Virus and host genomic, molecular, and cellular interactions during Marek's disease pathogenesis and oncogenesis

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ABSTRACT Marek's Disease Virus (MDV) is a chicken alphaherpesvirus that causes paralysis, chronic wasting, blindness, and fatal lymphoma development in infected, susceptible host birds. This disease and its protective vaccines are highly relevant research targets, given their enormous impact within the poultry industry. Further, Marek's disease (MD) serves as a valuable model for the investigation of oncogenic viruses and herpesvirus patterns of viral latency and persistence—as pertinent to human health as to poultry health. The objectives of this article are to review MDV interactions with its host from a variety of genomic, molecular, and cellular perspectives. In particular, we focus on cytogenetic studies, which precisely assess the physical status of the MDV genome in the context of the chicken host genome. Combined, the cytogenetic and genomic research indicates that MDV-host genome interactions, specifically integration of the virus into the host telomeres, is a key feature of the virus life cycle, contributing to the viral achievement of latency, transformation, and reactivation of lytic replication. We present a model that outlines the variety of virus-host interactions, at the multiple levels, and with regard to the disease states.

Key words: chicken, genome integration, Marek's disease, fluorescence in situ hybridization, telomere

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INTRODUCTION

Marek's Disease – Characteristics and History

Marek's disease (**MD**) is a complex, immunosuppressive disease characterized by paralysis, chronic wasting, lymphoma development in the viscera and musculature, and blindness in chickens (Davison and Nair, 2004; Jarosinski et al., 2006) infected by Marek's disease virus (**MDV**) (Churchill and Biggs, 1967). MD symptoms vary in severity based on virus strain as well as bird genotype and vaccination status with death occurring in susceptible, non-immunized chickens. In 1907, Jozsef Marek, a Hungarian veterinarian, first reported the common symptoms of "classic" MD, namely polyneuritis (Marek, 1907). Polyneuritis was later coupled with other symptoms as part of the same disease and named after the veterinarian in 1960 (Gimeno et al., 1999). MDV was classified as an alphaherpesvirus based on repeat structures identified through electron-microscopy (Cebrian et al., 1982) and confirmed by virus sequencing studies (Lee et al., 2000a; Tulman et al., 2000).

When first identified as a virus-induced disease, the characteristic feature was peripheral nerve inflammation. At present, more virulent strains of MDV induce a particularly severe range of symptoms, including strong immunosuppression, tumors, and neurological disorders (Gimeno et al., 1999). Increased MDV virulency induces dramatic thymic and bursal atrophy (Calnek et al., 1998) and macrophage death due to viral replication (Barrow et al., 2003). The disease is controlled with MD vaccines, which are either mono- or bivalent, as well as breeding for resistance and improved poultry management practices. Specific mechanisms of MDV immunization through use of vaccines are not completely clear. The innate immune response is induced, but the cytotoxic response is minimal as compared to response to other viruses (Markowski-Grimsrud and Schat, 2002). Ever-increasing virulence of MDV has led to growing concerns over potential breaks in vaccinal protection due to the fact that evolution towards higher virulency in response to vaccine usage has occurred in the past. This concern is elevated by the issue of reduced genetic diversity across much of the poultry industry (Nair, 2005; Muir et al., 2008). Improved

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Table 1. A review of Marek's disease virus serotypes and their features.

Serotype	Strain	Status	Genome size (kb)[g]	Annotated ORFs*	vTR gene[h,i,j]	vIl8 gene	pp38 gene	Meq oncogene	Telomeric repeats[k]
1	GA[a]	Virulent	174	103	+	+	+	+	+
1	Md5[b]	Very virulent	177.8	103	+	+	+	+	+
1	Md11[c,d]	Very virulent	178	99	+	+	+	+	+
1	Rispens/CV1988[d]	Attenuated	178.3	102	+	+/NF	+/NF	+/NF	+
2	SB-1[e]	Non oncogenic	165.9	75	−	+/NF	+/NF	−	+
3	HVT[f]	Non oncogenic	160	75	−	−	−	−	+

Open reading frames (**ORFs**), viral telomerase RNA (**vTR**), viral interleukin-8 (**vIL-8**), phosphoprotein 38 **(pp38),** + (plus) = present; − (minus) = absent; +/NF = present but non-functional.

[a]Lee et al., 2000a,b; [b]Tulman et al., 2000; [c]Niikura et al., 2006; [d]Spatz et al., 2007; [e]Spatz and Schat, 2011; [f]Kingham et al., 2001; [g]Wilson and Coussens, 1991; [h]Fragnet et al., 2003; [i]Fragnet et al., 2005; [j]Trapp et al., 2006; [k]Kishi et al., 1988.

vaccination strategies and understanding of the virus and the vaccines remain important areas of study to prevent or reduce outbreaks.

Marek's Disease Virus - Genome Structure

The human herpesvirus 6 (**HHV-6**) and Epstein-Barr virus (**EBV**) share a number of features with MDV (Delecluse et al., 1993b; Gomples and Macaulay, 1995; Arbuckle et al., 2010), including an association with lymphoproliferative cancers (Kaschka-Dierich et al., 1976; Daibata et al., 1998), emphasizing that the chicken is an important medical model organism for the study of human virus-induced diseases and cancers. This is, of course, in addition to the importance of MDV research, given its impact on chicken populations worldwide. MDV, also known as gallid herpesvirus 2, is a member of the genus *Mardivirus*. Three serotypes have been identified: 1) oncogenic serotype 1 strains (GA, Md11, Md5), 2) non-oncogenic serotype 2 strain (SB-1), and 3) serotype 3 herpesvirus of turkeys (**HVT**). MDV serotypes are identified by monoclonal antibodies produced against them by the host immune system after infection (Lee et al., 1983). Serotypes 2 and 3 are antigenically related to serotype 1 strains, but do not induce the disease phenotype or lymphomas in chicken (Kato and Hirai, 1985; Lee et al., 2000b). The first sequence-based assessment of the serotypes found that MDV serotype 1 and HVT had collinear genomes and similar structures to that of herpes simplex virus 1 (**HSV-1**) and varicella zoster virus (**VZV**), which indicated an *alpha*herpesvirus classification (Buckmaster et al., 1988). Several full-genome sequence and comparative genomic studies have been conducted since this initial sequencing work (Lee et al., 2000a; Tulman et al., 2000; Izumiya et al., 2001; Kingham et al., 2001; Spatz et al., 2007), providing a base for further comparative genomic studies in MDV research as well as tools for cytogenetic analyses of the virus (Table 1). Viral cloning, or the introduction of overlapping viral genomic fragments into a cosmid or bacterial artificial chromosome (**BAC**) vector, has proven to be an invaluable research tool for investigations of the MDV genome sequence as well as studies of MDV pathogenesis and tumorigenesis (Zelnik, 2003). MDV serotype 1 was the first viral serotype cloned and mutated to investigate a viral gene function; specifically, the gB genes were deleted and the impact on viral function was examined in vitro (Schumacher et al., 2000).

The MDV genome is 175 to 180 kb, depending on the strain, with an episomal or circular structure when independent of the host genome. The genome consists of several regions, namely the unique long ($\mathbf{U_L}$) and short ($\mathbf{U_S}$) regions flanked by terminal repeats long ($\mathbf{TR_L}$) and short ($\mathbf{TR_S}$) and internal repeats long ($\mathbf{IR_L}$) and short ($\mathbf{IR_S}$) (Figure 1). Genome structure and gene content of each region are similar among all MDV serotypes, but there exist key differences (Table 1). Oncogenic serotype 1 is defined by the presence of the *Meq* oncogene and other unique genes including pp38, vIL8, and vTR in repeat regions, particularly TR_L (Lee et al., 2000b; Nair, 2005). These genes also largely contribute to the unique biological features of MDV, such as targeting and transformation of activated T lymphocytes. The viral genes that are unique to each particular avian herpesvirus are located in the repeat regions of the genome as revealed by sequence comparisons (Zelnik, 2003). Sequence analysis of the serotype 1 MDV termini identified 2 different cleavage sites involved in the mature viral packaging process following genome replication. The "classical" cleavage site of MDV is upstream of the *DR1* motif site and results in a genome termini with telomeric repeats $(TTAGGG)_n$ (Volkening and Spatz, 2013).

