# DEVELOPMENT AND CHARACTERIZATION OF A SUSTAINABLE CHICK CELL LINE INFECTED WITH MAREK'S DISEASE VIRUS

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ABSTRACT

DEVELOPMENT AND CHARACTERIZATION OF A SUSTAINABLE CHICK CELL LINE INFECTED WITH MAREK'S DISEASE VIRUS

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Marek's disease virus (MDV) is an oncogenic, highly infectious, cell-associated avian herpesvirus. Fully productive MDV infections are restricted to feather follicle epithelium of afflicted birds. In cell culture, MDV infection of primary chick and duck fibroblast cells is semi-productive. Passage of MDV and production of MDV vaccines are limited to these primary cell systems. The limited life span of primary avian cell cultures has hampered efforts to use positive selection in generation of recombinant MDV and has complicated studies of temporal gene regulation.

We have developed a sustainable cell culture system (MDV OU2) using the nononcogenic, immortalized CHCC-OU2 chick cell line, which supports MDV replication. Southern blot and PCR analyses demonstrated that these cell lines do harbor MDV. MDV pp38 and pp14 expression was detected in sparse and confluent MDV OU2 cells by western blot analysis and IIFA staining, but expression of MDV structural glycoproteins (gB and gI) were detected only in confluent MDV OU2 cells. We also demonstrate using RT-PCR that MDV latency associated transcripts (LATs) are expressed at higher levels in sparse MDV OU2 cells than confluent monolayers and LAT

expression is down regulated in confluent cells. MDV ICP4 expression was inversely proportional to the level of MDV LAT expression. Presence of distinct plaques and expression of glycoproteins in confluent MDV OU2 cell monolayers is consistent with a cytolytic infection. Whereas the pattern of MDV LAT/ICP4 expression, and lack of glycoprotein expression in sparse MDV OU2 cells are consistent with a latent infection. Data presented in this dissertation suggest that MDV OU2 cells are latent when subconfluent and the virus is reactivated when cells become confluent.

MDV OU2 cells are capable of transferring MDV infection to CEF *in vitro* and can induce MD *in vivo*. PCR analysis and *in vivo* experiments also demonstrated that MDV genomes are stabilized in this cell culture system. Unlike MDV passaged in CEF cells, MDV OU2 cells are still oncogenic and can induce clinical symptoms of MD after more than two and a half years in active continuous culture.

To my Mother and Father for their love and support.

To my great wife Aida for her love and understanding.

and

to my great kids

Rawan, and Shadi.

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| | |
|---|---|
| **ADOL** | Avian Diseases and Oncology Laboratory |
| **AGP** | Agar-gel precipitin |
| **ALV** | Avian leukosis virus |
| **AVS** | Avian sarcoma virus |
| **BHV** | Bovine herepsvirus |
| **CEF** | Chicken embryo fibroblast |
| **CHX** | Cyclohexamide |
| **CKC** | Chicken kidney cells |
| **CPE** | Cytopathic effect |
| **CS** | Calf serum |
| **DEF** | Duck embryo fibroblast |
| **DMSO** | Dimethyl sulfoxide |
| **DR** | Direct repeat |
| **E genes** | Early genes |
| **EBV** | Epstein-Barr virus |
| **EHV** | Equine herpesvirus |
| **FBS** | Fetal bovine serum |
| **FFE** | Feather-follicle epithelium |

| | |
|---|---|
| **FITC** | Fluorescein-5'-isothiocyanate |
| **FPV** | Fowlpox virus |
| **gB/C/D/E/I/K** | Glycoprotein B/C/D/E/I/K |
| **HSV-1** | Herpes simplex virus type 1 |
| **HVT** | Herpesvirus of turkey |
| **IE** | Immediate early |
| **IIFA** | Indirect Immunofluorescence antibody |
| **IRL** | Inverted repeat long |
| **IRS** | Inverted repeat short |
| **IudR** | 5-iodo-2-deoxyuridine |
| **Kb** | Kilobasepairs |
| **KDA** | Kilo daltons |
| **L genes** | Late genes |
| **LATs** | Latenct associated transcripts |
| **LM** | Leibovitz-L15-McCoy 5A |
| **MATSA** | Marek's associated tumor surface antigen |
| **MD** | Marek's disease |
| **MDV** | Marek's disease virus |
| **ND** | Not determined |
| **Oct-1** | Octomer binding factor |
| **PAGE** | Polyacrylamide gel electrophoresis |
| **PBLs** | Peripheral blodd leukocytes |

