b, while Meq effectively pulled down c-Fos, very little, if any, c-Fos was found to associate with MeqGCN. Likewise, c-Jun interacts with Meq much more avidly than MeqGCN. This and the above experiment suggest MeqGCN preferentially if not exclusively, forms homodimer under physiological conditions.

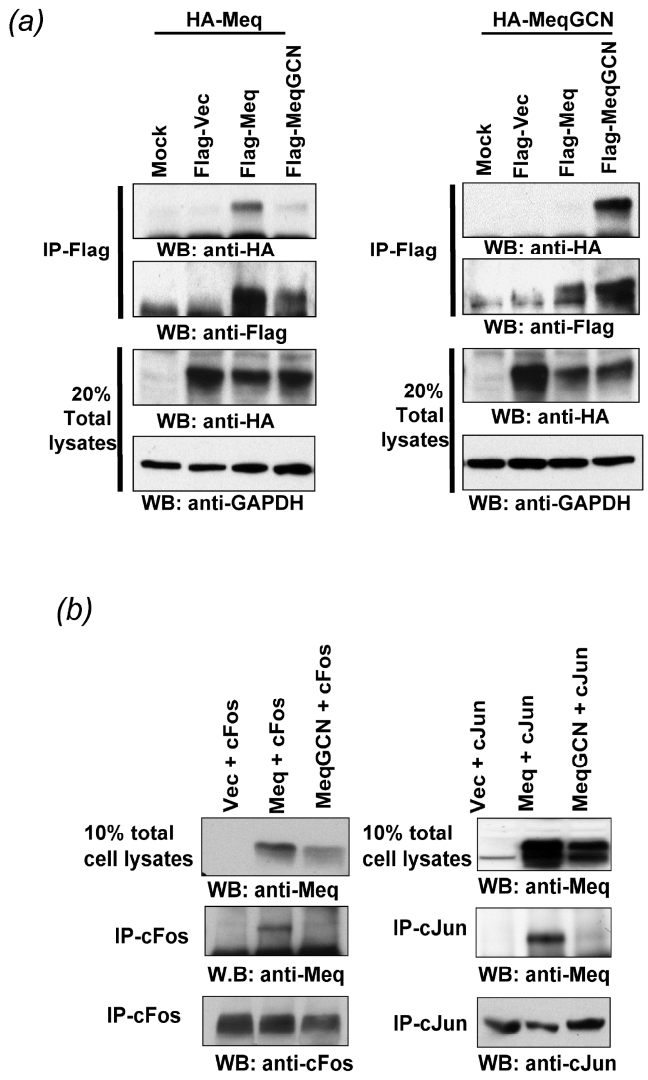

Fig. 3. Homo- and hetero-dimerization of Meq and MeqGCN proteins (a) Co-immunoprecipitation analysis of tagged Meq and MeqGCN proteins. Indicated plasmids were cotransfected in DF-1 cells. Twenty percent of total cell lysates that were used for coimmunoprecipitation were also included as controls. Meq coprecipitates only with Meq (left panel), and MeqGCN coprecipitates only with MeqGCN (right panel), demonstrating homodimerization. Membranes were reprobed with anti-Flag antibody as a control (b) MeqGCN had impaired ability to form heterodimer with c-Jun or c-Fos. The indicated plasmids were cotransfected in DF-1 cells, and immunoprecipitated with either anti-Fos or anti-Jun antibody, and blotted with anti-Meq antibody. Ten percent of total cell lysates that were used for immunoprecipitation were also included in the same gel as a control. Both c-Fos and c-Jun are effectively precipitated Meq but only weakly precipitate MeqGCN.

Meq homodimer mutant (MeqGCN) retains DNA binding, transactivation and repressive functions

Previous characterization of Meq has shown that Meq homodimers and heterodimers differ in their DNA binding affinities. Although both Meq homodimers and heterodimers bind AP-1 sequences, Meq together with c-Jun bound AP-1 sequences with greater affinity than Meq alone. In addition, Meq alone bound the MDV origin of replication (MDV Ori) located in the pp38/14 bidirectional promoter (42). Luciferase reporter assays indicated functional differences between Meq and Meq plus c-Jun in that although Meq activated the AP-1 containing *meq* promoter, Meq plus c-Jun resulted in higher activation. Furthermore, Meq expression was shown to repress the MDV pp38/pp14 bidirectional promoter which contains the MDV Ori (42). Therefore, EMSA and luciferase assays were performed to test if the DNA binding and transactivation/repression functions of MeqGCN were still functional. Recombinant Flag-Meq Flag-Meq and Flag-MeqGCN proteins were prepared from recombinant baculovirus infected cells. Recombinant proteins were incubated with ^{32}P labeled consensus AP-1 oligo or an oligo encompassing a portion of the MDV Ori. Both Meq and MeqGCN proteins bound both AP-1 and MDV Ori oligos and the intensity of band shifts for both Meq and MeqGCN decreased in the presence of specific competitor, indicating specific binding (Fig. 4).

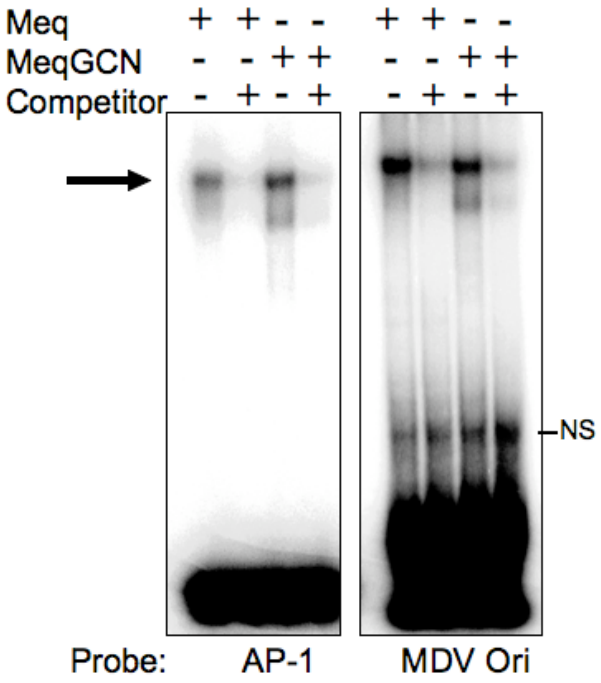

Fig. 4. Electromobility shift assay (EMSA) analyses of Meq and MeqGCN proteins were preformed to test the DNA binding capacity of MeqGCN. Purified baculovirus expressed proteins, Meq and MeqGCN, were incubated with radiolabeled oligonucleotide probes followed by gel retardation analysis. Two different probes were used: a consensus AP-1 oligo and ACACA probe derived from the MDV origin of replication (MDV Ori). Band shifts are observed by Meq and MeqGCN (black arrow), of which the intensity decreases in the presence of unlabeled competitor. NS, non-specific bands.

Luciferase assays were then performed to test the transactivation and repressive functions of MeqGCN compared to Meq. DF-1 cells were transfected with pcDNA empty vector, pcDNA-Meq or pcDNA-MeqGCN, together with pGL3- *meq* promoter reporter vector. As shown in figure 5, both Meq and MeqGCN activated the *meq* promoter, although MeqGCN activated the *meq* promoter to significantly lesser levels than Meq ($p<0.05$). Luciferase assays were also performed to test the ability of MeqGCN to repress the previously described pp38 and pp14 promoters. DF-1 cells were transfected with pcDNA, pcDNA-Meq, or pcDNA-MeqGCN, and either the pGL3-pp38 or pGL3-pp14 promoter reporter vectors. Although, pcDNA-Meq and pcDNA-MeqGCN repressed both, pp38 and pp14 promoters, repression by pcDNA-MeqGCN was significantly less ($p<0.05$) than parental pcDNA-Meq protein (Fig. 5). Nonetheless, both pcDNA-Meq and pcDNA-MeqGCN significantly repressed the pGL2-3X-Ori, which contains three copies of the sequence element found in the pp38/14 bidirectional promoter that Meq was previously reported to bind (42, 61).

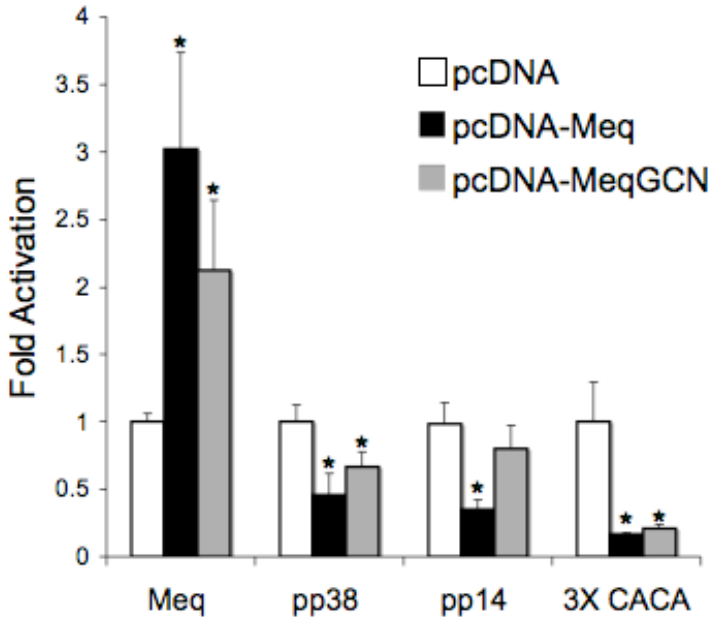

Fig. 5. Luciferase assays demonstrate that MeqGCN has functional transactivation and repression activities. DF-1 cells were transfected with *meq*-promoter, pp14 promoter, pp38 promoter or 3X-Ori luciferase reporter plasmids and pcDNA (empty vector), pcDNA-Meq or pcDNA-MeqGCN. Both Meq and MeqGCN activate the *meq*-promoter and repress the pp38/14 bidirectional promoters and 3X-Ori indicating MeqGCN maintains transactivation/repressive functions. Luciferase values are expressed as fold difference relative to pcDNA vector. Significance (*) equals $p \leq 0.05$. Error bars indicate SD.

Colony formation in soft agar

Colony formation in soft agar is a marker of transformation and Meq expression in fibroblasts has been shown to promote anchorage independent growth (41). Soft agar

assay was employed to test the ability of Meq homodimers to promote colony formation in soft agar. Meq and MeqGCN expression in selected DF-1 cells was confirmed by IFA (Fig. 6a top panel). As expected, selected DF-1 cells expressing the pcDNA-Meq construct formed large colonies in soft agar when compared to control cells selected with pcDNA empty vector (Fig. 6a bottom panel). Furthermore, cells expressing pcDNA-Meq had 23 X more colonies >100 μm in size compared to pcDNA control cells.

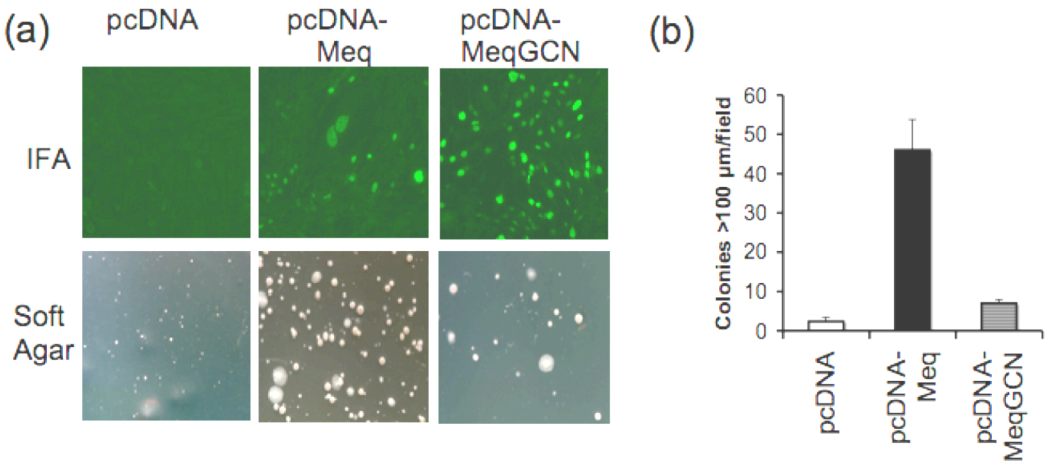

Fig. 6. In vitro soft agar assay demonstrating that MeqGCN has reduced transformation potential. (a) Top panel: Immunofluorescence analysis of Meq expression from pools of selected DF-1 cells transfected with pcDNA, pcDNA-Meq, pcDNA-MeqGCN. Bottom panel: Soft agar assay was performed using pcDNA, pcDNA-Meq, pcDNA-MeqGCN selected DF-1 cells to assess anchorage independent growth. (b) Number of colonies > 100 μm observed in cells expressing pcDNA, pcDNA-Meq, or pcDNA-MeqGCN. Average number of colonies counted from three random fields are shown. Error bars indicate SD.

In contrast, DF-1 cells expressing pcDNA-MeqGCN only had 3.5 X as many colonies >100 μm in size compared to empty vector control cells (Fig. 6b), indicating that Meq homodimers have a reduced potential for transformation in vitro.

Construction of a Meq-homodimer mutant virus rMd5-MeqGCN

A recombinant Md5 mutant virus in which the leucine zipper region of *meq* was replaced with the corresponding region of *GCN4* (rMd5-MeqGCN) was successfully constructed. The leucine zipper region of the *meq* gene was replaced with the leucine zipper region of *GCN4* by overlapping PCR, and the EcoQ fragment containing the chimeric *meqGCN* gene was cloned into the A6Δmeq and SN5Δmeq cosmids. The resultant cosmids A6-MeqGCN and SN5-MeqGCN together with cosmids P89, B40 and SN16 were cotransfected into DEF by the calcium phosphate method and a recombinant virus was subsequently recovered by homologous recombination. Southern blot analyses were performed to assess the integrity of the rMd5-MeqGCN viral genome. Genomic DNA from rMd5, rMd5-MeqGCN and rMd5ΔMeq infected DEF was digested with *Eco*RI or *Pst*I and probed with ^{32}P labeled Md5 comids or EcoQ fragment DNA, respectively. No differences were observed between rMd5 and rMd5-MeqGCN *Eco*RI digestion patterns confirming the integrity of the recombinant genomes (Fig. 7). DNA from rMd5, rMd5-MeqGCN and rMd5ΔMeq infected DEF was also digested with *Pst*I because there is a *Pst*I site located within the leucine zipper region of the *meq* gene which it is absent in the mutant *MeqGCN* gene. As expected, *Pst*I digested rMd5-MeqGCN DNA probed with ^{32}P labeled EcoQ fragment resulted in a single band

compared to two bands observed for rMd5 (Fig. 7). Like rMd5-MeqGCN, rMd5ΔMeq, also lacks the *Pst*I site due to the deletion of the *meq* gene, therefore a single yet smaller band was detected by Southern blot (Fig. 7).