MDV Infection Stages

Figure 2 provides an overview model of MDV infection, pathogenesis, and transformation, incorporating molecular, cellular (including cytogenetic), and genomics knowledge to-date. MDV infection begins with inhalation of infectious virions from shed feather dander. Within 24 h after contact in the lungs, the virus is present in the spleen, thymus, and bursa of the host bird (Schat et al., 1984). The viral transition from lung to lymphoid organs is attributable to infected macrophages (Calnek, 2001; Barrow et al., 2003), which transfer the virus to B lymphocytes, or direct

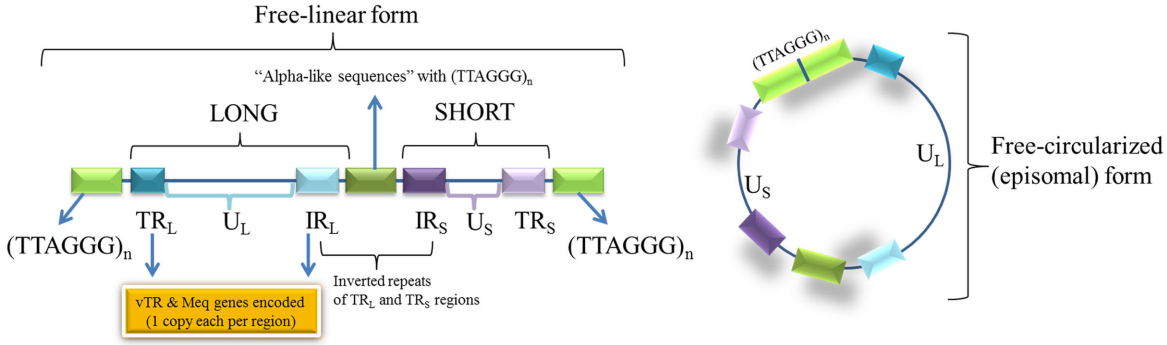

Figure 1. Schematic of 'free' Marek's disease virus: linear and episomal genomes. Diagrams of free (non-integrated) viral genome in the linear and circularized (episomal) forms are depicted. The MDV genome consists of double-stranded DNA (all serotypes) and is approximately 180kb (serotype 1). The MDV genome structure is divided into long and short sections, similar to other alpha-herpesviruses. Both the long and short (denoted by L and S subscripts, respectively) sections consist of: unique (U) sequence regions, blocks of sequences that are corresponding inverted repeats of one another (terminal (TR) and internal (IR)), telomeric DNA sequence ($(TTAGGG)_n$) blocks, and alpha-like sequence which includes telomeric DNA sequence (Kishi et al., 1988; Lee et al., 2000a; Tulman et al., 2000). The colored blocks indicate the locations of the $(TTAGGG)_n$ telomeric sequence repeats (green) and the blocks of inverted sequences within the long (dark blue, TR_L and light blue, IR_L) and short (dark purple, IR_S and light purple, TR_S) sections. The straight lines within the long and short sections represent the unique sequence regions (U_L and U_S). The $(TTAGGG)_n$ sequences, viral telomerase RNA (vTR) gene and *Meq* gene locations are indicated by arrows (see orange block notation). Notably, upon circularization of the genome, the two blocks of telomeric sequence repeats are brought together. Table 1 describes key differences and similarities between the MDV serotypes with regard to presence/absence and/or functionality of viral telomeric DNA sequence and the vTR and *Meq* genes.

infection of B lymphocytes (Baaten et al., 2009). All movement of MDV within an infected host bird is cell-associated, i.e., cell-free, unenveloped virus is not infective (Nazerian et al., 1968). Virulent MDV undergoes 4 overlapping infection stages, having critical consequences that contribute to viral persistence and pathogenesis in the host: early cytolytic, latent, late cytolytic, and transformation (Biggs, 1968; Adldinger and Calnek, 1973; Osterrieder et al., 2006). In the cytolytic stage, the virus replicates in macrophages and B and T lymphocytes. Following the lytic phase of infection, latent (non-replicating) infection is established around 7 d post-infection (**dpi**) (Arumugaswami et al., 2009; Baigent and Davison, 2004; Trapp et al., 2006). Latently-infected T lymphocytes are capable of being transformed, typically between 14 and 21 dpi, resulting in lymphomas in the visceral organs (Witter, 1997; Calnek, 2001; Burgess and Davidson, 2002; Nair, 2005; Osterrieder et al., 2006; Trapp et al., 2006).

MDV Transmission

Horizontal transmission is accomplished through shedding of infectious MDV virions in feather dander (Beasley et al., 1970). Viral shedding by an infected host bird occurs after latent MDV reaches and infects the feather follicle epithelium (**FFE**) via peripheral lymphocytes circulating in the blood (Johnson et al., 1975). The reactivated MDV in the FFE produces mature cell-free virions (Nazerian and Witter, 1970) that can persist in the external environment for wk to mo (Calnek, 2001; Osterrieder et al., 2006). Enveloped herpesvirus particles were first detected in the FFE in an electron microscopy study of JM-strain infected birds (Calnek et al., 1970). Infectious viral particles are first detected in shed dander around 8 dpi (Jarosinski, 2012) and viral load in dander increases up to 28 dpi. In vivo studies have demonstrated data that suggest that functional copies of the viral genes U_S2, U_L13, and glycoprotein C are all required for horizontal MDV transmission, although their exact functions are yet to be identified (Jarosinski et al., 2007).

MD Vaccines – Impact and Mechanisms

The 1960s were marked by a major increase in poultry loss due to MD (Witter, 1997). Remarkably, the discovery of the oncogenic herpesvirus etiology was followed by development of an effective vaccine, specifically live HVT (Churchill and Biggs, 1968; Churchill et al., 1969; Okazaki et al., 1970), in the early 1970s. Field strain virulency increased through the late 1970s resulting in more outbreaks until bivalent vaccines were employed (SB-1 + HVT) in the 1980s and later the attenuated serotype 1 Rispens/ CV1988 vaccine (Rispens et al., 1972; Petherbridge et al., 2003), all of which are cell-associated viruses. The MD vaccines were the first vaccines capable of preventing virus-induced cancer (Calnek, 1986). The attenuated Rispens vaccine was developed through serial in vitro passaging of serotype 1 MDV, a process known to reduce the ability of MDV strains to grow in vivo as well as to amplify tandem direct repeats in the IR regions of the viral genome (Maotani et al., 1986). The Rispens vaccine is the most effective against very virulent (**vv**) and very virulent plus (**vv+**) MDV to date, and is safe for inoculation in susceptible birds. Inoculation of birds with HVT and SB-1 also prevents MD, but is generally less effective against vv and vv+ strains. MD vaccines are