| | |
|---|---|
| **PCR** | Polymerase chain reaction |
| **PE** | R-phcoerythrin |
| **PI** | Propidium iodide |
| **PI** | Days post-infection |
| **pp14/24/38** | Phosphoprotein 14/24/38 |
| **PRV** | Pseudorapis virus |
| **RT** | Revesr Transcriptase |
| **SDS** | Sodium dodecyl-sulfate |
| **TK** | Thymodine Kinase |
| **TPB** | Tryptose phosphate broth |
| **TRL** | Terminal repeat long |
| **TRS** | Terminal repeat short |
| **UL** | Unique long |
| **US** | Unique short |
| **USDA** | Uinted State department of Agriculture |
| **VP16** | Virion protein 16 |
| **vvMDV** | very virulent MDV |
| **VZV** | Varicella-zoster virus |

# Chapter 1

# Literature Review

## 1.1 Introduction

Marek's Disease (MD) is a highly contagious lymphoproliferative disease of chickens, first described in 1907 by a Hungarian scientist, Joseph Marek (Marek, 1907). MD is often characterized by leg paralysis and lymphoid tumors (Payne, 1985). During the early 1960's, it was discovered that MD was caused by an oncogenic cell-associated herpesvirus, Marek's disease virus (MDV), (Churchill and Biggs; 1967; Nazerian et al., 1968; Solomon et al., 1968).

Prior to current vaccination practices, MD was responsible for tremendous losses to the poultry industry. Since the 1970s, MD has been effectively controlled by vaccination with antigenically related, non-pathogenic, serotype 2 MDV strains, serotype 3 herpesvirus of turkeys (HVT), and attenuated serotype 1 MDV strains (Witter, 1982; Witter and Lee 1984; Churchill et al., 1969; Powell, 1985). However, despite availability of vaccines, significant losses still occur. Frequent vaccine failures are attributed in part to appearance of very virulent strains of MDV, presence of maternal antibodies, and poor vaccination techniques. The continuous evolution of MDV strains and frequent vaccine failures have promoted significant interest in developing better methods for prevention and control.

Like other herpesviruses, MDV has the ability to establish, maintain, and reactivate from latency. This provides the virus with a unique ability to persist in its

natural host. MDV also offers a model for herpesvirus oncology in the natural host. MD is the first and only example of a naturally occurring malignant lymphomatous disease to be effectively controlled by vaccination. However, despite being efficiently controlled by vaccination, the molecular mechanisms of oncogenicity and vaccine-induced immunity are still unclear.

Despite the tremendous importance of MDV in both economic terms and as an excellent lymphoid tumor model in a natural host, characterization of MDV on the molecular level has lagged behind other herpesviruses. However, recognition of MDV as a model system for human herpesvirus induced neoplasia has promoted renewed interest in this virus system.

## 1.2 History of Marek's Disease

Marek's disease (MD) first described in 1907 (Marek, 1907), causes lymphomas involving gonads, muscle, skin, and several visceral organs (Payne, 1982; Calnek and Witter, 1991). The classical form of MD (characterized by impairment of neural function and cytolytic infection (Payne 1985)) was reported sporadically from many countries throughout the first half of the 20th century. Early in the 1950's the first acute case of MD was reported in Delaware (Benton and Cover, 1957) and eventually spread throughout the United States. The acute form of MD is marked by development of lymphomas resulting in tumors, approximately four to six weeks post infection. Production of live virus vaccines, in the early 1970's, resulted in a substantial decline in the incidence of MD (Purchase, 1973). However, within the last 15 years, increased

disease outbreaks in vaccinated chickens has been noticed. These outbreaks are thought to be due to either vaccine failure, appearance of very virulent strains of MDV, or poor vaccination techniques (Witter et al., 1980; Schat et al., 1981).

## 1.3 Biology of Marek's disease virus (MDV)

### 1.3.1 MDV classification and virion structure

MDV has been designated Gallid herpesvirus 1 and provisionally placed in the subfamily *Gammaherpesvirinae* of the family *Herpesviridae* (Matthews, 1979). The nucleocapsid measures about 100 nm, has 162 capsomers arranged in icosahedral symmetry (Nazerian and Burmester, 1968), and is surrounded by an amorphus tegument. MDV virions can be seen sometimes in the cytoplasm and, rarely, in the extracellular space. The predominant form of MDV virions are naked capsids, usually found in nuclei of infected cells. Only a subfraction of MDV virions are enveloped (Nazerian and Burmester, 1968). However, MDV tumor cells and their cell line derivatives contain few naked or enveloped virus particles. Enveloped virions are associated with the nuclear membrane of MDV infected cells and are restricted to feather-follicle epithelium (FFE) of infected chickens (Calnek et al., 1970). Enveloped virions are crucial for viral spread under natural conditions. These enveloped virions are shed to the environment with molted feathers or dander, and remain infectious for several months at 20 - 25 °C, thereby passing the infection to other chickens.