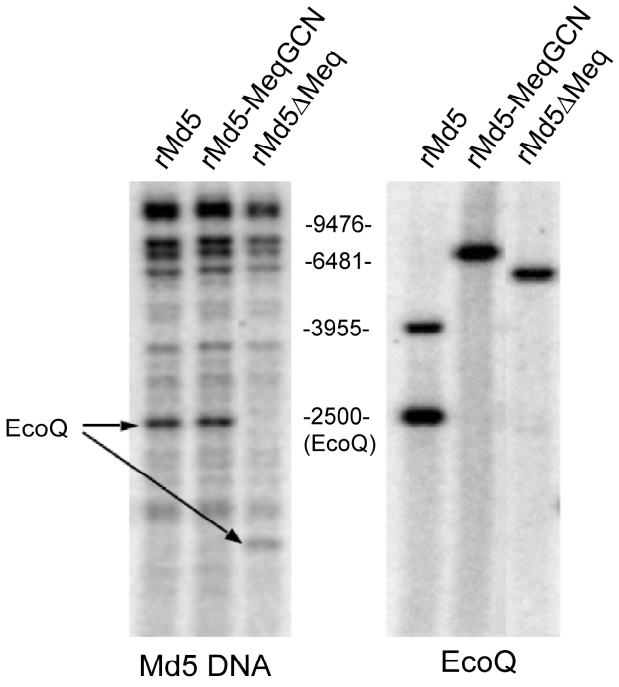

Fig. 7. Southern blot analysis of rMd5, rMd5-MeqGCN and rMd5ΔMeq. (A) DNA was digested with *Eco*RI and probed with total viral MDV DNA. The restriction profile of rMd5-MeqGCN is similar to rMd5, indicating no gross genome rearrangements incurred. The arrow indicates the location of the EcoQ fragment. Due to the *meq* deletion in the EcoQ fragment of rMd5ΔMeq this fragment migrates faster. B) DNA was digested with *Pst*I and probed with EcoQ fragment. The introduced LZ mutations in rMd5-MeqGCN resulted in a lost of a *Pst*I site, and therefore a single band is observed, in contrast to two bands for rMd5. Likewise, rMd5ΔMeq does not have a *Pst*I site and as a consequence of the *meq* deletion resulting in a faster migrating single band.

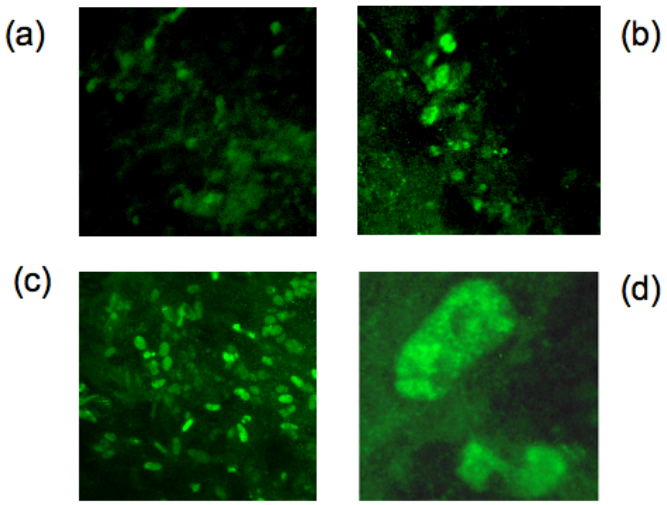

Fig. 8. Immunofluorescence analysis of DEF cells infected with recombinant viruses. (a) rMd5ΔMeq infected DEF (150 X), (b) rMd5-MeqGCN infected DEF (100 X) (c) rMd5 infected DEF (100 X) (d) rMd5-MeqGCN infected DEF (1000 X). Meq expression is observed in the nucleus of rMd5 and rMd5-MeqGCN infected DEF but not rMd5ΔMeq.

Immunofluorescence assay (IFA) was performed to evaluate Meq expression in virus infected DEF. Meq expression was observed in both rMd5 and rMd5-MeqGCN infected DEF but not in rMd5ΔMeq infected cells. In addition, magnification at 1000 X clearly showed that expression of MeqGCN localized to the nucleus (Fig. 8). While, less expression was observed in rMd5-MeqGCN compared to rMd5, this correlates with luciferase data, which indicated that MeqGCN activated the *meq* promoter to a lesser extent than parental Meq (Fig. 5).

In vitro and in vivo replication of rMd5-MeqGCN

The in vitro growth properties of rMd5-MeqGCN were tested, to assess if the leucine zipper mutations had any effect on virus replication in vitro. Our results indicate

that rMd5 and rMd5-MeqGCN virus replicated similarly at the time points tested (days 0, 2, 4, 6) (Fig. 9).

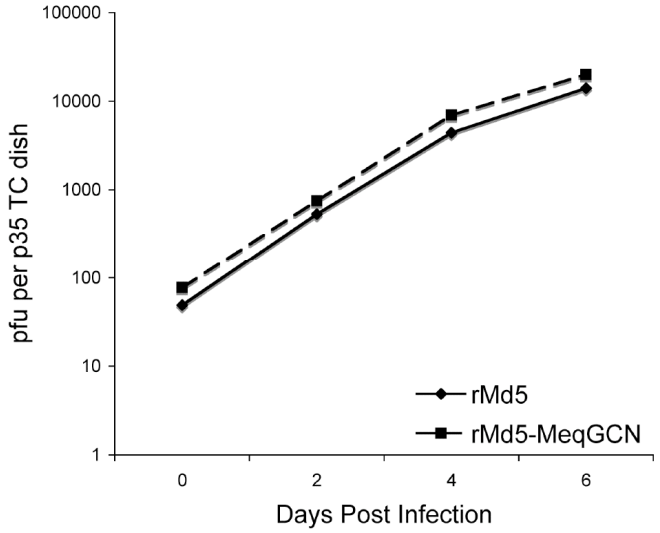

Fig. 9. In vitro growth properties of rMd5 and rMd5-MeqGCN. DEFs were infected with the indicated viruses, harvested on days 2, 4 and 6 after infection and titered on fresh DEF. Day 0 indicates the titer of the virus in the inoculum. The experiment was performed in duplicate, and the titer (logarithm of the mean number of plaque-forming units per dish) is indicated.

To test the role of Meq homodimers in in vivo replication, 4-week-old SPF chickens were inoculated with 3,000 pfu of rMd5, rMd5-MeqGCN or rMd5ΔMeq virus. Six days after inoculation two randomly selected birds were euthanized and lymphoid organs (thymus, spleen and bursa of Fabricius) collected and evaluated for viral lytic antigen (pp38) expression by IHC. Expression of pp38 was evident in bursa of Fabricius (Fig. 10) thymus and spleen (data not shown) of both rMd5 and rMd5-MeqGCN infected chickens, indicating that the leucine zipper mutations in the rMd5-MeqGCN virus did not affect early cytolytic infection.

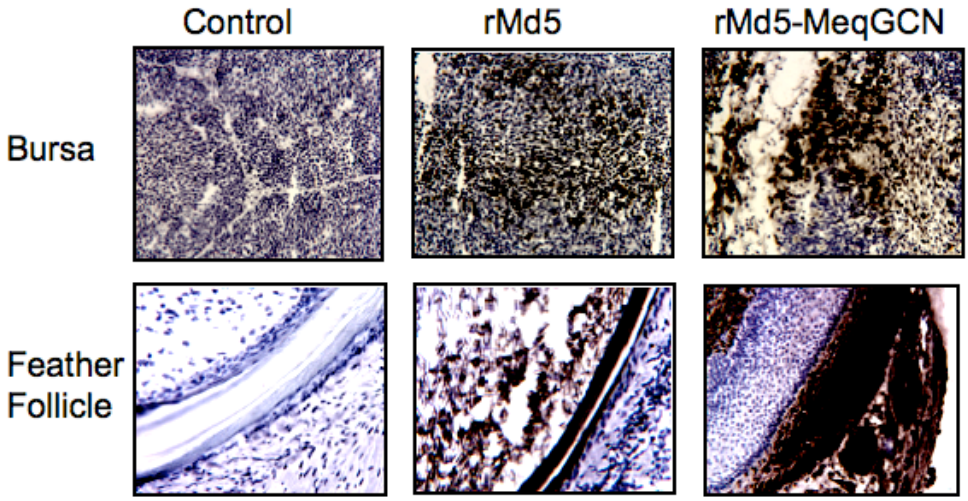

Fig. 10. Infection of lymphoid tissue and feather follicles. Immunohistochemistry of bursa and feather follicle 6 and 21 dpi, respectively with anti-pp38 monoclonal antibody (H19). Positive cells are indicated by brown precipitate, counterstaining was performed with hematoxylin.

To further assess the role of Meq homodimers in viral replication, lymphoid organ weight ratios were determined, from three birds from each group at day 14 PI, to evaluate lymphoid organ atrophy. As expected, splenomegaly as well as thymic and bursal atrophy was observed in chickens inoculated with rMd5, indicating high levels of cytolytic infection. Although, there were no significant differences between all groups, bursal and thymic atrophy were less evident in the rMd5-MeqGCN group compared to rMd5 (Fig. 11).

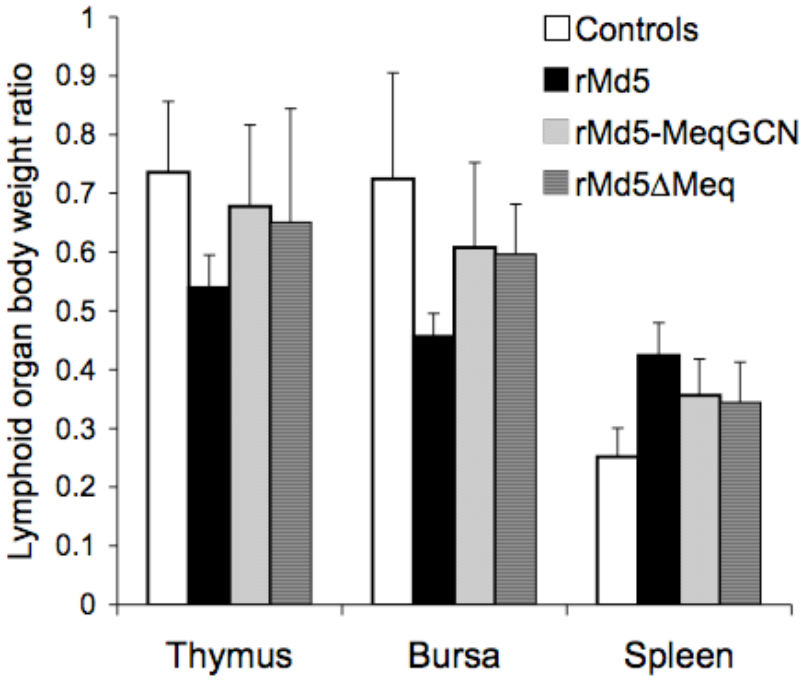

Fig. 11. Lymphoid organ/body weight ratios of rMd5, rMd5-MeqGCN, and rMd5ΔMeq infected chickens 14 dpi. Three birds from each group were euthanized, lymphoid organs collected and chickens and lymphoid organs weighed. Statistical analysis was performed using the Kruskal-Wallis test. Significance was set at $p<0.05$. Although, no significant differences were observed, less lymphoid organ atrophy was observed in chickens infected with rMd5-MeqGCN compared to rMd5.

It is well documented that MDV switches from an active cytolytic infection to a latent infection approximately 7-8 days PI and virus reactivation can be observed when latently infected lymphocytes are co-cultured with fibroblasts (11). To examine if Meq homodimers are involved in the establishment of latency and reactivation, peripheral blood lymphocytes (buffy coats) were obtained from three chickens from each group on days 14 and 21 PI and co-cultured with DEF. To confirm viral infection, portions of collected lymphocytes were fixed on microscope slides and IFA performed to detect viral antigen, pp38. As shown in Table 1, few or no plaques were obtained from PBMCs

of chickens infected with rMd5-MeqGCN and rMd5ΔMeq. Yet, at day 21, latently infected lymphocytes were positive for viral antigen pp38 (Fig. 12) suggesting that these viruses are defective in reactivation.

Table 1.
Virus reactivation from peripheral blood lymphocytes

Group	Day 14	Day 21
rMd5	45	120
rMd5-MeqGCN	1	0
rMd5ΔMeq	2.5	0

Reactivation assays were performed on days 14 and 21 after inoculation. The numbers represent the average number of pfu observed when10^6 peripheral blood lymphocytes were co-cultured with DEF

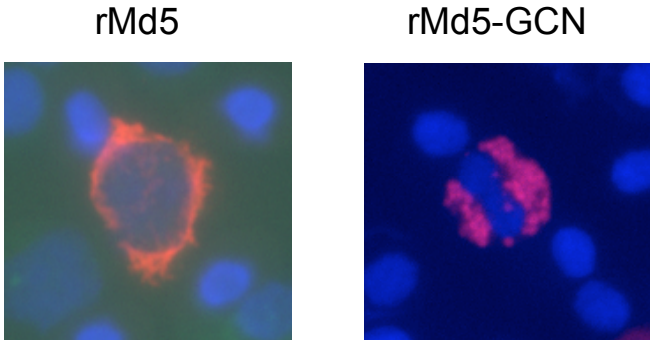

Fig. 12. Detection of MDV pp38 protein in latently infected cells 21 dpi. Immunofluorescence assay was performed with anti-pp38 monoclonal antibody (H19) on PBMC's collected from rMd5 and rMd5-MeqGCN infected chickens. Texas-red conjugated secondary antibodies were used to detect pp38 and nuclei were stained with DAPI.

Transmission of MDV occurs through replication of virus in the feather follicle epithelium (FFE) and release of dander containing infectious virus (13). Therefore, to determine if the rMd5-MeqGCN homodimer mutant was able to replicate in the FFE, expression of the lytic viral antigen pp38 was evaluated in three chickens from each inoculated group by IHC. Tissues from all inoculated groups tested positive for pp38 antigen indicating the ability of rMd5-MeqGCN, like rMd5, to replicate in the FFE (Fig. 10). To further assess the role of Meq homodimers in MDV transmission, day old SPF chickens were inoculated with 3,000 pfu of rMd5 or rMd5-MeqGCN and reared with three uninfected contact chicks. Buffy coats were collected from contact birds 8-weeks PI, DNA extracted and PCR for Meq performed. In both groups two out of three contact chickens tested positive for Meq or MeqGCN, respectively. Altogether these results confirm that rMd5-MeqGCN retains the ability to transmit horizontally by shedding through the feather follicle epithelium.