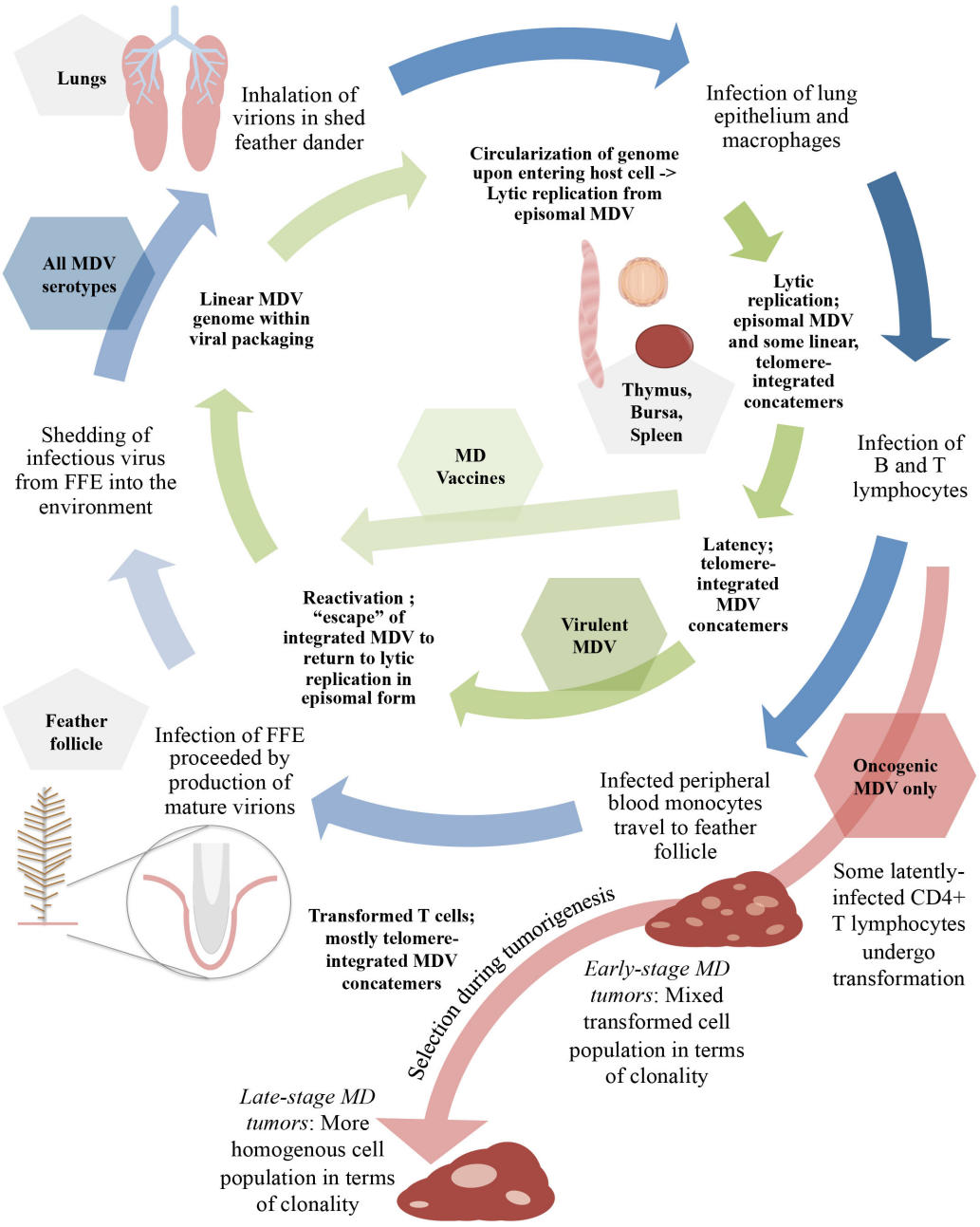

Figure 2. A model of Marek's disease virus and chicken host genomic, molecular, cellular, and tissue interactions during early infection, pathogenesis, and transformation. Three highly simplified, inter-connected routes are depicted by arrows: **green**, MDV-host genome status; **blue**, virus movement internally (host cells and tissues) and externally (dander in the environment); **light red**, transformation and tumorigenesis. Along these routes, the light gray pentagons with text and diagrams indicate the organs involved in the disease and the hexagons indicate MDV serotype pathways (**blue**, all MDV serotypes; **green**, virulent MDV vs. MD vaccine; **light red**, oncogenic MDV). The overlapping disease stages (cytolytic, latent, reactivated, transformed) and virus-host genome status (see main text for cytogenetic viral phenotype explanations) are indicated by the **bold font** along the routes: cytolytic, circularized-episomal MDV (cytogenetic chromosome-associated phenotype); cytolytic, episomal and linear telomere-integrated MDV (cytogenetic chromosome-associated and telomere-integrated phenotype); latency, telomere-integrated MDV (cytogenetic telomere-integrated only phenotype); reactivation or return to cytolytic phase; transformed cells, (mostly cytogenetic telomere-integrated only phenotype). In susceptible research chickens, tumors can develop as early as 21 d post-infection. See main text for outline of the overlapping timing of early cytolytic, latency, late cytolytic, and transformation stages. FFE = feather follicle epithelium.

typically administered shortly post-hatch or in ovo to allow chicks to become immunized (requires 7 to 14 d for full protection) before heavy exposure to the virus in adult chicken flocks. A major flaw of the current vaccination strategies is that they do not produce sterile immunity, i.e., virulent strain infection, replication, and transmission still occur in vaccinated birds (Nair, 2005).

The precise mechanism(s) of vaccine-related immunity is not known, but contributing factors may be reduced growth rate of MDV in the host (Purchase and Okazaki, 1971) and prevention of virulent MDV

immunosuppressive effects on the host (Islam et al., 2002). Infection of T and B lymphocytes by the vaccine strain(s) potentially interferes with the infection process of virulent MDV and contributes to the protective effect (Tischer et al., 2002); however, supplementary mechanisms are likely involved (Witter, 1984; Osterrieder et al., 2006). Lee and colleagues (1999) conducted in vivo studies to determine if MD vaccination impacted the distribution of virulent MDV among T lymphocytes, through PCR (viral load assessment) and plaque assays (infectivity). Serotype 1 virulent MDV was primarily found in CD4+ T lymphocytes, and lower titers of virulent MDV were detected in vaccinated birds up to one mo after infection, which encompasses both the lytic and latent stages of the viral life cycle. The MDV serotype 1 (Rispens) and 2 (SB-1) vaccines greatly differed in their overall titers and dissemination between CD4+ and CD8+ T lymphocytes. Furthermore, vaccinated and challenged birds showed higher levels of apoptosis among peripheral blood mononuclear cells as compared to challenged-only birds (Lee et al., 1999), suggesting that a component of the vaccine's protective mechanism is reduction in the number of CD4+ T lymphocytes.

It is currently understood that the Rispens/ CVI988 vaccine develops into mature virions at the host feather follicle and sheds into the environment in large quantities, in a similar manner to virulent strains, and transmits among chickens. Rispens was detected through qPCR in peripheral lymphocytes after vaccination (peak at 7 d post vaccination [**dpv**]) and in feather tips as early as 7 dpv (peak at 14 dpv). A steady decline of Rispens genome copy number in these tissues occurred after 14 dpv while the presence of the virus in shed dander steadily increased and peaked at 21 dpv (Islam et al., 2013). More recent studies have differentiated Rispens from oncogenic serotype 1 MDV by mismatch amplification mutation assay and demonstrated that insufficiently immunized (low dose vaccination) chickens have higher loads of oncogenic MDV in the blood and feather pulp as early as 21 dpi as compared to properly vaccinated birds (Gimeno et al., 2011, 2014). The HVT and SB-1 vaccines are also actively shed from host birds, starting around 7 dpv for HVT-vaccinated birds and 12 dpv for SB-1-vaccinated birds. The SB-1 and HVT strains continued to be shed with the feather dander for several months after vaccination and significantly reduced virulent MDV shedding in dander between 14 and 28 dpi (Islam and Walkden-Brown, 2007). Thus, another component of the vaccinal benefit may be interference with virulent MDV infection of the host FFE and resultant decreased viral transmission. Interestingly, a non-producer T-lymphoblastoid cell line, developed from the spleen of an HVT-vaccinated and MDV-infected healthy chicken, contained latent HVT and MDV genomes, and neither virus strain was reactivated (returned to lytic stage) by altering culture conditions (Hirai et al., 1981). The physical state of the HVT genome in the lymphoblastoid cell line was not determined. Two strains of HVT were found to be in the latent stage of infection, as indicated by low viral antigen expression, in the spleen of vaccinated chickens as well (Fabricant et al., 1982).

Integration into the Host Genome

Early studies of host telomeric integration by MDV Both linear and episomal forms of MDV are found in infected host cells. The episome is the host genome-independent form of the virus, and the linear form is typically integrated within the host genome (Delecluse and Hammerschmidt, 1993a). Latently infected (non-producer) cell lines exhibit very little to no extra-chromosomal circular or linear viral DNA and, rather, consist of integrated MDV (Kaschka-Dierich et al., 1979; Delecluse and Hammerschmidt, 1993a). However, free linear viral genome, or processed yet un-circularized MDV genomic DNA, is detected at low levels in some transformed low-producer cell lines (Kaschka-Dierich et al., 1979). Delecluse and Hammerschmidt (1993a) were the first to report, by cytogenetic methods, that MDV integrated into host chromosomal DNA in transformed cell lines developed from infected birds. Interestingly, the integration sites were mostly found to be distally located at the telomeres of the macrochromosomes (Delecluse and Hammerschmidt, 1993a). Integration into host DNA was supported by detection of doublet MDV fluorescence in situ hybridization (**FISH**) signals from sister chromatids, indicating viral DNA was replicated along with the host genome. Integration loci mapping was limited to the largest macrochromosomes due to a lack of identifying features for the microchromosomes. Integration profiles were recurrent from cell to cell within a line, yet differed from the profiles found in other cell lines.