### 1.3.2 MDV serotypes

MDV strains have been classified into three serotypes, based on agar gel precipitation (AGP) and indirect immunofluorescent antibody (IIFA) assays (Bulow and Biggs 1975a and b; Schat and Calnek, 1978), virus neutralization tests (King et al., 1981), and two dimensional polyacrylamide gel electrophoresis (PAGE) of viral polypeptides (Van Zaane et al., 1982). Serotype classification has been confirmed by reactivity of viral polypeptides with monoclonal antibodies (Lee et al., 1983), and by restriction enzyme pattern analysis of viral genomes (Ross et al., 1983).

MDV serotype 1 consists of all oncogenic strains and their attenuated derivatives. Serotype 1 MDV has been divided into very virulent (vvMDV), virulent, and attenuated strains based on their pathogenicity and oncogenicity in chickens. Md/5, Md/11, and RB1B strains are all classified as vvMDV and are responsible for causing many of the outbreaks in vaccinated chickens (Witter, 1985). GA, and JM strains are classified as virulent and can cause a high incidence of MD in unvaccinated chickens. Serial *in vitro* passage of vvMDV and virulent oncogenic MDV results in attenuation and loss of MDV tumorigenicity (Churchill and Chubb, 1969). These attenuated strains can cause tumors in only a few highly susceptible chickens (Schat, 1985). Serotype 2 consists of naturally occurring, nononcogenic MDV strains, and serotype 3 consists of apathogenic (in turkeys and in chickens), cell-associated, herpesvirus of turkeys (HVT).

### 1.3.3 MDV isolation

MDV replicates in a productive-restrictive manner in B-lymphocytes and cells growing in tissue culture. Fully productive infections with MDV are restricted to feather follicle epithelium where cell-free infectious virions may be readily isolated (Calnek et al., 1970; Schat, 1985). Cell-associated MDV can be isolated from peripheral blood leukocytes, spleen cells, kidney cells, and in the case of serotype 1, from lymphoma cells. MDV can be propagated in monolayers of primary or secondary chicken and duck embryo fibroblast (CEF, and DEF, respectively), chick kidney cells (CKC) (Churchill and Biggs, 1967), and CHCC-OU2 cells (Abujoub and Coussens, 1995). Cytopathic effect (CPE), characterized by formation of spherical cells loosely attached to the substratum and syncytia formation, occurs within 2-7 days post-infection. Due to the strict cell-associated nature of the virus, no infectious cell-free virus can be recovered from the tissue culture medium ( Calnek et al., 1970; Churchill and Biggs, 1967; Schat, 1985).

## 1.4 MDV vaccines

MDV is the first example of a naturally occurring tumor virus that can be effectively controlled by vaccination. Prior to development of vaccines against MDV, 20-30% mortality rates in commercial flocks were common (Pattison, 1985). One of the first vaccines developed against MDV was an attenuated serotype 1 (Churchill et al., 1969). However, the most widely used MDV vaccines in the United States are HVT based vaccines (Purchase et al., 1971; Purchase et al., 1972; Okazaki et al., 1970). HVT

is particularly useful as a vaccine because vaccines can be made from either cell-associated or cell-free preparation, and is apathogenic in chickens or turkeys (Payne, 1985). Serotype 2 MDV has also been used in vaccines against MDV ( Witter, 1992). The predominant serotype 2 MDV currently in use is SB-1, which spreads readily by contact and is protective against most virulent MDV strains (Bacon et al., 1989; Witter, 1985). SB-1 is commonly used in combination with HVT as a bivalent vaccine (Witter, 1992). The bivalent vaccine efficacy exceeds that of HVT alone (Pruthi et al., 1989). The pathogenicity of vvMDV strains has promoted renewal efforts to develop more efficacious vaccines. One vaccine includes the attenuated very virulent serotype 1 strain Md11/75c (Witter, 1982), and another attenuated virulent serotype 1 strain CVI 988 (Rispens et al., 1972). A trivalent vaccine containing HVT, SB-1, and Md11/75c has been shown to be 100% effective against challenge with vvMDV (Witter and Lee, 1984).