Oncogenecity of rMd5-MeqGCN

To determine if Meq homodimers are involved in transformation of T-cells, groups of nine SPF chickens were inoculated at day of age with 2,000 pfu of rMd5, rMd5-MeqGCN, or rMd5ΔMeq and observed for mortality for a period of 8 weeks. All chickens that died during the experiment or were euthanized at the termination of the experiment were evaluated for MDV-specific lesions. MDV-associated mortality was observed in rMd5-infected chickens starting at week 4 PI and none of the chickens survived the end of the experiment. In contrast, all of the rMd5-MeqGCN infected

chickens survived the 8-week experiment and microscopic examination of the vagus, brachial, and sciatic nerves showed no signs of Marek's disease. Similarly, as expected, the rMd5ΔMeq infected chickens also survived the duration of the experiment and were free of MDV specific lesions (Fig. 13 and Table 2).

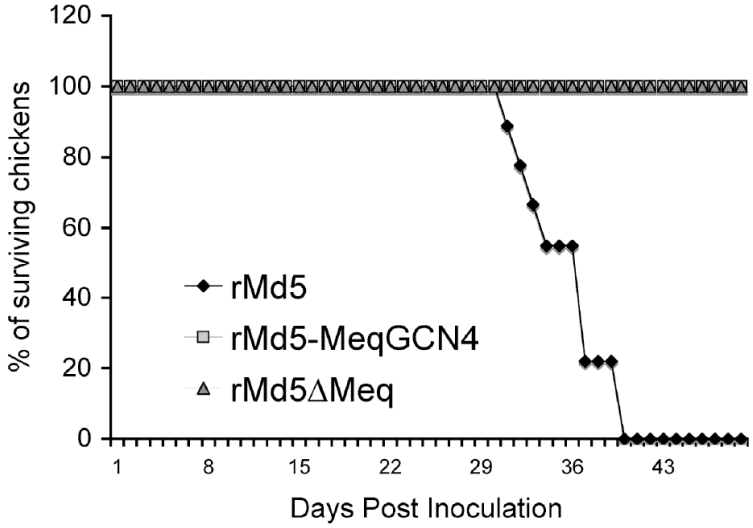

Fig. 13. Mortality in chickens inoculated with rMd5, rMd5-MeqGCN, and rMd5ΔMeq. Chickens were inoculated with 2,000 pfu of the indicated viruses at 1 day of age, maintained in isolation for 8 weeks and weekly mortality was recorded. Uninoculated chickens served as negative controls. Chickens that died during the experiment were evaluated for MDV-specific gross lesions.

Table 2.
Pathogenicity of rMd5, rMd5-MeqGCN, rMd5ΔMeq, and rMd5-MeqGCNR in SPF MDV maternal antibody-negative chickens

Virus[a]	Mortality (%)	Tumor Incidence (%)[b]
rMd5	9/9 (100)	9/9 (100)
rMd5-MeqGCN	0/9 (0)	0/9 (0)
rMd5ΔMeq	0/9 (0)	0/9 (0)
rMd5-MeqGCNR[c]	3/3 (100)	3/3 (100)
None	0/9 (0)	0/9 (0)

[a]All chickens were inoculated with 3,000 PFU of the indicated viruses.

[b]Chickens that were not positive for gross tumors were further evaluated for microscopic tumors

[c] Performed as an independent experiment

Construction and biological properties of revertant virus rMd5-MeqGCNR

To verify that the phenotypic changes observed with rMd5-MeqGCN were due to changes introduced in the leucine zipper region, a revertant virus was constructed. DNA obtained from rMd5-MeqGCN infected CEF was co-transfected with purified parental MDV EcoQ DNA, which contains the *meq* gene. A revertant virus, rMd5-MeqGCNR, was selected by plaque purification, and screened for the presence of both parental and MeqGCN-mutant *meq* genes by PCR. PCR revealed that of the 130 plaques tested, 3 contained, one copy of the parental *meq* gene restored in the viral genome. Three

chickens were infected with 3,000 pfu of rMd5-MeqGCNR and by week 5 PI all three were positive for MDV gross tumors, confirming that the attenuated phenotype of rMd5-MeqGCN was due to the mutations introduced in the leucine zipper of Meq. In addition PCR analysis of virus isolated from the rMd5-MeqGCNR infected chickens revealed that both copies of the parental *meq* gene had been restored.

Discussion

MDV is a ubiquitous, highly contagious, potent oncogenic virus, which is controlled by vaccination to prevent economic losses otherwise caused by Marek's disease. Due to widespread use of vaccines, MDV continues to evolve towards greater virulence and therefore, a better understanding of the molecular mechanisms of viral pathogenesis is warranted to control this economically important disease of poultry.

A number of genes unique to MDV are encoded within the repeat long region or at its junction with the unique long region, and have been associated with pathogenesis. For example, disruption of *pp38*, *vIL8*, or *LORF11* genes from oncogenic MDV resulted in reduced tumor incidence as a consequence of impaired lytic replication in chickens (17, 25, 40, 57). On the other hand, disruption of vTR (viral telomerase RNA) or *meq* gene did not appear to have an affect on lytic replication in vivo, but resulted in 60 and 100% reduction of tumor incidence, respectively (50, 79).

MDV-1 (GaHV-2) encoded Meq protein is consistently detected in MDV induced tumors and is a potentially multifaceted transcription factor which has been shown to directly interact with cell cycle regulator, cyclin-dependent kinase 2 (CDK2) and the co-repressor C-terminal-Binding Protein (CtBP) (49). Interestingly, abrogation

of the interaction of Meq with CtBP in an MDV-1 recombinant virus (pRB1B-Ct20) resulted in loss of transformation; however, the molecular mechanisms associated with this phenotype remain to be elucidated (8), although it can be speculated that Meq may interfere with the ability of CtBP to function as a co-repressor as has been described for adenovirus early region 1A (AdE1A) (65).

Meq shares homology with the Jun/Fos bZIP family of transcription factors, of which viral counterparts v-Jun and v-Fos are oncogenic, therefore implying a similar oncogenic potential (73). In vitro transformation studies and the loss of transformation observed in vivo with rMd5ΔMeq provides convincing proof that Meq is essential for transformation by MDV. However, the mechanisms of Meq transformation remain unknown. In vitro characterization of Meq DNA binding and transactivation/repression activities, have provided support for the hypothesis that Meq homodimers and heterodimers have distinct roles in MDV-1 pathogenesis (33, 41, 42, 61); however, the role of Meq homodimers and heterodimers in MDV-1 pathogenesis has not been characterized.

In this study, a recombinant virus in which the leucine zipper of the Meq protein was replaced with the leucine zipper of the yeast GCN4 protein was successfully generated. It has been well established that the leucine zipper region of bZIP proteins determines their dimerization specificity, which in turn affects their DNA binding properties (37, 84). The mutant Meq protein we generated containing the dimerization domain of GCN4 retained both DNA binding and transactivation/repression functions in vitro. However, rMd5-MeqGCN expressed MeqGCN to a lesser extent than parental

rMd5 virus expressed Meq in infected DEF (Fig. 8). This correlates with in vitro luciferase data, which showed that parental Meq activates the *meq* promoter at significantly greater levels than MeqGCN (Fig. 5), supporting a previous report that Meq, together with c-Jun, has a greater binding affinity for AP-1 sequences and higher transactivation activity on the *meq* promoter than Meq alone (42). Since MeqGCN transactivates the Meq promoter to a lesser extent than parental Meq, it is possible that MeqGCN may also have decreased transactivation of other AP-1 containing promoters. This decrease in transactivation potential could contribute to the decreased transformation observed by MeqGCN in the soft agar assay (Fig. 6). However, transactivation activity does not always correlate with transformation as has been observed in studies with v-Jun mutants that are strong transactivators and poor transformers and *vice versa*. (27). We also found that MeqGCN homodimers had a reduced ability to transrepress the pp38/14 promoter although MeqGCN repressed MDVOri sequences found within the pp38/14 promoter at levels comparable to parental Meq. It is possible that MeqGCN homodimers in the context of the full pp38/14 promoter are not able to bind the pp38/14 promoter as well as parental Meq or bind other elements in the promoter with different affinity therefore decreasing transrepression. It has been shown that v-Jun-GCN4 leucine zipper chimeric proteins are more stable than parental v-Jun homodimers and it is therefore possible that the stability of MeqGCN homodimers be different than that of parental Meq homodimers.

The contribution of other MDV proteins or cellular factors to MDV transformation cannot be dismissed either. Disruption of Meq contact with CtBP resulted

in a loss of oncogenicity showing the importance of Meq and CtPB interactions (8). In addition, Meq has been shown to associate with cell cycle regulators, CDK2 and p53 (38, 49), although, the contribution of these interactions in MDV transformation have not been defined. It remains possible that MeqGCN homodimers have altered interactions with these or other unknown cellular factors. However, taken together, our data demonstrate that MeqGCN homodimers are not sufficient to induce transformation of T-cells in chickens. Further investigations with Meq-heterodimer mutants, might aid in further defining the role of Meq in MDV transformation.

The role of Meq homodimers in MDV-1 pathogenesis was determined by evaluating both cytolytic infection and transformation in vivo. In vitro, rMd5-MeqGCN replicated similar to parental virus and in vivo, cytolytic infection was evident in lymphoid organs and feather follicles as assessed by expression of MDV-1 lytic protein pp38 (Fig. 10). However, evaluation of effects of MeqGCN on cytolytic infection is difficult because Meq expression is not essential for early cytolytic infection (50). Meq is not always detected early in infection and expression of Meq in vivo, is variable and has been shown to depend on the virus strain and chicken line used (24, 56). As the FFE is the site of fully productive infection and source of horizontal transmission (13), we evaluated the role of Meq homodimers on transmission by IHC (Fig. 10) and contact transmission. Our results show that Meq homodimerization did not interfere with either replication in the FFE or transmission to contact chickens.

Atrophy of thymus and bursa of Fabricius and splenomegaly is a marker of MDV-1 lytic infection and is a good indicator of the virulence of MDV-1 pathotypes.

Atrophy is evident as early as 8 days post infection with no significant differences observed among MDV-1 pathotypes. However, at day 14 PI, although lymphoid organ atrophy is less than at day 8 PI, significant differences between MDV-1 pathotypes are evident. By day 14 PI less atrophy is observed with less virulent pathotypes indicating the affected tissues are able to recover. On the other hand, a greater degree of atrophy at day 14 PI is observed with the more virulent pathotypes. The greater degree of atrophy may be a result of prolonged cytolytic infection or an able to infect a greater number of cell types or if they maintain a therefore causing more damage to tissue than less virulent strains (14). To assess the virulence of rMd5-MeqGCN, lymphoid organ/body weight ratios were determined for rMd5-MeqGCN, rMd5 and rMd5ΔMeq infected chickens. As expected, lymphoid organ atrophy and splenomagly were observed at day 14 PI in the rMd5-infected group, while rMd5-MeqGCN infected chickens displayed less lymphoid organ atrophy and were more similar to uninfected controls (Fig. 11). Although, no significant differences were observed at day 14 among all groups, the statistical power of the time point was limited due to the small number of chickens examined and may not accurately reflect the biological differences between these viruses. However, these results suggest that rMd5-MeqGCN is less virulent than parental rMd5.

One of the goals of this study was to dissect the Meq functions, in the hope to separate its transforming potentials from the in vivo replication/latency functions. A perhaps oversimplified model is that Meq/Jun heterodimers, like v-Jun, imparts transforming function, whereas Meq/Meq homodimers are involved in latency entry and/or reactivation. To test this model, the replicative properties of rMd5-MeqGCN

virus were assessed in vivo by evaluating early cytolytic infection and reactivation from latency. Although early cytloytic infection was not impaired (Fig. 10) the virus appeared to be defective in reactivation (Table 1). A switch from lytic to latent infection in MDV-1 normally occurs approximately 7 days PI, and virus from latently infected cells can be reactivated when co-cultured with fibroblasts in vitro (11). In our study, few viral plaques were detected at day 14 and none at day 21 for both rMd5-MeqGCN and rMd5ΔMeq groups. However, although not quantitative, the presence of latently infected cells in the chickens was confirmed by the detection of viral antigen pp38 (Fig. 12) and PCR amplification of the MDV-1 genome (data not shown) in all infected groups. Since both rMd5-MeqGCN and rMd5ΔMeq viruses appear to be defective in reactivation it may be argued that Meq heterodimers might play an important role in virus reactivation from latency.

Inoculation of chickens with rMd5-MeqGCN also showed that this recombinant virus is apathogenic. During the 8-week experiment none of the chickens infected with rMd5-MeqGCN developed gross or microscopic tumors whereas in the rMd5 group, all the chickens that died or were euthanized suffered from Marek's disease (Fig. 13). Like rMd5ΔMeq, rMd5-MeqGCN did not transform T-cells, further supporting in vitro data and implicating Meq heterodimers in MDV-1 transformation. In order to rule out the possibility that tumor formation was inhibited by the production of antibodies directed towards the GCN leucine zipper, we tested convalescent chicken serum on cells expressing Meq and MeqGCN by IFA. None of the sera obtained from rMd5 or rMd5-MeqGCN infected chickens reacted positively with cells expressing either Meq or

MeqGCN (data not shown). These results are further supported by previous work with retrovirus constructs expressing v-Jun GCN4 leucine zipper mutants, which remained oncogenic in infected chickens (27, 34). This suggests that immune responses to MeqGCN did not contribute to the lack of oncogenecity observed in rMd5-MeqGCN infected chickens. Importantly the phenotypic differences observed in this study for rMd5-MeqGCN can be attributed to the leucine zipper mutations, as the Md5 phenotype was fully restored in the revertant virus, rMd5-MeqGCNR.

In summary, this work provides the first in vivo evidence that Meq homodimers are not sufficient for MDV-1 transformation reinforcing the notion that the participation of Meq/Jun may be key to its transforming ability. In order to better characterize the Meq transcriptional pathways involved in MDV-1 pathogenesis, current studies with recombinant Meq heterodimer virus are underway to investigate the role of Meq-Jun heterodimers in MDV transformation in vivo.

CHAPTER III

HETERODIMERIZATION AND HOMODIMERIZATION OF MAREK'S DISEASE VIRUS ENCODED MEQ PROTEIN CONTRIBUTES TO T-CELL TRANSFORMATION IN CHICKEN

Introduction

Marek's disease virus serotype 1 (MDV-1) is a potent oncogenic avian herpesvirus and the etiologic agent of Marek's disease (MD). MDV elicits a rapid onset of malignant T-cell lymphomas within several weeks of infection, resulting in mortality. Although MDV shares biological characteristics with gammaherpesviruses, such as its ability to transform lymphocytes MDV is classified as an alphaherpesvirus, based on viral genome sequence and organization and is collinear with HSV and VZV (67, 74). Three Marek's disease viruses have been described, MDV-1, Marek's disease virus serotype 2 (MDV-2) and Meleagrid herpesvirus 1 (MeHV-1) however only MDV-1 is oncogenic. In addition, MDV-1 continues to evolve and a number of different pathotypes exist within MDV-1 ranging from mild to very virulent plus, varying in both cytolytic and oncogenic potential (12, 88).