Host Genome Acquisition The MDV genome contains several homologous host genes and telomeric repeats, which were presumably acquired from the chicken genome during the evolution of the gallid herpesvirus (Tulman et al., 2000; Petherbridge et al., 2004; Niikura et al., 2006). These findings from virus sequencing studies provided further evidence of physical virus-host genomic interactions. Niikura and colleagues (2006) found that a serotype 1 MDV strain, specifically an infectious Md11 BAC clone propagated on duck embryo fibroblasts, contained a region from the duck genome within the TR_S region of its viral genome. This sequence was likely acquired during viral lytic replication, perhaps by recombination, as a latent infection was not established in the duck fibroblasts. Further lytic replication in vitro was not hindered by the acquired duck genome sequence, as the duck substitution MDV strain persisted through multiple passages in chicken embryo fibroblasts (Niikura et al., 2006). Presumably, most acquired chicken sequences that have persisted in the current MDV field strains confer an

advantage (selection favoring the modified MDV) in viral infection and/or transmission.

AVIAN CELLULAR AND MOLECULAR IMMUNOLOGY

Immune Cells Targeted by MDV and the Host Immune Response

MDV replication occurs in macrophages as well as B and T lymphocytes (Calnek et al., 1984). However, the virus achieves latency and transformation primarily in CD4+ T cells (Lee et al., 1999; Robinson et al., 2010, 2014, and references therein), although there is some evidence for latent virus in B, CD4-CD8- or CD8+ T lymphocytes. Viral integration into the host genome is detectable in cells early post infection from the bursa, thymus, and spleen—organs with large B and/or T lymphocyte populations (Robinson et al., 2014). The adaptive response is highly important to lytic virus clearing post infection, due to the highly cell-associated nature of MDV. However, the innate immune response of the host to challenge has been found to play a critical role in studies of inbred chicken lines with differing MD resistance (Kaiser, 2010).

All MDV serotypes are known to reduce surface expression of the Major Histocompability Complex (**MHC**) B class I glycoproteins during viral lytic replication, which is thought to aid in immune response evasion (Hunt et al., 2001). This reduction of cell surface MHC molecules does not persist into latency, perhaps because minimal viral gene expression and integration into host telomeres are sufficient for evasion of host responses.

Resistance to Marek's Disease - Bird Genotypes and Host Gene Involvement

MDV is highly effective at inducing fatal tumor development in susceptible birds within a few wks after infection, unless the host is protected by in ovo or at-hatch vaccination and/or is a highly resistant genotype (Calnek, 2001). Selection methods for MD resistance in commercial chickens that avoid inbreeding effects include mass selection after exposing a flock to a high dose of the virus (infected environment or inoculation) and family selection (Bacon et al., 2001). Family selection with inbreeding, which involves sibling crosses and offspring-parent crosses after testing for disease resistance, is the approach often used to develop MD resistance/susceptibility research lines (Bacon et al., 2001). The inbreeding results in a near identical genome sequence within each line, which is a useful feature for genetic investigations. A few prime examples of lines resulting from this breeding method are the MD resistant line 6_3 and MD tumor susceptible line 7_2 as well as line 15I developed at the USDA Avian Disease and Oncology Laboratory (**ADOL**) (Waters and Fontes, 1960; Crittenden et al., 1972).

MHC congenic lines, consisting of birds with 99.9% identical genomes and different MHC-B haplotypes, are often used to understand the role of the different haplotypes on MD pathogenesis. For example, UC Davis (**UCD**) congenic lines share the UCD 003 inbred genetic background but each line has a unique B haplotype (Abplanalp et al., 1992; Bacon et al. 2001). Congenic lines are crossed to identify loci with a role in certain traits related to MD resistance. Recombinant congenic lines from crosses of ADOL lines 6_3 and 7_2 each contain a unique segment from line 7_2. These congenics have been tested for MD tumor incidence to identify non-MHC genes with an impact on viral oncogenesis (Yonash et al., 1999).

Genetic studies of MD resistance employing inbred research lines uncovered a few major genes involved in the resistance. It is well known that MHC genes contribute, as explained by the impact of MHC genotype on cell-mediated and humoral immune response and the specificity of immune response to MDV and MD transformed cells (Bacon et al., 2001). The MHC gene family encodes immunity-related cell surface molecules that are primarily involved in antigen presentation and regulation of T lymphocytes. Importantly, the chicken MHC-B and RfpY haplotypes are linked on GGA 16, but segregate independently (Miller et al., 1996), as they are separated by a large GC-rich region of unknown sequence (Delany et al., 2009). Numerous studies have established the influence of B haplotype on MD resistance in chicken (Briles and Olsen, 1971; Briles et al., 1977; Schat et al., 1994; Miller et al., 1996). However, non-MHC genes are influential as shown by studies of lines 6_3 and 7_2, which have the same MHC B^*2 haplotype (Hunt and Fulton, 1998). There are mixed results on the influence of the MHC RfpY haplotype on MD resistance (Bacon et al. 1996; Wakenell et al., 1996; Vallejo et al., 1998).

Other non-MHC genes are also thought to contribute to MD resistance through cellular interactions, variations in MDV-targeted cells, innate immunity, or cytokine regulation. Notably, MD-resistant and -susceptible lines have similar prevalence of CD4+ T lymphocytes during the lytic and latent stage of MDV infection, but during the tumor development stage CD4+ T lymphocytes remain predominant in susceptible bird lesions and CD8+ T cells become predominant in resistant bird lesions. These data indicate that gene(s) involved in CD8 T cell response to tumors may aid in MD suppression (Burgess et al., 2001). Studies of Cornell MD susceptible (S) and resistant (K) bird lines also indicated the involvement of the ALVE chicken endogenous viral genes on resistance (Aggrey et al., 1998).

It was established as early as the 1970s that MD-resistant chickens with particular MHC haplotypes were generally better immunized by the MD vaccines (Spencer et al., 1974). Significant genotype by vaccine interactions has been demonstrated when

commercial (sufficiently high) doses of vaccine were administered (Bacon and Witter, 1994). More specifically, the vaccine's protective efficiency was demonstrated to be higher in resistant line 6_3 birds as compared to line 7_2 genotype birds (Chang et al., 2014).

Advances in chicken genomics and genomic technology (sequencing, association studies, etc.) have allowed researchers to comb the entire genome for genes and pathways influencing MD resistance. QTL mapping (Bumstead et al., 1997; Vallejo et al., 1998; Yonash et al., 1999) and subsequent comparative genomics for loci identification (Burt et al., 1999) as well as DNA microarrays (Heidari et al., 2010; Hu et al., 2015) have all been employed to identify loci relevant to MD. Vaccination and selective breeding approaches for chicken immunization and disease resistance are beginning to exploit data on avian immune responses, the MHC, and cytokines as well as genomic data to optimize efficiency (i.e., innate immunity enhancement and modification) and scope (i.e., optimize number of strains and/or pathogens protected against) (Kaiser, 2010). However, further research and testing of these genomic and immunology data-enhanced approaches will be necessary to protect chickens against more virulent MDV strains and other pathogens (Nair, 2005; Gimeno, 2008).

MDV LIFE CYCLE – MOLECULAR AND CELLULAR DYNAMICS

Introduction to the Herpesvirus Life Cycle

Herpesviruses are double-stranded DNA viruses and mature virions consisting of linear genomes contained within an icosahedral capsid. Upon infection of target host cells, the linear genome becomes a covalently closed circular episome and proceeds to a lytic replication stage, consisting of high gene expression and production of viral progeny, or to latency, in which gene expression is minimal and virus is not actively produced.

Cytolytic Replication – Mechanisms, Gene Expression, and Genome Configuration

For most herpesviruses, genome circularization occurs within 30 min of infecting a host cell and is followed by lytic replication (Boehmer and Nimonkar, 2003; Morissette and Flamand, 2010). Rolling circle replication produces tandem head-tail viral genome concatemers. The initiation of this process relies on the presence of signaling sequences or origins of replication. The generated concatemers in the nuclei are cleaved and packaged into pre-formed capsids (Boehmer and Nimonkar, 2003). The peak of the lytic replication stage after MDV infection occurs around 3 to 7 dpi (Shek et al., 1983; Osterrieder et al., 2006, and references therein). Important cytolytic-expressed viral genes include the interleukin 8 homolog (**vIL8**) gene, which functions as a chemoattractant of chicken peripheral mononuclear cells in chicken (Parcells et al., 2001), and the viral lipase homolog (**vLIP**) gene, which contributes to efficient viral replication in infected cells (Kamil et al., 2005). Infection with mutant MDV strains with deletions of vIL8 leads to decreased lytic replication and oncogenicity, although some MD tumors developed. The vIL8-deleted MDV is still able to transition into latency, indicating that it contributes to the lytic stage but is not required for latency or transformation (Parcells et al., 2001).

Hunt and colleagues (2001) detected reduced cell surface MHC class I (**BF**) glycoprotein expression in 60% of infected UA04 cells undergoing MDV lytic replication as well as all UA04 and MSB1 tumor cells with reactivated virus. However, intracellular BF protein levels were not significantly altered in the MDV-infected cell lines, thus interference with BF transport to the cell membrane was predicted to be the mechanism behind reduced surface expression (Jugovic et al., 1998). This effect was not observed in latently infected chicken cells, perhaps because reduced overall gene expression and viral integration into the host DNA during latency (along with other mechanisms) are sufficient to evade a host immune response (Hunt et al., 2001).

Latency – Timing and Gene Expression

A common feature of herpesviruses is latency, which is defined by viral infection without replication or production of infectious virions (Stevens, 1989). MDV-challenge in vivo studies indicate the presence of latent virus in host cells around 7 dpi, primarily in CD4+ T lymphocytes (Lee et al., 1999). MDV latent infection can continue indefinitely in T lymphocytes throughout the host while cytolytic replication and virion production occur concurrently in the FFE. Most MDV latency genes are located in the long and short repeat regions of the genome (Sugaya et al., 1990), which encodes *Meq*, vTR, the LAT family, and numerous MDV-1 microRNAs (**miRNAs**) (Burnside et al., 2006). Latency-associated transcripts (**LATs**) are characteristically expressed by latent MDV; however, there exist no clear data on the level of LAT expression or number of transcripts (Osteriedder et al., 2006, and references therein). The LAT family is also known to be highly expressed in MD lymphomas (Ross et al., 1997). Both latently infected and transformed cells possess histone modifications (repressive) at the origin of lytic replication and (activating) at the *Meq* oncogene and miRNA cluster (Brown et al., 2012). For many herpesviruses, including MDV, reactivation of latent virus can lead to a return of disease symptoms.

Cellular Transformation by MDV

The precise relationships of the latency and tumorigenesis stages are currently unknown (Nair, 2013),

and an important question persists: must MDV latency precede transformation? There is ample scientific evidence of temporal and behavioral overlap between these 2 stages, and they also have the shared feature of telomere-integrated MDV. Additionally, it has been suggested that only some latently-infected CD4+ T lymphocytes undergo transformation and even fewer will generate the predominant transformed cell lineages found in a late-stage MD lymphoma (Calnek, 2001; Robinson et al., 2010, 2014). However, mystery remains regarding the switch from latent infection to T-lymphocyte transformation in the visceral organs of susceptible, unvaccinated host birds (Nair, 2005).

As discussed above, research suggests MDV integration into the host genome is involved in tumorigenesis. In other systems, hepatitis B and papilloma virus 18 also integrate into the genomic DNA of host cells before transformation is detected (Popescu et al., 1990). Host telomere integrated MDV is a key feature of MD lymphomas (Robinson et al., 2010, 2014). Cytogenetic studies of early- and late-stage MD tumors found that 92.8% of the observed dividing cells contained only the telomere-integrated form of MDV, indicating a significant relationship between viral integration and MD-induced tumors/tumorigenesis (Robinson et al., 2014). Furthermore, birds challenged with mutant- or deleted-telomeric repeat MDV strains, which lack the ability to integrate into the host telomeres, had significantly decreased lymphoma development. This result provided further evidence of a link between MDV integration into host telomeres and achievement of cellular transformation (Kaufer et al., 2011a).

MDV Genes Involved in Tumorigenesis Several viral genes involved in oncogenesis have been identified within the MDV genome (Table 1). MDV miRNAs, mapping to genes such as *Meq* (Burnside et al., 2006), are known to be expressed in both MDV-induced tumors and lymphoblastoid cell lines. Integration of MDV into the host genome also appears to play a role in the viral latency and tumorigenesis stages, rather than being an incidental occurrence (Desfarges and Ciuffi, 2012; Robinson et al., 2014).

The *Meq* oncogene is one of the most thoroughly studied MDV genes and is known to play a key role in MDV-induced T cell lymphomagenesis, in combination with other transcription factors, through host and viral gene expression modifications (Brown et al., 2009). *Meq* encodes an abasic leucine zipper transcriptional factor with favored dimerization with the *cJun* oncoprotein (*cJun-Meq*) and itself (*Meq-Meq*) (Qian et al., 1996). The dimerized form of *Meq* impacts expression of cellular anti-apoptotic factors and viral transformation-associated genes and binds to cell cycle control factors and MERE promoter sites to up-regulate its own expression (Liu et al., 1999; Osterrieder et al., 2006). *Meq* is expressed in MD tumors and lytically infected T lymphocytes (Jones et al., 1992). When birds are infected with *Meq*-deleted (**Δ*Meq***) MDV (lacking both copies), tumors do not develop (Silva et al., 2010) and viral telomeric integration is not observed in the primary immune tissues (Robinson et al., 2014). A functional copy of the *Meq* gene is absent in MDV serotypes 2 and 3 and, thus, these strains do not express the oncoprotein upon infection, replication, or latency. When comparing the *Meq* gene of the attenuated Rispens/ CV1988 MDV vs. serotype 1 oncogenic MDV, a 178bp sequence (domain duplication) was found to be inserted into the CVI988 genome, which led to a frameshift mutation of a transactivation domain (Lee et al., 2000b).

Another MDV gene that contributes to oncogenesis is the vIL8 homolog, encoded in the U_L region of the genome. vIL8 functions as a chemoattractant of chicken peripheral mononuclear cells, which includes lymphocytes, monocytes, and macrophages. In vitro studies indicated that vIL8 induces chemotaxis of B lymphocytes, as well as CD4+ and CD25+ T lymphocytes, all of which are targets of MDV infection (Engel et al., 2012). Within in vivo studies, a vIL8-deleted mutant MDV strain resulted in decreased lytic replication and oncogenicity in the host, although some MD tumors developed (Parcells et al., 2001).

The viral homolog of the chicken telomerase RNA subunit (**TR**), known as **vTR**, is located within the IR_L/TR_L regions of the MDV genome (Delany and Daniels, 2003; Fragnet et al., 2003). The high degree of homology between vTR and chicken TR is interpreted to be evidence of selective pressure to maintain the TR sequence. There is evidence of vTR contributing to MDV-induced cellular transformation (Trapp et al., 2006) and speculation that vTR expression may have anti-apoptotic properties in latently infected cells (Osterrieder et al., 2006). A vTR-knockout and a vTR-mutated (with non-typical telomere repeats) MDV strain were severely impaired in their ability to induce lymphomas in vivo, as indicated by greatly reduced (>60%) tumor incidence and size (Kaufer et al., 2011b). Lytic replication was not altered as compared to wild-type MDV. Reversion of the vTR sequence to wild-type, which restores its interactions with telomerase reverse transcriptase (**TERT**), led to a restoration of viral-induced tumorigenesis in host birds. Thus, it was concluded that telomerase activity must be a necessary component to transformation by MDV. In vitro, the DF-1 chicken fibroblast cell line adopted a transformation-like phenotype when vTR was constitutively expressed. Interestingly, vTR in combination with chicken TERT (**chTERT**) leads to greater telomerase activity than chTR with chTERT (Fragnet et al., 2005). Notably, the non-oncogenic Rispens vaccine contains a mutation in the vTR gene, which leads to reduced function (Fragnet et al., 2005). It remains of interest to establish the extent to which host telomere biology, including telomere maintenance (described below for chicken), is involved in vTR-associated transformation (Trapp et al., 2006).