The highly oncogenic nature of MDV suggests the presence of a viral oncogene(s). Initial investigations in the search for candidate viral oncogenes focused on transcriptionally active regions of the MDV genome, genes expressed in transformed cells, and identification of genes unique to the MDV-1. Genome comparisons of

oncogenic MDV-1, non-oncogenic MDV-2, and MeHV-1 confirmed the Marek's disease putative oncogene *meq* is unique to the MDV-1 genome. The *meq* gene is named after the EcoQ fragment where it is located, "Marek's EcoQ", and two copies are found in the viral genome within the terminal repeat long (TR_L) and internal repeat long (IR_L) regions (60, 66, 67, 74, 80). Meq is a 339 amino acid nuclear phosphoprotein that is abundantly expressed in MDV transformed cells. The structure of Meq is well defined. Meq contains nuclear and nucleolar localization signals a bZIP (basic-region leucine zipper) domain and a proline rich transactivation domain (38, 46, 62). The bZIP domain of Meq shares significant homology, with the protooncogene c-Jun, a transcription factor of the AP-1 (activating protein) complex (33). AP-1 transcription factors are well-described proteins that include the Jun, Fos and ATF/CREB families. AP-1 proteins are characterized by their ability to bind and regulate sequence specific gene elements, AP-1 sites (5'-TGAG/CTCA-3'), which are found in many genes associated with cell proliferation (73). Transformation by deregulated expression of c-Jun, c-Fos and their viral counterparts v-Jun and v-Fos is well documented. The shared homology between Meq and c-Jun suggests Meq may transform through similar mechanisms (53, 84, 86).

A considerable amount of in vitro data supports the oncogenic nature of Meq, which has been shown to promote anchorage-independent growth, cell-cycle progression, and is anti-apoptotic (48, 49). More recently, in vitro expression of Meq was shown to upregulate genes similar to those upregulated by v-Jun, suggesting that Meq transforms *via* a v-Jun transforming pathway (41). Yet, the most convincing evidence for Meq's oncogenic property in MDV transformation was the characterization

of a Meq null recombinant MDV virus (rMd5ΔMeq), which replicated in chickens but did not induce tumors. The essential role of Meq in MDV transformation has been further supported by generation of Meq mutant viruses, such as Meq C-terminal binding mutant (pRB-1B-Ct20) and more recently Meq homodimer mutant virus (rMd5-MeqGCN) both of which are non-oncogenic (8, 77).

Like c-Jun, Meq can both homodimerize and heterodimerize and has the ability to dimerize with c-Jun, Fos, Creb and ATF members (42). It is well known that the leucine zipper region of bZIP proteins determines the dimerization partners of bZIP proteins, which in turn are important determinants of DNA binding specificity and therefore transcriptional regulation. For example, different c-Jun dimers have been shown to play distinct roles in transformation, i.e. anchorage or serum independent growth. (84). Again, similarly to c-Jun, the DNA binding properties of Meq depend on its dimerization partner. Previous characterization of the in vitro DNA binding properties of Meq revealed Meq/c-Jun heterodimers bind AP-1 sequences with greater affinity than Meq homodimers (42, 61). In addition, Meq and c-Jun were shown to bind AP-1 sequences, but only Meq was shown by electrophoretic mobility assay (EMSA) to bind the sequences contained in the MDV origin of replication (Ori). Transcriptional analysis of Meq on the AP-1-like containing *meq* promoter, and the MDV lytic *pp14/38* bidirectional promoter which contains the MDV Ori, revealed that Meq transactivates the *meq* promoter but represses the bidirectional *pp14/38* promoter. Also, Meq has been shown to bind the immediate early ICP4 promoter region by chromatin immuoprecipitation analysis therefore potentially regulating another lytic gene. This,

together with luciferase reporter data, suggests that Meq heterodimers activate AP-1 containing promoters, therefore potentially activating genes associated with cell proliferation, while Meq homodimers may repress genes associated with lytic infection and consequently may be involved in regulating lytic or latent infection. Collectively this data points to a role of Meq heterodimers in transformation and Meq homodimers in regulation of viral replication.

In order to delineate the functions of Meq and address the role of Meq homodimers in MDV pathogenesis, we recently reported a recombinant Meq mutant virus (rMd5-MeqGCN) expressing a chimeric *meq* gene (*MeqGCN*), which allowed for only Meq homodimer formation. Although this virus replicated in vitro and in vivo, the ability to transform T-cells was lost supporting a role for Meq heterodimers in MDV transformation.

In order to evaluate the role of Meq/c-Jun heterodimers in MDV pathogenesis, a recombinant virus was constructed that allowed for the exclusive formation of Meq/c-Jun heterodimers by substituting the parental Meq leucine zipper with that of the leucine zipper of c-Fos (MeqFos). The leucine zipper region of c-Fos allows for the formation of only heterodimers thus conferring heterodimerization of MeqFos. The DNA binding and transactivation/repression properties of MeqFos were tested in vitro. Recombinant virus expressing MeqFos was studied in vitro *and* in vivo and compared to parental rMd5. An additional virus was recovered rMd5-MeqFos/GCN, which contains one copy of the previously described *meqGCN* gene and one copy of *meqFos* and was also included with in vivo studies. rMd5-MeqFos replicated similarly to parental rMd5 in vitro; however,

infection of chickens with rMd5-MeqFos did not result in gross tumor formation but pre-neoplastic nerve lesions were observed. Interestingly, paralysis and neoplastic MDV lesions were observed with chickens infected with rMd5-MeqFos/GCN. We, therefore, present the first in vivo evidence that both Meq homodimers and heterodimers contribute in MDV transformation.

Materials and methods

Cells and viruses

Primary duck embryonic fibroblasts (DEF) were used for virus propagation, virus reactivation assay, growth curves and DNA transfections. Cosmids derived from a very virulent MDV strain, Md5 were used to generate recombinant viruses (64). Revertant virus (rMd5-MeqFosR) was recovered in chicken embryonic fibroblasts (CEF). The DF-1 cell line was used for luciferase assays, chromatin immunoprecipitation (ChIP) and co-immunopreciptation (co-IP) experiments (35). Both DEF and CEF were maintained in Leibowitz-McCoy (LM) media supplemented with 5% bovine calf serum (BCS) and penicillin-streptomycin at 37°C. DF-1 cells were maintained in LM media supplemented with 4% fetal bovine serum (FBS) and penicillin-streptomycin at 37°C.

Cosmids

Previously described cosmids, SN5, P89, SN16, A6 and B40, encompassing the entire genome of the very virulent strain Md5 (64) were used to generate a recombinant Md5 (rMd5) virus and a recombinant Md5 with a chimeric Meq gene containing the

leucine zipper from c-Fos (rMd5-MeqFos) (Fig. 1).

Mutations in the *meq* gene were generated as described previously (77) with the following only exception that the primers used for overlapping PCR were specific for the *c-Fos* leucine zipper. Briefly, *meq-Kpn*I, which is located within the EcoQ fragment of the Md5 genome and contains the coding sequence for *meq* nucleotides 1- 385, was mutated by a series of subcloning steps and overlapping PCR. Overlapping PCR was performed in a combination of three PCR reactions to replace the leucine zipper region of *meq* with the leucine zipper region of *c-Fos*. Two primary PCR reactions were performed to generate the 5' and 3' ends of *meqFos-Kpn*I fragment. The 5' reaction was performed using primers M13R (5'- CAG GAA ACA GCT ATG AC -3') and SR1194-*Fos* Leucine Zipper Reverse (5'- CTC CGC CTG CAG AGC GGA CTT CTC CTC CTC CAG CTG GTC CGT CTC CGC CTG CAG TTT GTC TAC ATA GTC CGT CTG CTT CCT -3'). The 3' end PCR reaction was performed using primers SR1190 (5'- GAC CGA GAT AGG GTT GAG TG -3') and SR1195-*Fos* Leucine Zipper Forward (5'- GAG AAG TCC GCT CTG CAG GCG GAG ATA GCC AAC CTG CTG AAG GAG AAG GAG AAG CTG CGT GTA CAG TTG GCT TGT CAT GAG CCA-3'). Both amplicons were gel purified, mixed together and used as templates in a third PCR reaction with primers M13R and SR1190, generating a full *meq-Kpn*I fragment containing the *Fos* leucine zipper in place of the *meq* leucine zipper (*meq-Fos-Kpn*I). The *meqFos-Kpn*I fragment was cloned into the EcoQ fragment, generating EcoQ-MeqFos, which was subsequently cloned into cosmids A6ΔEcoQ and SN5ΔEcoQ using

recA assisted restriction endonuclease digestion (RARE) yielding cosmids SN5-MeqFos and A6-MeqFos.

Transfections

Viral inserts were released from parental and mutant cosmid DNA by digestion with *Not*I, and purified by phenol chloroform extraction and ethanol precipitation before transfection. The calcium phosphate procedure was used to transfect 1.2 x10^6 DEF in 60 mm dishes with 500 ng of cosmids P89, SN16, and B40 in combination with SN5 and A6, or SN5-MeqFos and A6-MeqFos, or SN5-MeqFos and A6-MeqGCN, to generate rMd5, rMd5-MeqFos, and rMd5-MeqFos/GCN, respectively. Five days after transfection, cells were trypsinized and seeded onto DEF monolayers and monitored for cytopathic effects. Viral stocks of recovered viruses were subsequently made and titrated in DEF for further analysis.

Southern blot

DNA from rMd5 and rMd5-MeqFos infected DEF was isolated by proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation. Three micrograms of each DNA sample was digested with either *Eco*RI or *Pst*I, separated on a 1% agarose gel, and transferred to nylon membranes. ^{32}P-dCTP-labeled probes representing the complete MDV genome (cosmids SN5, P89, SN16, A6 and B40) or EcoQ fragment (2,456 bp) were generated by random priming using High Prime DNA Labeling Kit (Roche) and were used to hybridize to viral DNA, using standard protocols

(68).

RT-PCR

PCR was performed with primers SR1118 and SR3074 or SR3075 in order to confirm expression of the presence of *meqFos* and *meqGCN* genes from DEF infected with rMd5-MeqFos/GCN. Total RNA was extracted using RNAqueous®-4PCR (Ambion). The reverse transcriptase reaction was performed using equal amounts of RNA followed by PCR. Four μl of cDNA were used for subsequent PCR reactions using primers SR1118-*meq* start primer (5'-GAT CCC GGG GAG ATG TCT CAG GAG CCA GAG C-3') and leucine zipper specific primers SR3075-*Fos* leucine zipper (5'-TTC AGC AGG TTG GCT ATC -3'), or SR3074-*GCN4* leucine zipper (5'- CTA ATT TCT TTA ATC TGG CAA C -3').

Revertant virus

CEF were inoculated with rMd5-MeqFos to obtain viral DNA used for transfections. Gel purified parental Md5 EcoQ fragment together with proteinase K digested and phenol-chloroform extracted rMd5-MeqFos genomic DNA, were used to co-transfect CEF by the calcium phosphate procedure. After viral plaques were evident, individual viral plaques were isolated as described (77).The presence of parental *meq* or *Fos* leucine zipper sequences from individual plaques were detected by PCR using the following primers: SR1118-*meq* start primer and leucine zipper specific reverse primers SR3073-*meq* leucine zipper (5'- GTC CTT AGA TCT CGA ATT TCC -3') or SR3075-

Fos leucine zipper, for parental and *Fos* leucine zippers, respectively.

Indirect immunofluorescence assay (IFA) and immunohistochemistry (IHC)

Confluent DEF monolayers were infected with rMd5 or rMd5-MeqFos, and when viral plaques were apparent, cells were fixed with ice-cold acetone/alcohol (6:4) and Meq expression evaluated by IFA using rabbit polyclonal anti-Meq antibodies (1:300). Lymphocytes collected for reactivation assays were deposited on a microscope slide using a cytospin centrifuge, fixed with ice-cold acetone/alcohol (6:4) and expression of pp38 evaluated by IFA using the H19 pp38 specific monoclonal antibody (1:400). For IHC, lymphoid organs (thymus, spleen and bursa of Fabricius), and feather follicles from infected and uninfected chickens were embedded in optimal cutting temperature compound (Tissue-Tek, OCT, Sakura Finetek, Torrance, CA), immediately frozen in liquid nitrogen and stored at -80°C until use. Six- to 8-μm-thick cryostat sections of tissue were prepared and fixed with cold acetone at -20°C for 5 min, and air-dried. Immunostaining was carried out using H19 pp38 monoclonal antibody (1:3000) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions.

Luciferase assays

The transactivation/repression activity of the MeqFos protein on MDV promoters was evaluated by luciferase assay. The previously described *meq*, pp38, pp14 and MDV Ori promoter constructs (77) were used for luciferase assays. *Meq*, *MeqFos*, and *c-Jun*

genes were cloned into the pcDNA 3.1 Zeo vector (Invitrogen) to generate expression vectors, pcDNA-Meq, pcDNA-MeqFos, and pcDNA-Jun. DF-1 cells ($1X10^5$) were seeded in 12-well plates 16 hr prior to transfection and incubated at 37°C under standard conditions. Transfections were performed using siPORT XP-1 (Ambion), 500 ng of pcDNA (empty vector control), pcDNA-Meq, pcDNA-MeqFos, or 250 ng of pcDNA-Jun with either 250 ng of pcDNA-Meq or pcDNA-MeqFos, and either 250 ng of pGL3-*meq*, pGL2-Ori, or pGL3-pp14 or pGL3-pp38 promoters. Cell lysates were harvested 48 hours post-transfection with active lysis buffer (Promega) and luciferase assay performed using a Biotek Clarity luminometer (Biotek, Winooski, VT). The protein concentration in each transfected sample was measured by Bradford assay (Bio-Rad, Hercules, CA) and luciferase activity was normalized to protein concentration. Assays were performed in triplicate and three independent experiments were performed for each reporter vector tested. Transactivation or transrepression activity was expressed as the fold differences relative to empty pcDNA vector control. Results of all three experiments were analyzed by one-way ANOVA followed by Tukey HSD test using the SPSS®, Version 14.0 software (SPSS Inc., Chicago, IL, USA). For all analysis, $p \leq 0.05$ was considered statistically significant.