Reactivation of MDV Lytic Replication in Latently Infected Cells

Reactivation of MDV involves a recurrence of high viral gene expression and the viral genome replication process in a formerly latently infected host lymphocyte. In host birds, MDV reactivation from latency may naturally occur around 2 wk post-infection, which is a particularly important event in migratory T lymphocytes near the feather follicles. MDV-reactivation aids the infection of FFE, from which mature virions will develop and shed with the host dander.

During latency, lytic replication-inducing gene expression is suppressed and apoptosis of the host, latently infected T lymphocyte, is blocked by viral mechanisms. As discussed, the *Meq* oncogene plays a critical role in activation of transformation/latency genes and repression of lytic genes. During reactivation, *Meq* splice variants that lack the domains involved in transactivation and -repression are expressed, permitting a return to rolling circle replication and high gene expression in the host cell. It is also known that the cytoplasmic phosphoprotein pp38 is highly expressed during lytic replication and reactivation (Baigent et al., 1998), particularly in MD susceptible birds. It also has been found that the presence of telomeric repeats in the MDV genome is crucial for MDV reactivation (Kaufer et al., 2011a). The trigger mechanism of reactivation is not fully understood, but may involve reduction of cytokines (Parcells et al., 2003).

TELOMERES AND TELOMERASE – ROLES IN THE HOST CHICKEN AND MDV INFECTION

Telomeres and Telomerase

Telomeres are nucleoprotein caps on the ends of linear chromosomes in eukaryotes (Zakian, 1995). They serve as the solution to the problem of DNA-end loss after each round of cell division. The eukaryotic telomeric repeats are often 5 to 10 nucleotides and GC-rich, and serve as binding sites for the proteins that cap the DNA ends (De Lange, 2005). The enzyme telomerase, which consists of the TERT and a TR subunit (that provides the telomere template) functions to elongate the telomeric sequence by a specialized form of reverse transcription and counter telomeric erosion that occurs in each round of DNA replication (Greider and Blackburn, 1987; Greider and Blackburn, 1989; Blackburn, 2001). TERT and TR expression levels impact the presence and activity levels of telomerase (Fragnet et al., 2005). Telomerase is not detected in somatic cells but is highly active in stem cells, germ line cells, and lymphocytes in humans (Hiyama et al., 1995). Telomerase activity and changes in telomere biology also are associated with malignant transformation and immortalization (Kim et al., 1994; Artandi and DePinho, 2010). In fact, telomere stabilization through elevated telomerase activity and disruption of the "normal telomere clock" can play a significant role in metastatic tumor cell immortality (Harley et al., 1994; Shay et al., 2001).

Chicken Host Telomere Biology

In chicken, the telomeres consist of repeated $TTAGGG_n$ DNA sequences (Delany et al., 2003). Three overlapping classes of chicken telomeric sequence have been identified that vary by length and chromosomal location—namely, Class 1 (0.5 to 10 kb), Class 2 (10 to 40 kb), and Class 3 (40 kb to 2 Mb). Despite its smaller genome size, the chicken genome has 10 times more telomeric sequence than the human genome and includes a set of ultra-long (aka mega-telomere) arrays with the longest telomeres observed in any vertebrate species, making avian telomere biology a particularly interesting field of research (Delany et al., 2003). Cytogenetic (Nanda and Schmid, 1994; Solovei et al., 1994; Delany et al., 2007) and molecular analyses (Delany et al., 2000) revealed the length variation of chicken telomeres as well as the existence of interstitial telomeres and the unique mega-telomeres. In chicken, the telomeres were found to shorten after multiple cell divisions as in human, and this correlated with down-regulation of telomerase activity in post-natal somatic tissues (Delany et al., 2000; Taylor and Delany, 2000; Swanberg et al., 2010). Telomere shortening or irregular maintenance of telomeres in chicken cells is associated with tumorigenesis or transformed cell types (Swanberg et al., 2010, and references therein).

Telomeric Repeats and the Viral Telomerase RNA Gene in the MDV Genome

Viral terminal repeats and cellular telomeres are similarly crucial for genome maintenance and stability, particularly during replication processes. In fact, viral terminal repeats play a role in genomic DNA stability for many linear DNA viruses. Terminal repeats also contribute to a range of other factors, including viral integration and packaging (Deng et al., 2012 and references therein).

MDV harbors 2 forms of telomeric repeat arrays (consisting of the $TTAGGG_n$ sequence) in its genomic termini (upon cleavage to the linear form), specifically multiple (consisting of up to 100) and short (exactly 6) repeats (Kaufer et al., 2011a; Greco et al., 2014). Both the short and longer repeat arrays are located between the R_L and R_S segments of the MDV genome (Figure 1) and the cleavage site of concatemeric MDV is adjacent to the short telomeric repeats region (Volkening and Spatz, 2013). The role of the viral telomeric repeats is hypothesized to be 1) service as a mini-chromosome cap or 2) contribution to the capacity of the viral genome to be packaged into the host genome via recombination or a telomerase-based pathway. There is evidence

that the latter role applies to MDV (Osterrieder et al., 2014) and that telomeric integration allows the MDV genome to be replicated and distributed to progeny cells during host cell proliferation. Telomere sequence in the MDV genome also appears to be key in the processing of replicated concatemers of viral DNA (Kishi et al., 1991). Notably, the absence of MDV telomeric repeats severely decreases viral replication, integration, tumorigenesis, and reactivation of latent MDV in vivo (Jarosinski et al., 2006; Kaufer et al., 2011a; Greco et al., 2014).

The Mechanism of MDV Telomeric Integration MDV integrates into the host genome, likely in the form of a long concatemer of viral genomes as indicated by cytogenetic and molecular studies (Delecluse and Hammerschmidt, 1993a; Kaufer et al., 2011a; Robinson et al., 2010, 2014). The precise details of the MDV integrating mechanism, including whether it exploits the host telomere extension pathway and/or homologous recombination, are yet to be determined. However, there is a growing amount of data on the necessary viral components in the integration process (Osterrieder et al., 2014). The lack of a cell population with the telomere-integrated only phenotype upon infection by the Δ*Meq* MDV strain (see phenotypes described below) may indicate a role for the *Meq* oncogene in establishment of this state of MDV in activated lymphocytes (Robinson et al., 2014). Also, a vTR-mutated MDV strain lost the ability to induce tumors in vivo, leading to speculations over the role of vTR in telomeric integration of MDV (Osterrieder et al., 2006).

The MDV integration mechanism likely involves the viral telomeric repeat arrays (Kaufer et al., 2011a; Greco et al., 2014). MDV strains with a mutated or deleted version of the telomeric repeats in their genome failed to integrate at the host telomeres, as indicated by Southern blot and FISH analyses (Kaufer et al., 2011a). Greco and colleagues (2014) found that mutated short telomeric repeats reduced viral replication and integration efficacy in vivo. However, these effects on the viral life cycle were not observed as strongly in an in vitro system, and a precise wild-type repeat length, but not sequence, was necessary for replication to occur. Telomeric repeat sequence alteration also impacted MD tumor incidence and transmission in vivo, and in the few tumors that developed the number of MDV integration sites were reduced by 2-fold as compared to the wild-type and revertant virus (Greco et al., 2014). It should be noted that multiple telomeric repeats (vs. short) were found to play a more significant role in integration.

Telomere and Telomerase Changes Induced by MDV Infection The changes in host telomere biology or telomerase activity that result in cellular transformation can be induced by viral infection (Counter et al., 1994; Horikawa and Barrett, 2003). In MDV infection, telomeric integration has a causal link to tumorigenesis, perhaps relating to telomere dysfunction among other factors (Deng et al., 2012). The MDV vTR has a functional promoter and, resultantly, induces telomerase activity in TR-/- knockout murine cells and is naturally expressed in the peripheral blood lymphocytes of MDV challenged and vaccinated chicken. The telomerase activity in the knockout line is particularly compelling as it provides evidence of the functionality of vTR in a heterologous telomerase complex. vTR-knockout MDV was severely impaired in its ability to induce lymphomas in vivo, as indicated by greatly reduced tumor incidence and size in chickens (Trapp et al., 2006). It also has been determined that vTR presence in the MDV genome can lead to lymphoma development in a telomerase complex independent manner, i.e., cells expressing a mutant-vTR that does not functionally complex with TERT were transformed in vivo, as well (Kaufer et al., 2010).

CYTOGENOMIC MDV-HOST INTERACTIONS

Overview of MDV Cytogenetic Methods

Molecular and cytogenetic studies of MD have been significantly enhanced by the availability of viral and chicken genomic DNA clones (Schumacher et al., 2000; Lee et al., 2003; Petherbridge et al., 2003; Hillier et al., 2004; Petherbridge et al., 2004; Baigent et al., 2006; Silva et al., 2010) and genome sequence data (Lee et al., 2000a; Izumiya et al., 2001; Kingham et al., 2001; Spatz et al., 2007). The clones provide a tool for strain- or region-specific labeling of DNA while the sequence data allow more in-depth exploration of genetic content, regulation, and interactions, as well as advances comparative genomics. The primary cytogenetic technique employed to investigate viral and host genome interactions has been FISH, applied to host mitotic metaphase chromosomes. Southern blot hybridization has its own advantages for integration studies but can miss low copy number integration, while FISH offers a more sensitive and accurate viral detection approach that is reliable and cell-specific (Leenman et al., 2004; Reisinger et al., 2006; Haugg et al., 2014).

MDV Phenotypes and Infection Stages

Our understanding of the genomic interactions between MDV and its host and how these interactions impact viral pathogenesis, transmission, and tumorigenesis has been improved by cytogenetic studies. Robinson et al. (2014) characterized 4 distinct viral genome status phenotypes (see Figure 3) in dividing lymphocytes of the primary and secondary immune organs between one and 21 dpi employing FISH with a specific MDV-BAC probe. The 4 phenotypes were 1) MDV-null, lacking MDV signals in the nucleus or around the chromosomes, 2) MDV chromosome-associated, MDV fluorescence signals surrounding the host chromosomes, 3) MDV chromosome-associated/integrated, presence of associated signals as well as MDV signals integrated into the sister chromatids at one or more telomeres,

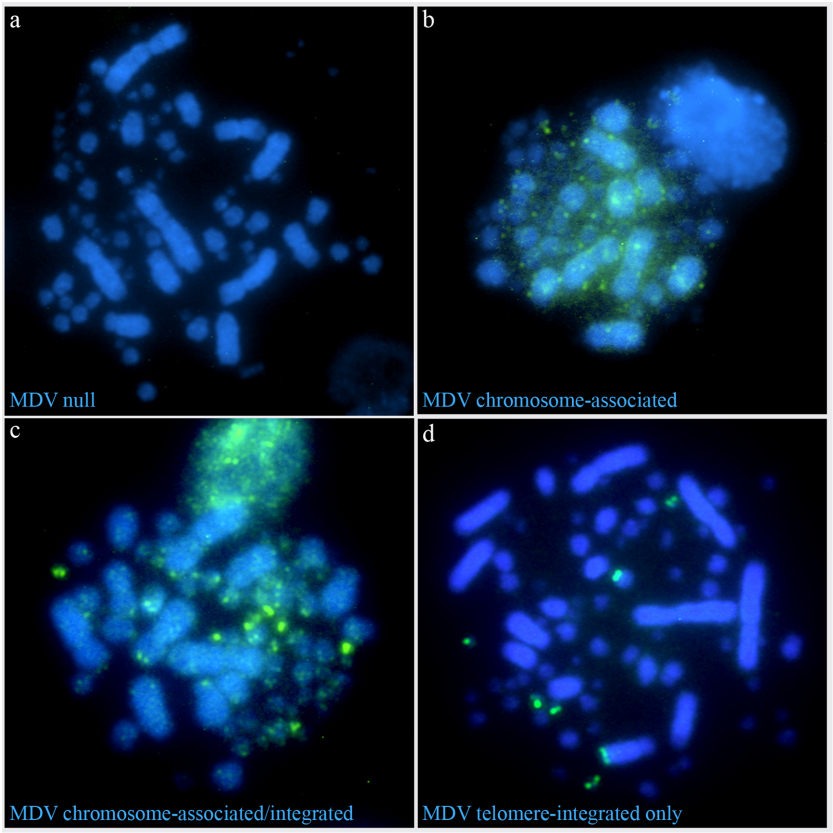

Figure 3. Marek's disease virus-chicken host genome interaction phenotypes. The phenotypes shown indicate the interactions and status of the herpesvirus with regard to the chicken host genome, in mitotically dividing cells from the immune organs of MDV-challenged birds (see text for additional descriptions). (a) MDV-null phenotype, lacking MDV FISH signals (FITC, **green**) in the nucleus or around the chromosomes (DAPI, **blue**). (b) MDV chromosome-associated phenotype, consisting of diffuse MDV fluorescence signals surrounding the host chromosomes. (c) MDV chromosome-associated/integrated phenotype, defined by the presence of diffuse, host DNA associated MDV signals as well as bright and punctate signals, signifying MDV integrated into host sister chromatids at one or more telomeres. (d) MDV chromosome-integrated only phenotype, consisting of the distinct, punctate MDV FISH signals at the telomeres with no other signals detected.

and 4) MDV chromosome-integrated only, presence of the distinct, punctate signals at the telomeres with no other MDV signals.

The relevant timepoints and stages of infection in the serotype 1 MDV life cycle are well described (Osteriedder et al., 2006; Schat and Nair, 2008), which enables correlation of MDV-host phenotypes to the known temporal disease stages and viral "activities." MDV-null cells lack MDV (episomal or integrated). The MDV chromosome-associated phenotype suggests cells are in the cytolytic stage (virus replicating independently of the host genome). The MDV chromosome-associated/telomere-integrated phenotype reflects the presence of replicating episomal virus simultaneously existing in cell(s) with viral DNA that has integrated into the host genome and, finally, the integrated-only MDV phenotype represents a latently infected or transformed cell, cleared of replicating virus with the host chromosomes harboring linear MDV genomes (Figures 2 and 3). In a time course study (1 to 21 dpi, Robinson et al., 2014), the telomere-integrated only viral phenotype (latency and transformation associated) generally increased over time in all immune tissues, particularly in the spleen, while the chromosome-associated phenotype decreased over time in all tissues. There was remarkable heterogeneity in chromosomal location and number of MDV integrations initially (in non-tumor cells of the spleen), but all were telomeric and typically at only one telomere arm per chromosome. Southern blot analyses showed DNA-junction fragments containing both host-telomeric and MDV DNA from infected lymphoblastoid cells, confirming viral integration (Kaufer et al., 2011a).

Temporal Dynamics of MDV in the Host

Chromosomal Association During Timing of Cytolytic Replication During the early cytolytic infection, the virus is actively spreading among host immune cells, and the MDV genome is replicated and packaged into infectious virion particles. The viral load in the immune tissues peaks during this initial stage (shortly after initial exposure to the infectious dander). Robinson and colleagues (2014) found evidence of serotype 1 MDV lytic replication throughout the immune organs via cytogenetic detection as early as one dpi, which is earlier than previously reported (4 dpi by Baigent and Davidson, 1999). This finding emphasizes that FISH detection of viral genome status is highly sensitive

(Robinson et al., 2014). The thymic and bursal immune tissues, which consist of populations of primarily T and B lymphocytes, respectively, exhibit peak levels of MDV chromosome-associated phenotype cells around 4 dpi, which continuously declined by 14 and 21 dpi. These results are consistent with data indicating a peak of lytic replication between 4 and 7 dpi (Baigent and Davison, 2004). Evidence that MDV integrates prior to the latent stage (7 dpi) of MD pathogenesis supports our knowledge that indeed stages overlap within and among cells.