Chromatin immunoprecipitation assay (ChIP)

DF-1 cells (1.5×10^6) were seeded in 100 mm dishes 16 hrs prior to transfection with pcDNA (empty vector control), pcDNA-Meq, or pcDNA-MeqFos and pGL2-3XOri and pGL3-*meq* promoter using siPORT XP-1. ChIP assays were carried out essentially

as described (52) with the following modifications. Briefly, cells were trypsinized, trypsin was neutralized with DF-1 growth media, and cells were pelleted by centrifugation. Cell pellets were resuspended in 10 ml fresh media and cell suspensions were cross-linked in 1% formaldehyde (v/v) at 37°C for 10 min. The cross-linking reaction was stopped by the addition of glycine at a final concentration of 125 mM for 5 minutes at RT. The fixed cell suspensions were pelleted by centrifugation and washed two times in PBS. After the second wash, cell pellets were resuspended in SDS-lysis buffer (50 mM Tr-s-HCl, pH8.1, 1% SDS, 10 mM EDTA), sonicated on ice, and diluted with ChIP dilution buffer (16.7 mM Tris-HCl, pH8.1, 0.01% SDS, 1.1 % Triton X-100, 1.2 mM EDTA, 167 mM NaCl) containing 1x Complete Protease Inhibitor Mixture (Roche). Chromatin was cleared twice by incubation with Protein G agarose/salmon sperm DNA bead slurry (Millipore, Billerica, MA) at 4 °C for 30 min, followed by centrifugation. Ten µl of anti-Meq antibody or 1 µg of rabbit-IgG was added to the cleared chromatin and incubated at 4 °C overnight with mixing. Chromatin was precipitated with Protein G agarose/salmon sperm DNA bead slurry 1 h at 4 °C with mixing. The agarose beads were recovered and washed successively for 5 min at 4 °C in low salt, high salt, LiCl and TE buffers. Chromatin was eluted in freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$) at room temperature for 15 min. Samples were treated with RNase A and cross-linking was reversed by incubation at 65 °C for 4 h. Chromatin was ethanol precipitated and proteinase K digested. The precipitated DNA was purified using Purelink PCR purification kit (Invitrogen) and eluted in 50 µl of sterile water. PCR detection of pGL2-3XCACA and pGL3-MSP plasmids was performed in 25-µl reactions

containing 2 µl of chromatin and primers pGL2-ChIP-F1 (5'-CCC CCT GAA CCT GAA ACA TA-3') and pGL2-ChIP-R1 (5'-GCC TTA TGC AGT TGC TCT CC-3'); pGL3-ChIP-F1 (5'-GTG GCT TTA CCA ACA GTA CC-3') and pGL3-ChIP-R1 (5'-GCT CTC CAT CAA AAC AAA AC- 3'), respectively. PCR amplification was performed with an initial denaturation at 95 °C for 3 min followed by 23 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s followed by a final elongation step at 72 °C for 5 min.

Colony formation in soft agar

DF-1 cells were transfected with pcDNA, pcDNA-Meq or pcDNA-MeqFos using siPORT XP-1 (Ambion). Approximately 48 hours after transfection, the transfected DF-1 cells were selected with 300 µg/ml zeocin (Invitrogen) for 4 weeks. Expression of Meq was confirmed by IFA using rabbit anti-Meq antibodies as described above. Pools of resistant cells (5 $X10^3$) were seeded in 0.33% agarose containing LM media with 150 µg/ml zeocin and 10% FBS overlaid on a 0.5% agarose in a 35-mm plate. After 3 weeks of culture, colonies were examined under a light microscope and photographed using 12 X magnification. Three different fields were randomly selected and colonies greater than 100 µm were counted. Two independent experiments were performed and each experiment was performed in triplicate.

In vitro growth kinetics

To compare the growth characteristics of rMd5 and rMd5-MeqFos, DEF seeded on 35 mm plates were inoculated with approximately 50 plaque forming units (PFU) of each virus. On days 2, 4 and 6 post inoculation, the infected cells were trypsinized, serial

dilutions inoculated onto 35 mm plates seeded with fresh DEF and plaques at the different dilutions counted 7 days later.

Pathogenesis experiments

Experiment 1

To study the effect of Meq heterodimers on viral replication, 4-week-old SPF chickens (Hy-Vac, Adel, Iowa) were randomly sorted into experimental groups of nine chickens each. One group remained as a non-inoculated control, whereas the other groups were inoculated subcutaneously with 3,000 PFU of rMd5 or rMd5-MeqFos. At 14 and 21 days post infection (dpi), blood samples, from randomly selected chickens, were collected in heparin for reactivation assays (see below), and were subsequently euthanized for tissue sample collection. At 6 and 21 dpi lymphoid organs and feather follicles were collected for IHC, respectively.

Experiment 2

To study the role of the Meq heterodimers on oncogenesis, SPF day old chicks (ten per group) were inoculated subcutaneously with 2,000 PFU of rMd5, rMd5-MeqFos, or rMd5-Meq/Fos/GCN and reared in modified Horsfall-Brauer isolation units for 8 weeks. Weekly mortality was recorded and all chickens were necropsied at time of death or at termination of the experiments (8 weeks) and evaluated for MD specific lesions in the viscera and the nerves. Blood was also collected from three randomly selected chickens from each group for reactivation assays at day 37 dpi.

Experiment 3

To study the role of Meq heterodimers on horizontal transmission, six SPF (Charles River) day old chicks were inoculated subcutaneously with 3,000 PFU of rMd5 or rMd5-MeqFos while three additional uninoculated chicks were reared with each group and served as contacts to assess horizontal transmission. At 8 weeks post inoculation buffy coats were obtained from heparinized blood by centrifugation at 500 X g. DNA was extracted using Purelink Genomic DNA extraction kit (Invitrogen) and PCR was performed using MDV specific Meq 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') to detect the presence of MDV genome.

Reactivation assay

Buffy coats were collected as described above at 14, 21, and 37 dpi. DEF monolayers seeded in 35 mm plates were inoculated with 10^6 lymphocytes in duplicate and viral plaques counted 7 days post inoculation.

Results

Meq heterodimer mutant (MeqFos) retains DNA binding, transactivation and repressive functions

Like the Jun family of bZIP transcription factors, Meq has the ability to form homodimers and heterodimers (42). It is well known that the specific amino acid residues within the leucine zipper of bZIP proteins determine their dimerization

properties. Among these transcription factors, c-Fos has been extensively studied and it is known that the leucine zipper of c-Fos exclusively forms heterodimers with the Jun family of transcription factors (26, 37, 63). Similar to other studies (15, 26, 27, 34, 63), we utilized the leucine zipper region of c-Fos in place of the parental leucine zipper of Meq to study the function of Meq/Jun heterodimers in MDV pathogenesis. The leucine zipper of *meq* was successfully "swapped" with the leucine zipper of *c-fos* using overlapping PCR, resulting in a Meq heterodimer mutant, MeqFos (Fig. 14).

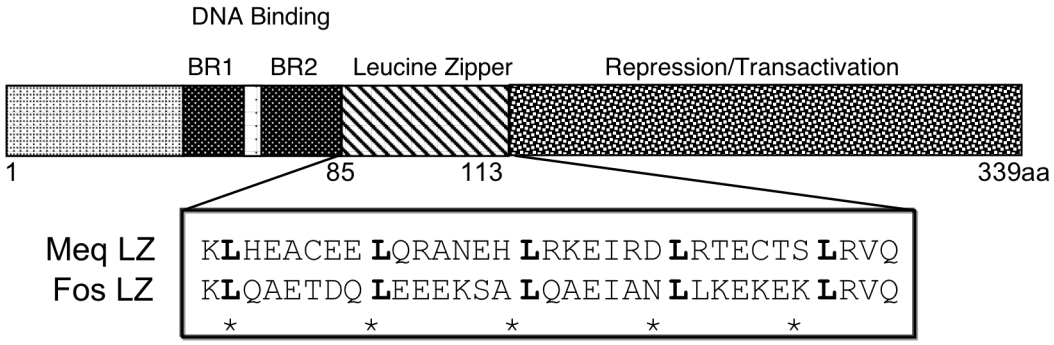

Fig. 14. Schematic representation of the *meq* gene and *meq* and *fos* leucine zippers (LZ). The DNA binding basic regions (BR1 and BR2) and transactivation domains, are depicted. The LZ sequence of parental *meq* and c-*Fos* used to replace parental *meq* LZ in rMd5-MeqFos are shown. Asterisks indicate the conserved leucine sites.

The open reading frames of *meq* and *meqFos* were cloned into the pcDNA expression vector. Previous work has shown that Meq homodimers and heterodimers have different DNA binding affinities. Although both Meq homodimers and

heterodimers bound AP-1 sequences, Meq together with c-Jun bound AP-1 sequences with greater affinity than Meq alone. In addition, Meq alone bound the MDV origin of replication (MDV Ori) located in the pp38/14 bidirectional promoter by EMSA analysis (42). Luciferase reporter assays indicated functional differences between Meq and Meq plus c-Jun in that although Meq activated the AP-1 containing *meq* promoter, Meq plus c-Jun resulted in higher activation. Furthermore, Meq expression was shown to repress the MDV pp38/pp14 bidirectional promoter which contains the MDV Ori (42). Therefore, chromatin immunoprecipitation (ChIP) and luciferase assays were performed to test the DNA binding and transactivation/repression functions of MeqFos.

Luciferase assays were utilized to test the transactivation and repressive functions of MeqFos compared to parental Meq. DF-1 cells were transfected with pcDNA empty vector, pcDNA-Meq, pcDNA-MeqFos, pcDNA-c-Jun, or a combination of pcDNA-Meq, pcDNA-MeqFos, and pcDNA-c-Jun, together with reporter vectors pGL3-*meq*, pGL3-*pp14*, pGL3-*pp38*, or pGL2-3X-Ori. As shown in figure 15, both Meq and MeqFos activate the *meq* promoter, however MeqFos required co-transfection with pcDNA-c-Jun for transactivation, therefore indicating heterodimerization MeqFos with c-Jun is required for transactivation. Unexpectedly, both Meq and MeqFos repressed *pp38* and *pp14* promoters (Fig. 15). In addition, both Meq and MeqFos repressed the pGL2-3X-Ori reporter, which contains three copies of the sequence element found in the pp38/14 bidirectional promoter that Meq homodimers was previously reported to bind (42, 61).

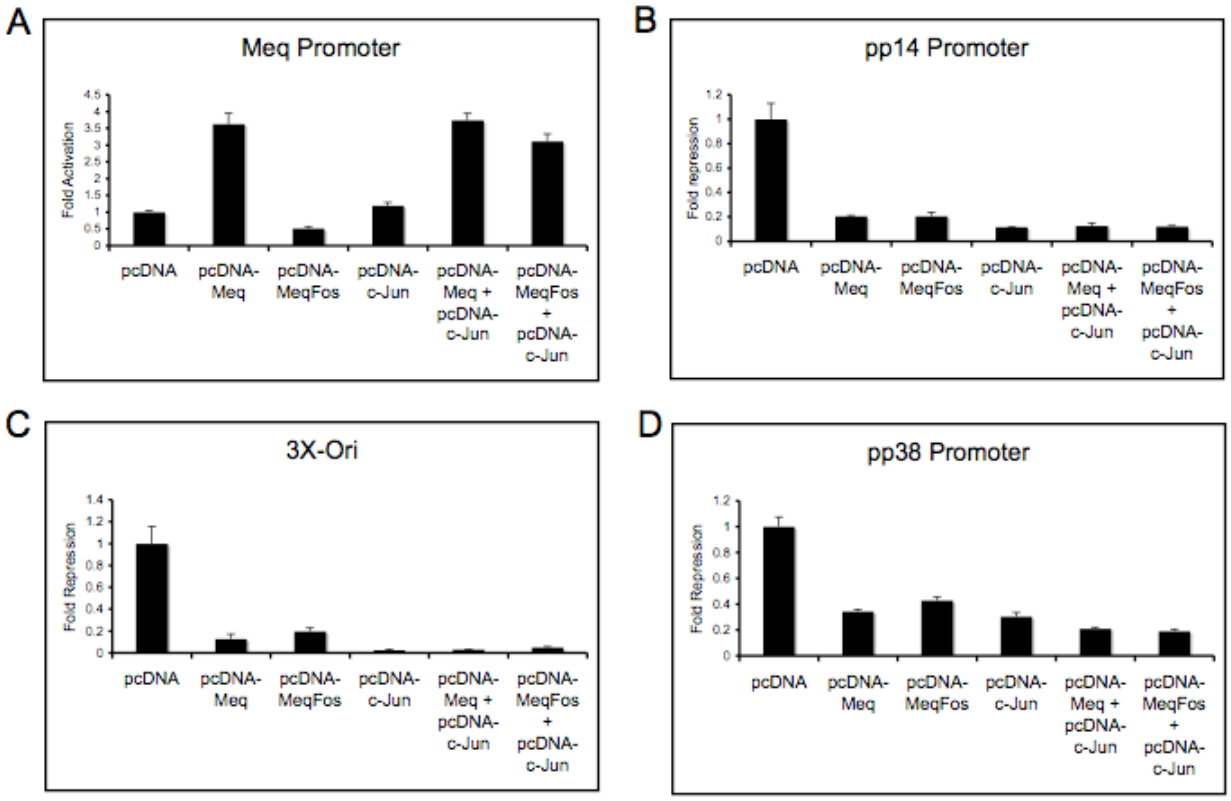

Fig. 15. Luciferase assays demonstrate that MeqFos has functional transactivation and repression activities. DF-1 cells were transfected with *meq*-promoter, pp14 promoter, pp38 promoter or 3X-Ori luciferase reporter plasmids and pcDNA (empty vector), pcDNA-Meq, pcDNA-MeqFos, or pcDNA c-Jun, with or without c-Jun. Both Meq and MeqFos activate the *meq*-promoter and repress the pp38/14 bidirectional promoters and 3X-Ori. However, activation of the meq promoter by MeqFos requires c-Jun. Luciferase values are expressed as fold difference relative to pcDNA vector. Significance (*) equals $p \leq 0.05$. Error bars indicate SD.

In order to evaluate the DNA binding capacity of Meq and MeqFos ChIP assays were performed. DF-1 cells were transfected simultaneously with pGL3-*meq* promoter and pGL2-3X-Ori, along with pcDNA empty vector, pcDNA-Meq or pcDNA-MeqFos with or without pcDNA-c-Jun. As expected, Meq bound both the *meq* promoter and MDV Ori and MeqFos also bound the meq promoter. However, in contrast to previous

EMSA data MeqFos was also able to bind the 3X-Ori. In addition, DNA binding by both Meq and MeqFos was enhanced when cells were transfected with pcDNA-c-jun (Fig.16).

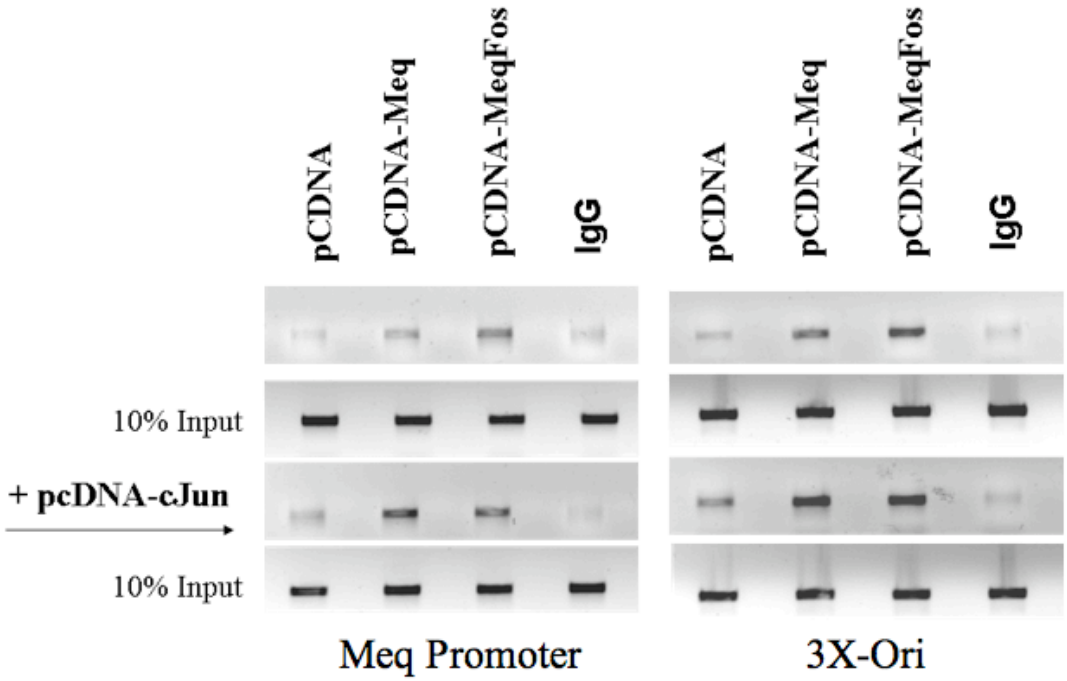

Fig. 16. Chromatin Immunoprecipitation (ChIP) analysis was performed to test the DNA binding capacity of of Meq and MeqFos on the *meq* promoter and 3X-Ori construct, which contains three repeats of the CACA Meq binding site. Both Meq and MeqFos were recruited the *meq* promoter and 3X-Ori, and transfection with pcDNA-c-Jun increased binding.

Colony formation in soft agar

Colony formation in soft agar is a marker of transformation and Meq expression in fibroblasts has been shown to promote anchorage independent growth (41). Soft agar assay was employed to test the ability of Meq heterodimers to promote colony formation in soft agar. Meq and MeqFos expression in selected DF-1 cells was confirmed by IFA

(Fig. 17a top panel). As expected, selected DF-1 cells expressing the pcDNA-Meq construct formed large colonies in soft agar when compared to control cells selected with pcDNA empty vector (Fig. 17a bottom panel). Furthermore, cells expressing pcDNA-Meq had 25 X more colonies >100 µm in size compared to pcDNA control cells. In contrast, DF-1 cells expressing pcDNA-MeqFos only had 3.5 X as many colonies >100 µm in size compared to empty vector control cells (Fig. 17b), indicating that MeqFos heterodimers have a reduced potential for anchorage independent growth.

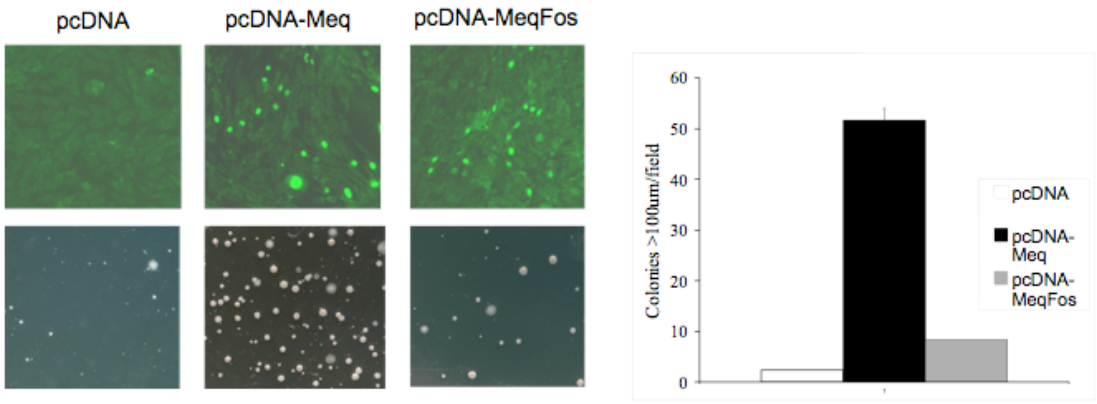

Fig. 17. In vitro soft agar assays demonstrating that MeqFos has reduced transformation potential. (a) Top panel: Immunofluorescence analysis of Meq expression from pools of selected DF-1 cells transfected with pcDNA, pcDNA-Meq, pcDNA-MeqFos. Bottom panel: Soft agar assay was performed using pcDNA, pcDNA-Meq, pcDNA-MeqFos selected DF-1 cells to assess anchorage independent growth. (b) Number of colonies > 100 µm observed in cells expressing pcDNA, pcDNA-Meq, or pcDNA-MeqFos. Average number of colonies counted from three random fields are shown. Error bars indicate SD.

Construction of a Meq-heterodimer mutant virus rMd5-MeqFos

A recombinant Md5 mutant virus in which the leucine zipper region of *meq* was replaced with the corresponding region of *Fos* (rMd5-MeqFos) was successfully

constructed. The leucine zipper region of the *meq* gene was replaced with the leucine zipper region of *Fos* by overlapping PCR, and the EcoQ fragment containing the chimeric *meqFos* gene was cloned into the A6Δmeq and SN5Δmeq cosmids. The resultant cosmids A6-MeqFos and SN5-MeqFos together with cosmids P89, B40 and SN16 were cotransfected into DEF by the calcium phosphate method and a recombinant virus was subsequently recovered by homologous recombination. In addition, rMd5-MeqFos/GCN was recovered by cotransfection of cosmids P89, B40, SN16, SN5-MeqFos and previously described cosmid A6-MeqGCN. Southern blot analyses were performed to assess the integrity of the rMd5-MeqFos viral genome. Genomic DNA from rMd5 and rMd5-MeqFos infected DEF was digested with *Eco*RI or *Pst*I and probed with ^{32}P labeled Md5 comids or EcoQ fragment DNA, respectively. No differences were observed between rMd5 and rMd5-MeqFos *Eco*RI and *Pst*I digestion patterns confirming the integrity of the recombinant genomes (Fig. 18a). RT-PCR was performed to confirm mRNA expression of MeqFos and MeqGCN from DEF infected with rMd5MeqFos/GCN (Fig. 18b).

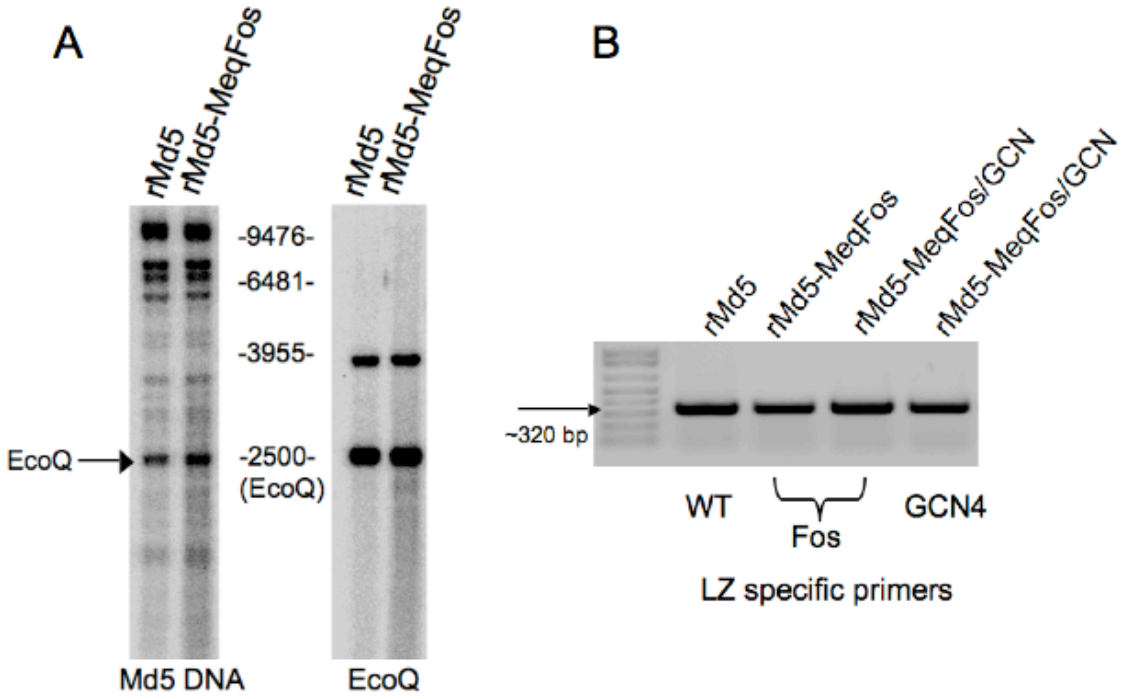

Fig. 18. Southern blot and RT-PCR analysis of recombinant viruses. a) Southern blot analysis of rMd5 and rMd5-MeqFos. Both rMd5 and rMd5-MeqFos DNA was digested with *Eco*RI, or *Pst*I and probed with total viral MDV DNA, or EcoQ fragment, respectively. The restriction profile of rMd5-MeqFos is similar to that of rMd5, indicating no gross genome rearrangements incurred. b) RNA isolated from rMd5, rMd5-MeqFos, and rMd5-MeqFos/GCN infected DEF was isolated and RT-PCR with Meq start and leucine zipper specific primers performed, generating a 320 amplicon.

Immunofluorescence assay (IFA) was performed to evaluate Meq expression in virus infected DEF. Meq expression was detected in both rMd5, rMd5-MeqFos, and rMd5-MeqFos/GCN infected DEF but not in uninfected cells (Fig. 19).

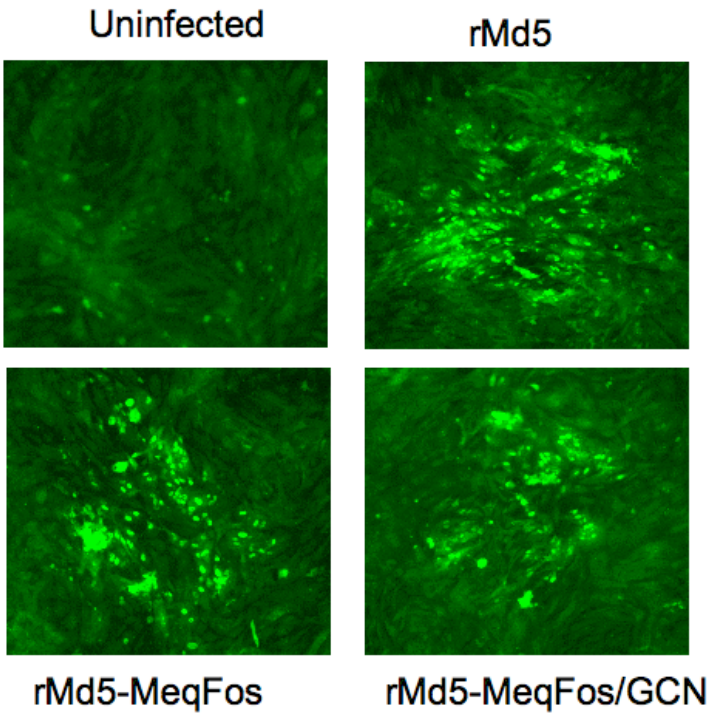

Fig. 19. Immunofluorescence analysis of DEF cells infected with recombinant viruses. Uninfected DEF, rMd5-Meq infected DEF, rMd5-MeqFos infected DEF, rMd5-MeqFos/GCN infected DEF, at 100X magnification.

In vitro and in vivo replication of rMd5-MeqFos

The in vitro growth properties of rMd5-MeqFos were tested, to assess if the leucine zipper mutations had any effect on virus replication in vitro. Our results indicate that rMd5 and rMd5-MeqFos virus replicated similarly at the time points tested (days 0, 2, 4, 6) (Fig. 20).

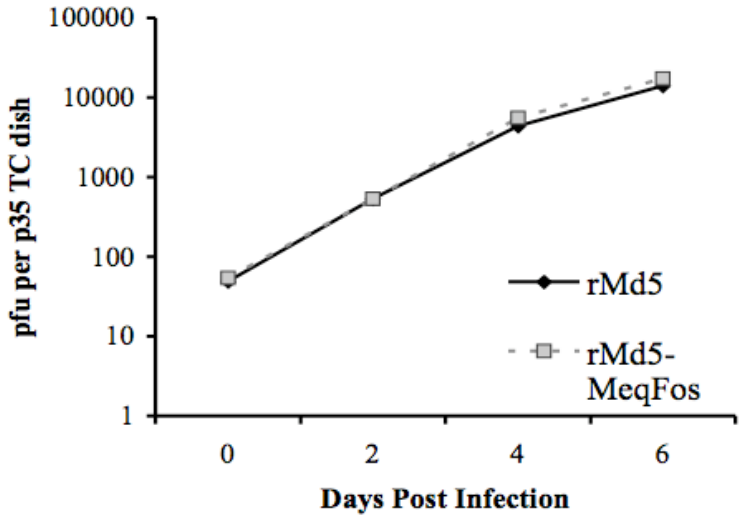

Fig. 20. In vitro growth properties of rMd5 and rMd5-MeqFos. DEFs were infected with the indicated viruses, harvested on days 2, 4 and 6 after infection and titered on fresh DEF. Day 0 indicates the titer of the virus in the inoculum. The experiment was performed in duplicate, and the titer (logarithm of the mean number of plaque-forming units per dish) is indicated.

To test the role of Meq heterodimers during in vivo replication, 4-week-old SPF chickens were inoculated with 3,000 pfu of rMd5 or rMd5-MeqFos. Six days after inoculation two randomly selected birds were euthanized and lymphoid organs (thymus, spleen and bursa of Fabricius) collected and evaluated for viral lytic antigen (pp38) expression by IHC. Expression of pp38 was present in bursa of Fabricius (Fig. 21) thymus and spleen (data not shown) of both rMd5 and rMd5-MeqFos infected chickens, indicating that the leucine zipper mutations in the rMd5-MeqFos virus did not affect early cytolytic infection.