Telomeric Integration During Timing of Latency Integration is a characteristic feature of the MDV life cycle; however, the necessity of integration to the various aspects of the viral life cycle is difficult to establish exactly. Important questions include: Is integration required for viral latency and does such contribute to persistence? Is integration essential for transformation and tumorigenesis? Integration of viral DNA appears not to be an incidental occurrence but, rather, has a role in the viral latency and tumorigenesis stages (Desfarges and Ciuffi, 2012). In MDV there is clearly a temporal overlap between the established timing of latency (around 7 dpi, but involving temporal overlap with the lytic stage) from molecular data (Lee et al., 1999; Nair, 2013 and references therein) and MDV telomere-integration from cytogenetic data (Robinson et al., 2014). This suggests a relationship between integration of the virus and the lytic to latency transition. Furthermore, the timing of the latency to tumorigenesis (14 to 21 dpi) transition during MDV infection correlates with a reduction of the associated/integrated viral phenotype (such reduction related to the clearing of the virus) and an increase in the chromosome-integrated only phenotype among the dividing immune cells (Robinson et al., 2014). The cells of the spleen exhibited particularly high levels of the MDV integrated only virus phenotype after 7 dpi. Spleen cell populations consist of B and T lymphocytes, as well as macrophages and other lymphoid and non-lymphoid cells (Olah et al., 2014); however, it has not been determined if there is a bias of MDV integrating into chromosomes of T vs. B lymphocytes. The overlap in timing and events established by the molecular and cytogenetic data are intriguing, but it is not yet known whether the act of integration into host telomeres is necessary to achieve latency or if viral integration requires latency-related events to occur (chicken vs. the egg).

MDV Integration and Oncogenesis Both in vitro and in vivo studies indicate an integral biological relationship between MDV integration and cellular transformation or MD lymphoma formation in susceptible hosts. Almost the entire population of mitotically dividing lymphocytes (>90%) analyzed from primary MD-lymphomas have the MDV telomere-integrated only phenotype, consisting of integrations at multiple telomeric site(s) (Robinson et al., 2010, 2014). Minimal amounts of free episomal virus (i.e., lytically replicating) were identified in tumors. Furthermore, birds infected with a non-integrating, mutant MDV strain had decreased to no lymphoma development—further demonstration of an association between MDV integration and achievement of cellular transformation (Kaufer et al., 2011a,b).

Analysis of the unique telomeric integration sites of MDV within and among tumors suggested that both monoclonal and polyclonal tumors (Robinson et al., 2010) form in challenged, susceptible birds. A mode of 5 MDV telomere-integration sites per nuclei was identified in the tumors of MD-susceptible heterozygous line (USDA-ADOL $151_5 \times 7_1$ F_1) chicks, with a range of one to 8 integrations (Robinson et al., 2010). Doublet virus FISH signals were commonly detected at the chromosome ends, indicative of telomere-integrated MDV in both sister chromatids and suggesting that the virus replicates along with the host chromosomes (Robinson et al., 2010). Fluorescence signal strength variation among different viral integration loci was detected within and among individual cells and lymphomas, indicating that a variable number of tandemly repeated viral genomes in the host DNA occurs from one integration event to the next. However, there was signal consistency for a particular integration locus within analyzed lymphoma. Data from the Robinson et al. (2010) study did not support a preference of MDV towards integration at the mega-telomeres, although integration always occurs within or near the telomeres. Also, aneuploid and tetraploid tumor cells were observed similar to that reported for many human leukemia and lymphomas.

With regard to whether integrations are random or if there exists preferred or targeted integration into specific host chromosomes, a statistically-supported "preferential chromosome" for MDV integration(s) was not detected among the analyzed lymphomas. However, there were strong tendencies towards MDV integration into the telomeres of GGA 9 (encodes chicken TR) as well as GGA 4 (encodes chicken IL-8 gene), GGA 6, GGA 12 and GGA 20 (Robinson et al., 2010).

The question of clonality (i.e., lineage relationship of cells within or among tumors) was addressed through examination of the profile of chromosomal integrations among cells of a tumor and in other tumors from the same bird. The result was mixed; a majority of tumors exhibited profiles indicating monoclonal tumor origins, yet in other cases polyclonal tumors were detected (Robinson et al., 2010). By another method, T lymphocyte spectratyping of MD lymphomas, limited clonality was indicated (Mwangi et al., 2011). MD is well known to be a highly individualistic disease with a great degree of inter-individual variation; thus, these results are not entirely surprising for a virally-induced cancerous condition.

The MDV integration profiles of early- to late-stage MD tumors from GA-challenged birds were compared (Robinson et al., 2014) and 3 categories of tumor phenotypes based on their viral integration profiles were uncovered. These included 1) highly heterogeneous

(5 to 8 different integration profiles wherein less than 50% of cells displayed the most frequent profile), 2) moderately heterogeneous (5 to 7 different profiles with 50 to 70% of cells exhibiting the most frequent profile), and 3) homogenous (2 to 5 different profiles wherein greater than 70% of cells shared the most frequent profile). Interestingly, early-stage tumors, collected at 21 dpi, exhibited the heterogeneous phenotype. Conversely, late-stage tumors, collected at 61 and 73 dpi, had only the homogenous tumor phenotype. Our proposed model is that early-stage tumors represent a collection of recently transformed T lymphocytes, many of which do not persist or contribute to the cell lineage of a late-stage tumor. The increased uniformity of cells, in terms of viral integration phenotype, in late-stage tumors (i.e., one lineage tends to dominate during tumor growth and metastases) is indicative of a selection process during tumorigenesis.

Reactivation and MDV Phenotype Although MD lymphomas consist principally of latently-infected and transformed cells, increased numbers of cells with lytically replicating virus (free linear form) are detected from MD primary lymphomas upon different culture conditions (Dunn and Nazerian, 1977; Calnek et al., 1981; Delecluse et al., 1993c; Robinson, 2013). Cytogenetic analysis of the latently infected UA04 T lymphocyte line (Dienglewicz and Parcells, 1999) following incubation with bromodeoxyuridine showed multiple, speckled FISH signals surrounding the MDV-integrated chromosome (absent in control cells). This starburst of dispersed viral FISH signals is interpreted as escaped, replicating MDV genomes (Robinson, 2013; Schmid et al., 2015 Figure 2 *in the section entitled "Host-Viral Genome Interactions in Marek's Disease"*). Thus, MDV may not only use integration as a means for avoiding immune detection (latency), but also as a mode of persistence in the host with the means to "escape" from the host genome and return to the replication phase of its life cycle, i.e., reactivation (Figure 2 **model**). The ability to return to a state of productive replication after integration presumes that the MDV genome integrated intact and without rearrangement into the host DNA. Clearly, further research is necessary to support or refute such a model.

Novel Findings in MDV Cytogenomic Dynamics

MD Vaccine Interactions with the Host Genome The in vivo interactions of MD vaccines, specifically Rispens/ CV1988, SB-1 and HVT, with the host genome during the first 3 wk after vaccination also have been investigated to understand if the vaccines can integrate (McPherson et al., in prep). The chromosome-associated/integrated phenotype was detected post vaccination in spleen cell preparations, indicating that all 3 MD vaccine serotypes are capable of integrating into host telomeres. This result was noteworthy considering that the viral telomeric repeats are intact within all MD vaccine genomes, but the *Meq* and vTR genes, which are known to contribute to MDV integration, are absent from SB-1 and HVT genomes (Fragnet et al., 2003; Kingham et al., 2001; Fragnet et al., 2005; Spatz and Schat, 2011) and lack functional copies in the Rispens genome (Trapp et al., 2006; Spatz et al., 2007). Interestingly, the MDV telomere-integrated only phenotype was not observed as found for serotype 1 viruses, similar to a *Meq*-deleted MDV strain (see below).

ΔMeq MDV Interactions with the Host Genome A non-oncogenic Δ*Meq* MDV strain (Silva et al., 2010) maintains a markedly high and sustained level of MDV chromosome-associated signals in host immune tissues (suggesting continuous replication) and does not establish the telomere-integrated only phenotype in host cells between one and 21 dpi (Robinson et al., 2014). Thus, this unique deletion strain is indeed capable of telomeric integration, yet does not transition to integrated-only, nor is it known to transform host lymphocytes in vivo or possess a capacity for latent infection. These results provided evidence that *Meq* expression and a transition to telomere-integration profiles alone (no episomal form) by MDV in host nuclei are integral to the establishment of the latent infection and MD-induced transformation. However, neither of these viral activities is required for infiltration of the host immune cells, lytic replication, or transmission.

CONCLUSION

MD is a significant, ongoing health issue for poultry that has persisted despite the enormous body of high-quality fundamental and applied research discoveries. MD vaccines have resulted in a large degree of disease control, yet we know MDV field strains are evolving. It is important for continued research and resource efforts to eradicate and manage this disease for the benefit of this high-value agricultural resource. Simultaneously, research efforts around MDV can contribute to the body of knowledge for cancer-causing human viruses and herpesvirus-associated diseases.

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