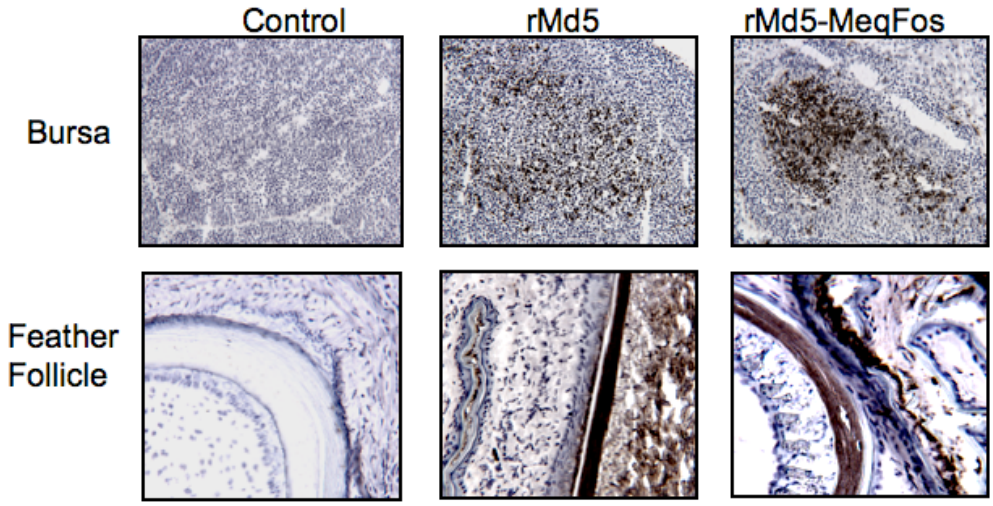

Fig. 21. Infection of lymphoid tissue and feather follicles of chickens infected with rMd5 and rMd5-MeqFos. Immunohistochemistry of bursa of Fabricius and feather follicles, 6 and 21 dpi, respectively using anti-pp38 monoclonal antibody (H19). Positive cells are indicated by brown staining, counterstaining was performed with hematoxylin.

MDV switches from an active cytolytic infection to a latent infection approximately 7-8 days PI and virus reactivation can be observed when latently infected lymphocytes are co-cultured with fibroblasts (11). To examine if Meq homodimers are involved in the establishment of latency and reactivation, peripheral blood lymphocytes (buffy coats) were obtained from three chickens from each group on days 14 and 21 PI and co-cultured with DEF. To confirm viral infection, portions of collected lymphocytes were fixed on microscope slides and IFA performed to detect viral antigen, pp38 (Fig. 22).

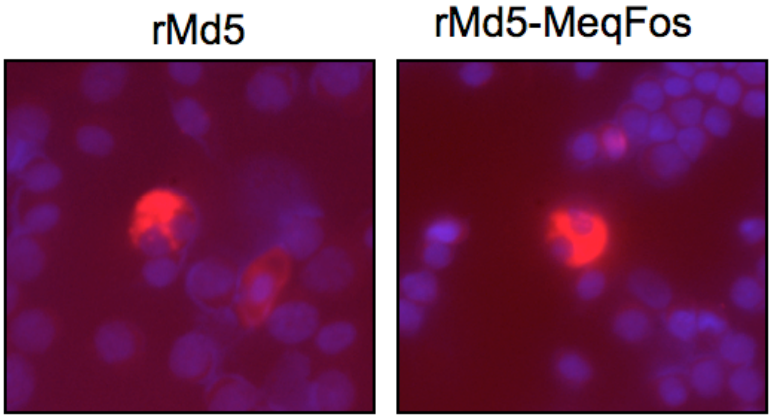

Fig. 22. Detection of MDV pp38 protein in latently infected cells 21 dpi. Immunofluorescence assay was performed with anti-pp38 monoclonal antibody (H19) on PBMC's collected from rMd5 and rMd5-MeqFos infected chickens. Texas-red conjugated secondary antibodies were used to detect pp38 and nuclei were stained with DAPI.

IFA confirmed expression of pp38 from infected lymphocytes at day 21 PI (Fig. 22) although reactivated virus was not recovered from co-culture with DEFs. PBMC's were also collected for reactivation assays at day 37 PI from infected chickens used for experiment 3. As shown in Table 3, none or few reactivated viral plaques were obtained from PBMCs of chickens infected with rMd5-MeqFos days 14 days and 21 PI. However, reactivated virus comparable to rMd5 was obtained at day 37 PI from one out of three chickens from both rMd5-MeqFos and rMd5-MeqFos/GCN infected chickens (Table 3).

Table 3.
Virus reactivation from peripheral blood lymphocytes

Group	Day 14	Day 21	Day 37*
rMd5	121 181 75	28 120 123	116.5 118.5 129
rMd5-MeqFos	0 1 5	0 0 0	2.5 8 133.5
rMd5-MeqFos/GCN	ND	ND	1.5 4 158

Reactivation assays were performed on days 14, 21 and 37* after inoculation. The numbers represent the average number of pfu observed when10^6 peripheral blood lymphocytes were co-cultured with DEF from each chicken in duplicate. * Samples were collected from chickens from experiment 2.

Transmission of MDV occurs after viral replication in feather follicle epithelium (FFE) and release of infectious dander (13). Therefore, to assess if the rMd5-MeqFos heterodimer mutant was able to replicate in the FFE, expression of the lytic viral antigen pp38 was evaluated in three chickens from each inoculated group by IHC. Tissues from both rMd5 and rMd5-MeqFos infected groups tested positive for pp38 antigen indicating the ability of rMd5-MeqFos, like rMd5, to replicate in the FFE (Fig. 21). To further evaluate MDV transmission, day old SPF chickens were inoculated with 3,000 pfu of rMd5 or rMd5-MeqFos and housed with three uninfected contact chicks. Buffy coats were collected from contact birds 8-weeks PI, DNA extracted and PCR for Meq performed (data not shown). Transmission to contact birds was confirmed by PCR in two chickens infected with rMd5 and all three contact chickens infected with rMd5-MeqFos were PCR positive for *meq* (data not shown). Altogether these results confirm

that rMd5-MeqFos retains the ability to transmit horizontally by shedding through the FFE.

Oncogenecity of rMd5-MeqFos

To determine if Meq-Jun heterodimers are sufficient for in vivo transformation of T-cells, groups of nine SPF chickens were inoculated at day of age with 2,000 pfu of rMd5, rMd5-MeqFos, or rMd5-MeqFos/GCN and observed for mortality for a period of 8 weeks. All chickens that died during the experiment or were euthanized at the termination of the experiment were evaluated for MDV-specific lesions. MDV-associated mortality was observed in rMd5-infected chickens starting at week 4 PI and none of the chickens survived to the end of the experiment. Preneoplastic lesions only were observed in the rMd5-MeqFos group while 20% of chickens infected with rMd5-MeqFos/GCN contained neoplastic lesions and 60% contained preneoplastic lesions. Paralysis was observed in rMd5-MeqFos/GCN infected chickens containing neoplastic nerve lesions. In addition one out of three contact chickens from the rMd5-MeqFos/GCN infected group contained preneoplastic nerve lesions while none were observed in the contact chickens of the rMd5-MeqFos group.

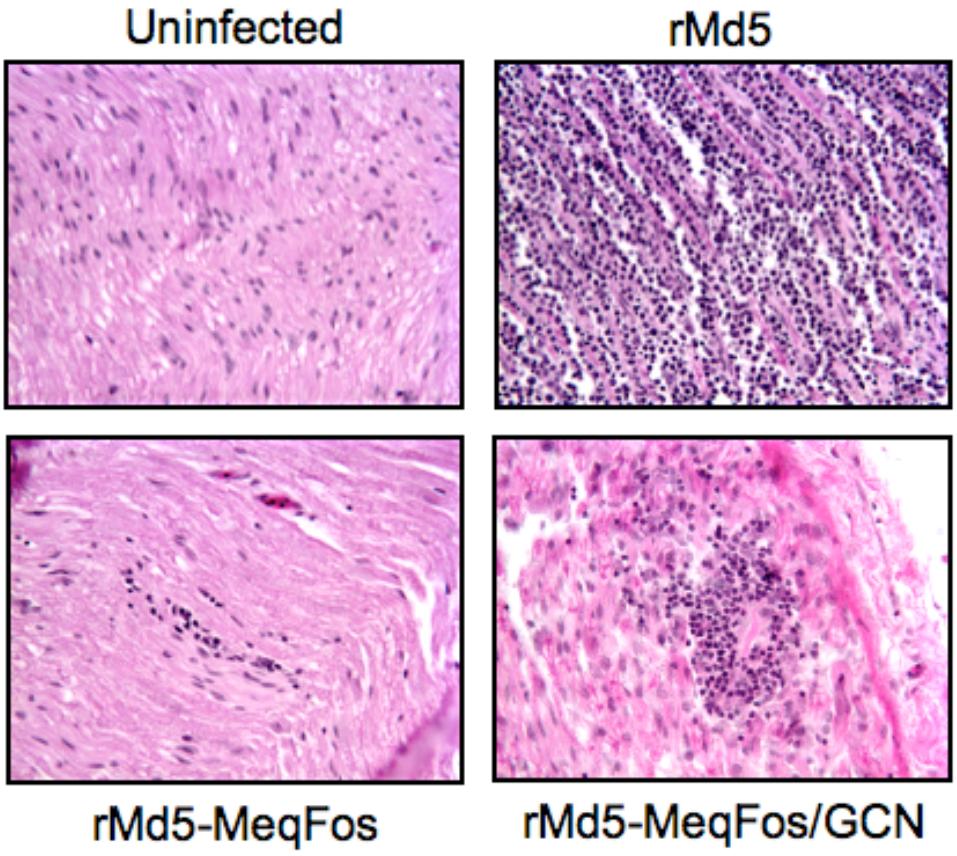

Fig. **23**. MDV induced preneoplastic and neoplastic nerve lesions from chickens infected with rMd5, rMd5-MeqFos and rMd5-MeqFos/GCN. Neoplastic nerve lesions were observed in rMd5 and rMd5-MeqFos/GCN infected chickens while preneoplastic lesions were observed in chickens infected with rMd5-MeqFos.

Table 4.
Pathogenicity of rMd5, rMd5-MeqFos, rMd5-MeqFos/GCN and rMd5-MeqFosR in SPF MDV maternal antibody-negative chickens

Virus[a]	MDV Mortality (%)	Histological Lesions (%)[b]	
		Preneoplastic	Neoplastic
rMd5	10/10 (100)	ND	ND
rMd5-MeqFos	0/10 (0)	4/8 (50)	0/0 (0)
rMd5-MeqFosR[c]	3/3 (100)	ND	ND
rMd5-MeqFosGCN	2/10 (20)	6/10 (60)	2/10 (20)
Uninfected	0/8 (0)	0/3 (0)	0/3 (0)

[a]All chickens were inoculated with 3,000 PFU of the indicated viruses.

[b]Chickens that were not positive for gross tumors were further evaluated for microscopic tumors

[c] Performed as an independent experiment

Construction and Biological Properties of Revertant Virus rMd5-MeqFosR

To verify that the phenotypic changes observed with rMd5-MeqFos were due to changes introduced in the leucine zipper region, a revertant virus was constructed. DNA obtained from rMd5-MeqFos infected CEF was co-transfected with purified parental MDV EcoQ DNA, which contains the *meq* gene. A revertant virus, rMd5-MeqFosR, was selected by plaque purification, and screened for the presence of both parental *meq* and mutant *meqFos* genes by PCR. PCR revealed that of the 102 plaques tested, one contained one copy of the parental *meq* gene restored in the viral genome. Three

chickens were infected with 3,000 pfu of rMd5-MeqFosR and by week 5 PI all three were positive for MDV gross tumors, confirming that the attenuated phenotype of rMd5-MeqFos was due to the mutations introduced in the leucine zipper of Meq.

Discussion

In this study, the role of Meq-Jun heterodimers in MDV transformation was investigated to complement our recent investigations evaluating Meq/Meq homodimers in MDV pathogenesis using a recombinant Meq homodimer (rMd5-MeqGCN) virus. We found that rMd5-MeqGCN is non-oncogenic, supporting the concept that Meq/c-Jun heterodimers cooperate in transformation. The results of the current study further dissect the functions of Meq homodimers and heterodimers in MDV pathogenesis by evaluating the Meq-Jun partnership in vivo and we demonstrate that both Meq homodimers and heterodimers contribute in MDV transformation.

In order to assess the functions of Meq-Jun heterodimers in MDV pathogenesis, a recombinant virus in which the leucine zipper of the Meq protein was replaced with the leucine zipper of c-Fos was successfully generated. It is well established that the leucine zipper region of bZIP proteins determines their dimerization specificity, and that c-Fos exclusively heterodimerizes with the Jun family of transcription factors (37, 63, 84). In addition, the modular nature of bZIP proteins allows the leucine zipper region to be "swapped" among bZIP proteins generating chimeric proteins that allow for the characterization of specific bZIP dimers (15, 27, 34, 63, 72).

The DNA binding, transactivation/repression, and transformation properties of

MeqFos were evaluated in vitro. Previous in vitro characterization of Meq DNA binding and transactivation/repression activities have indicated that Meq homodimers and heterodimers differ in DNA binding and therefore transcriptional activity, thus implying Meq homodimers and heterodimers may have distinct roles in MDV-1 pathogenesis (33, 41, 42, 61). Therefore, the DNA binding and transactivation/repression activities of MeqFos were tested in vitro.

As expected parental Meq alone or with c-Jun significantly activated the *meq* promoter. In addition, MeqFos together with c-Jun activated the *meq* promoter, thus supporting that the leucine zipper mutations conferred heterodimerization with c-Jun (Fig. 15). In addition, ChIP assays confirmed that MeqFos was recruited to the *meq* promoter, and binding was enhanced by cotransfection with c-Jun (Fig. 16). This implies that Meq homodimers were able to activate the Meq promoter in the absence of exogenous c-Jun, or that Meq partnership with ATF/CREB members may activate the Meq promoter. Nonetheless, Meq/c-Jun heterodimers have a synergistic effect on activation of the Meq promoter, as levels of activation were comparable to Meq alone even though half the amount of pcDNA-Meq DNA was used for cotransfection, and pcDNA-c-Jun did not activate the Meq promoter independently (Fig. 15). We recently described that the vaccine strain of Meq, CVI-Meq, required exogenous c-Jun for activation of the Meq promoter. This observation may reflect that even though the leucine zipper is identical between Md5 Meq and CVI-Meq, other differences in CVI-Meq, such as phosphorylation, contribute to preferentially dimerization with c-Jun or that CVI-Meq homodimers are inefficient transactivators due to amino acid differences

in their transactivation domain (1).

Unexpectedly MeqFos and c-Jun significantly repressed both pp38 and pp14 promoters and the 3X-Ori (Fig. 15). Previous characterization of Meq-Jun DNA binding sequences (20) demonstrated that Meq together with c-Jun did not bind the MDV Ori (42, 61), which is located in the pp38/14 bidirectional promoter, therefore it was expected that repression of these reporters was due to Meq homodimers. A number of putative transcription factor binding sites have been described in the pp38/14 promoters including AP-1, SP-1, CAAT, and c-myc (20). It is possible that Meq and c-Jun may repress these promoters by competition for AP-1 sites by other AP-1 dimers (54). However, MeqFos and c-Jun repressed the 3X-Ori, although this construct is not a relevant promoter, however it contains three tandem repeats of the previously characterized Meq binding site located within the MDV Ori (42, 61). Therefore, this construct was used to evaluate Meq binding under physiologic conditions by ChIP assay. Previous EMSA data using bacterially expressed Meq and c-Jun protein, showed that only Meq homodimers bound the "CACA" sequence. However, our observations indicate that MeqFos under physiologic conditions is recruited to the 3x-Ori (Fig. 16). Nonetheless, our data shows that MeqFos retains transactivation and repressive functions, and that transactivation requires excess c-Jun, implying that MeqFos exclusively heterodimerizes.

The ability of MeqFos to promote anchorage independent growth as a marker for transformation was evaluated in vitro by soft agar assay. Although numerous colonies were observed in DF-1 cells expressing Meq, reduced colony formation was observed in

DF-1 cells expressing MeqFos (Fig. 17). This together with previous observations that MeqGCN also had reduced colony formation in vitro suggests that both Meq homodimers and Meq heterodimers may promote anchorage independent growth or that Meq heterodimerization with other AP-1 members outside the Jun family also contribute to transformation in vitro. Complementing pathways have been observed for c-Jun transformation in vitro. Although, in the case of c-Jun, GCN4 and EB1 Jun chimeric proteins transform in vitro and in vivo, the chimeric proteins exhibit increased stability over wild type c-Jun proteins and enhance the transforming ability of c-Jun homodimers (27, 34). In addition to homodimers contributing to c-Jun transformation, heterodimers of c-Jun/Fos and c-Jun/ATF2 activate complementing pathways involved in c-Jun transformation (81). Meq also has the ability to dimerize with ATF/CREB family members, however the possible contribution of ATF/CREB family members in MDV transformation has not been defined (42) but may be of consideration as ATF/CREB members have also been implicated in oncogenesis. Upregulation of CREB has been described in acute myeloid leukemia and clear cell carcinoma, and is necessary for transformation by human T-lymphotropic virus type 1 (HTLV-1) oncogene tax (76), and ATF-2 has been shown to promote serum independence in vitro, and contribute to growth and progression of mouse skin tumors (83).

The role of Meq heterodimers in Md5 pathogenesis was investigated by evaluating both viral replication, latency/reactivation and transformation properties of rMd5-MeqFos in vivo. In vitro, rMd5-MeqFos replicated similar to parental virus and in vivo, cytolytic infection was apparent in lymphoid organs and feather follicles as

assessed by expression of MDV-1 lytic protein pp38 (Fig. 21). As the FFE is the site of fully productive infection and source of horizontal transmission (13), we evaluated replication of rMd5-MeqFos in the FFE by IHC (Fig. 21) and transmission to contact chickens. Our results show that Meq heterodimerization did not interfere with either replication in the FFE or transmission to contact chickens. These results are not unexpected as Meq is not required for replication in lymphoid organs or feather follicle epithelium (50).

We previously reported the rMd5-MeqGCN appeared to be defective in reactivation and speculated, therefore, that Meq heterodimers may play a role in reactivation from latency (77). The switch from lytic to latent infection in MDV-1 generally occurs approximately 7 days PI, and virus from latently infected cells can be reactivated when co-cultured with fibroblasts in vitro (11). Therefore, reactivation assays were performed. Like rMd5-MeqGCN, rMd5-MeqFos appeared to have impaired reactivation. As none or few viral plaques were reactivated at days 14 and 21 PI (Table 3) even though the presence of viral infection was confirmed by IFA (Fig. 19). Therefore, these results do not support that Meq/c-Jun heterodimers are involved in reactivation.

However, since our assessment was not quantitative, we cannot dismiss the possibility of decreased viral loads. Intriguingly, our IFA results show viral pp38 expression was only detected in dividing cells from PBMC's. This observation is similar to latent infection with EBV in Burkitt's lymphoma where viral antigen expression was only detected in dividing memory B-cells thus supporting a true latent infection where

viral proteins are not expressed (28). Interestingly, at day 37 PI one chicken from rMd5-MeqFos and rMd5-MeqFos/GCN had viremias comparable to parental rMd5 (Table 3). To assess if any mutations in the MeqFos and MeqGCN genes incurred which may have contributed to higher reactivation numbers observed with these two recovered viruses, the MeqFos and MeqGCN genes were sequenced. However, no mutations were detected (data not shown). In addition, we evaluated if an association between MDV lesions and reactivation was present, but this was not clear as the rMd5-MeqFos infected chicken had preneoplastic nerve lesions and only the rMd5-MeqFos/GCN infected chicken had neoplastic nerve lesions. Therefore, we can only speculate that host factors may have contributed to reactivation in these two chickens. However, a worthwhile follow up study would be infection of chickens with the rMd5-MeqFos and rMd5-MeqFos/GCN recovered viruses with high reactivation and evaluate if they are more pathogenic, than the original inoculums.

In order to assess the role of the Meq-Jun partnership in MDV transformation in vivo, SPF chickens were infected with rMd5-MeqFos and evaluated for MDV specific lesions for an eight-week period. An additional virus, rMd5-MeqFos/GCN, which contains one copy of MeqFos and one copy of MeqGCN was also included in order to investigate if both Meq homodimers and heterodimers contribute to MDV transformation in vivo. Preneoplastic nerve lesions were detected in both rMd5-MeqFos and rMd5-MeqFos/GCN infected chickens, and 20% of chickens infected with rMd5-MeqFos/GCN contained neoplastic nerve lesions. However, visceral tumors were not observed in either groups, and paralysis was only observed in rMd5 and rMd5-

MeqFos/GCN infected chickens. These data support that both Meq homodimers and heterodimers contribute to T-cell transformation. However, rMd5-MeqFos/GCN clearly displayed a phenotype distinct from rMd5, as visceral tumors were not observed and MD mortality was significantly reduced. Therefore, Meq homodimers and Meq/Jun heterodimers are not sufficient to restore the Md5 phenotype. It is possible that interactions of Meq with bZIP proteins such as ATF/CREB contribute to MDV pathogenesis. Also interaction of Meq with other unknown cellular factors may have been abrogated by replacement of the parental leucine zipper with Fos and GCN leucine zippers. Further studies with point mutations in the leucine zipper region rather than gross changes may help in further delineating the function of Meq homo and heterodimers.

In summary, this work provides the first in vivo evidence that both Meq homodimers and Meq-Jun heterodimers contribute to MDV transformation. Further work investigating the role of Meq and ATF/CREB members will help in developing a better understanding of the transforming pathways in MDV pathogenesis.

CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS

Summary of research

The ratinale behind these studies was based on previous characterization of the bZIP properties of Meq and the association between Meq and c-Jun in MDV tumor cells. In vitro observations indicated differential roles for Meq homodimers and Meq/c-Jun heterodimers since they displayed differences in DNA binding based on EMSA analysis (42, 61). Further support for the Meq-Jun partnership in MDV transformation was demonstrated by upregulation of v-Jun genes by cells expressing Meq, and attenuated colony formation in cells expressing Meq when c-Jun is silenced (41). Therefore the hypothesis evaluated was that Meq heterodimers are involved in Meq induced transformation via a c-Jun activation pathway and that Meq homodimers are involved in regulation of viral latency and reactivation. This hypothesis was tested by the generation of recombinant Meq mutant viruses (rMd5-MeqGCN and rMd5-MeqFos) expressing chimeric *meq* genes (*MeqGCN* and *MeqFos*) that exclusively form homodimers or heterodimers, respectively. In addition, a third recombinant virus was recovered rMd5-MeqFos/GCN containing one copy of each *MeqGCN* and *MeqFos* in order to study the contribution of both Meq homodimers and heterodimers in MDV pathogenesis. The DNA binding, transactivation/repression and transforming properties of the chimeric *meq* genes were tested in vitro and cytolytic and transforming infection of the recombinant viruses evaluated in vivo.

Both MeqGCN and MeqFos retained DNA binding and transactivation/repressive functions in vitro. However, MeqFos required exogenous expression of c-Jun to transactivate the *meq* promoter while Meq and MeqGCN did not. This supports that MeqFos dimerization was restricted to c-Jun. In addition, while MeqGCN retained the ability to repress the pp38/14 promoter and 3X-Ori, repression was less than Meq. Unexpectedly, MeqFos was able to repress pp38/14 promoter and 3X-Ori, even though Meq/c-Jun dimers were reportedly unable to bind the Ori sequence (42). ChIP assays confirmed that MeqFos bound the 3X-Ori. However, since previous characterization of Meq binding sites utilized bacterial expressed proteins and EMSA analysis (42), cooperation of other unknown cellular factors may have contributed to the ability of MeqFos to bind the 3X-Ori. Furthermore, although other Meq binding sites within the pp38/pp14 promoter have not been defined by site-directed mutagenesis, AP-1 sites are reported to be located in the bidirectional promoter, providing a potential binding site for MeqFos and c-Jun. Therefore, transcriptional repression by Meq may involve displacement of activating factors at the bidirectional promoter by binding these elements.

Both MeqGCN and MeqFos exhibited reduced transforming potential in vitro as assessed by colony formation by soft agar. Interestingly, Meq and MeqFos expression was reduced in selected cells compared to MeqGCN, implicating that Meq and MeqFos expression may have unfavorable effects and only a subset of cells can support high Meq expression levels. More importantly, although rMd5-MeqGCN, rMd5-MeqFos and rMd5-MeqFos/GCN replicated in infected chickens, they are less pathogenic than

parental rMd5. Recombinant virus Md5-MeqGCN was non-oncogenic while rMd5-MeqFos induced preneoplastic nerve lesions in 50% of infected chickens. Although rMd5-MeqFos/GCN was more oncogenic with 20% and 60% of infected chickens containing neoplastic and preneoplastic nerve lesions, respectively, visceral tumors were not observed. Furthermore, chickens infected with rMd5 had to be euthanized or died on average four-five weeks PI. In contrast, all rMd5-MeqFos chickens survived the 8-week experiment except for two early nonspecific deaths. While only two chickens exhibited paralysis from the rMd5-MeqFos/GCN group and were euthanized 5 and 7 weeks PI while the remaining chickens survived the duration of the experiment.

This indicates that although Meq homodimers and heterodimers contribute to MDV transformation, the mutations in the leucine zipper resulted in viruses that are not as pathogenic as parental Md5. Revertant viruses from both rMd5-MeqGCN and rMd5-MeqFos had restored parental phenotypes, confirming that the phenotypic differences observed with rMd5-MeqGCN and rMd5-MeqFos were due to the leucine zipper mutations. Revertant viruses that were recovered by plaque purification contained one copy of the mutant gene either *meqGCN* or *meqFos* and the parental *meq* gene. Interestingly, the revertant virus isolated from an rMd5-MeqGCNR infected chicken (only one was tested) contained both parental *meq* genes, while virus isolated from three out of three chickens infected with rMd5-MeqFos retained one copy of each *meqFos* and *meq*, thus implying that the *meqGCN* retained no advantage for the replicating virus, rMd5-MeqGCNR, while the *meqFos* gene still retained some function and was retained in the viral genome of rMd5-MeqFosR.

Since Meq has been shown to dimerize with ATF/CREB family members (42) and since the leucine zipper region of MeqFos should be restricted to interactions with Jun family members, it is possible that Meq dimerization with ATF/CREB family is also involved in MDV pathogenesis. As it has been shown that Jun/ATF2 interactions are one component involved in c-Jun transformation along with Jun/Jun and Jun/Fos dimers (27, 34, 81). In addition, p53 and Rb are reported to interact with Meq and both of these putative binding sites are near the leucine zipper (36, 45). However, soft agar assays were performed in DF-1 cells that do not express p53 (36) therefore at least the loss of this interaction would not have influenced anchorage independent growth in DF-1 cells. However, the putative binding site for Rb is adjacent to the leucine zipper and although this motif was not changed, it remains possible that the interaction could have been disrupted.

Lastly, we evaluated reactivation from latency from chickens infected with rMd5, rMd5-MeqGCN, rMd5-MeqFos and rMd5-MeqFos/GCN. All viruses but rMd5 showed some deficiency in reactivation. However, these results cannot be fully confirmed without some assessment of viral load. But interestingly, pp38 expression was detected in PBMC's from infected chickens and was only detected in dividing cells. This observation is interesting because in latent infection none to only a subset of viral proteins are expressed. In some cases, as has been described in latent EBV infected memory B-cells only EBNA1 is detected in dividing cells. EBNA1 is responsible for ensuring that the viral genome is segregated to daughter cells, and is localized in the nucleus (28). The pp38 expression was observed primarily in the cytoplasm of PBMC's

so it is unclear as to it's function in dividing cells but detection of it's expression supports that these cells were latently infected and not reactivating virus.

Future studies

Even though the c-Fos leucine zipper is well characterized allowing dimerization with the Jun family of bZIP proteins only, further evaluation of the dimerization properties of MeqFos should be pursued. Luciferase assays indicate that MeqFos heterodimerizes with c-Jun, but future experiments designed to test if MeqFos exclusively heterodimerizes should be performed. EMSA analysis of in vitro transcription/translated products or bacterial expressed proteins has been utilized to study the DNA binding and dimer formation for other bZIP proteins (15, 26, 63). This same approach would be useful for testing the dimerization properties of MeqFos because it would test if MeqFos/MeqFos dimers form and bind the Ori sequence independently of other cellular factors, aiding in the interpretation the described ChIP assay results.

This work demonstrated that both MeqGCN and MeqFos have reduced ability to transform and suggests that other cellular factors, ATF/CREB, p53, Rb, or other unknown cellular factors are necessary for full transformation by Meq. As a first step, the expression of CREB/ATF members in MDV transformed cells could be evaluated and coimmunoprecipitation assays performed to test possible dimerization between Meq and CREB/ATF members. Similar approaches with p53 and Rb could be performed. If an association is found, these experiments could be followed by using cells expressing

Meq, in proliferation assays, soft agar assays, assays to assess apoptosis, and siRNA targeting the proposed cooperating protein.

Global analysis of Meq binding sites within the MDV genome and chicken genome would be a great tool for identifying Meq target genes. Chicken genome ChIP arrays are now available and an MDV tailored ChIP array could be produced. This would be a useful tool to detect Meq binding sites in MDV transformed cells, and could be applied to MDV transformed cells treated with sodium butyrate to induce reactivation. Comparisons of the Meq binding sites in latent/transforming infection and lytic infection would provide a global assessment of Meq binding sites.

Further work characterizing the transcriptional region of Meq would expand current knowledge of MDV transformation. Another possibility for the decrease in pathogenicity observed in rMd5-Meq/GCN and rMd5-Meq/Fos viruses is that another contributing viral factor may have been altered due to the leucine zipper mutations. Recently, a novel Meq splice variant has been identified in the Meq region (55). For this particular splice variant, the splice donor site is located the leucine zipper region and the splice acceptor site is at the C-terminus of Meq (55). Although Meq expression predominates over this splice variant in MDV transformed cells it may also have a contributing role in MDV transformation or may have a role in reactivation, as the leucine zipper mutant viruses described in this work displayed impaired reactivation.

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