## 1.5 Pathology and Pathogenesis

### 1.5.1 virus-cell interactions

MDV is contracted from the environment via the respiratory system (Payne, 1985). Once MDV enters a chicken, three virus-cell interactions are recognized: 1) productive infection, 2) non-productive latent infection, and 3) non-productive transforming infection (Schat, 1985; Calnek and Witter, 1991). These interactions may produce two distinct pathological forms of MD: classical and acute. Classical MD predominantly affects peripheral nerves and causes cytolytic infection. The acute form of MD is marked by lymphoproliferation which results in tumor formation. The most

common clinical signs of MD are leg paralysis due to lymphocytic infiltration and nerve demyelination. In addition, MDV can cause depigmentation of the iris, resulting in blindness. Infected visceral organs, particularly the gonads, liver, and lungs, may have diffuse or grayish-white lymphoid tumors (Calnek and Witter, 1991). In addition, severe atrophy of the bursa of Fabricius often occurs in infected chickens. The final pathogenesis of MDV infection is affected by many factors such as: virus strain and dose, age, sex, genetic strain, and immune status of the host (Calnek and Witter, 1991).

#### 1.5.1.1 Productive infection

Productive infections are cytolytic, and are characterized by viral DNA replication, and viral antigen synthesis. Productive infection can be further divided into productive-restrictive and fully-productive infection. A productive-restrictive infection occurs mainly in B-lymphocytes, some epithelial cells, and in cultured cells. The virus particles produced by these cells are naked nuclear virions, and infectivity remains cell-associated (Calnek and Witter, 1991). Fully productive infection occurs only in feather follicle epithelium, and results in production of a large number of enveloped cell-free virions (Calnek et al., 1970).

#### 1.5.1.2 Non-productive infection

##### 1.5.1.2.1 Latent infection

Latent infections are detected at the end of early cytolytic infection. Latent infection is predominantly in T-lymphocytes (Shek et al., 1983), but has also been

detected in B-lymphocytes (Calnek et al., 1981b). During latent infections MDV genomes persist in cells with limited expression of viral genes (Maray et al., 1988; Sugaya et al. 1990; Silver et al., 1979; Schat et al., 1989). Latent infections can persist for the entire life of the bird without the production of infectious virus progeny, unless the virus is reactivated (Dunn and Nazerian, 1977).

#### 1.5.1.2.2 Transforming infection

In transformed cells, viral genomes persist in cells with limited transcriptional activity. To date, no viral antigens have been associated with transformed cells, but an activated T-cell marker, Marek's associated tumor surface antigen (MATSA) is expressed at a higher level in transformed cells (McColl et al., 1987). Transforming infection seems to only occur in T-cells, since all lymphoblastoid cell lines are comprised of transformed T-lymphocytes (Calnek and Witter, 1991). The majority of these T-cells, are activated T-helper $CD4^+$ $CD8^-$. However, some of the cell lines established from experimentally induced lesions are $CD8^+$ and $CD4^-$ $CD8^-$ T-cells (Schat et al., 1991). The relationship between latency and transformation is not well understood, but current evidence suggests that latent infection is a prerequisite for malignant transformation and neoplastic disease.

### 1.5.2 Pathogenesis

Pathogenesis has been well defined in experiments with MDV infections of susceptible chickens. MDV infection can be divided into four stages: 1) early cytolytic infection, 2) latent infection, 3) late cytolytic infection or permanent immunosuppression,

and 4) transformation. MDV infections usually occurs via the respiratory tract, where MDV is phagocytized by macrophages (Calnek, 1985) and then disseminated from the lung via lymphoid cells (Calnek and Witter, 1991). Early cytolytic infection, starts 3 to 5 days post-inoculation, and affects primarily B-lymphocytes, and to a lesser degree activated T-lymphocytes. The cytolytic infection results in macrophage and granulocyte infiltration of specific organs, which leads to necrosis and an inflammatory response. A consequence of these events is spleen degeneration and atrophy of the thymus and bursa of Fabricious.

The next stage of infection occurs 5 to 7 days post-inoculation when the infection changes from a cytolytic infection of primarily B-lymphocytes to a latent infection of predominantly T-lymphocytes (Shek et al., 1983). This switch is associated with immunosuppression of the host. Latently infected T-cells can persist for the life of the chicken. However, latent infection is not restricted to lymphoid tissues, but also includes nonmyelinating Schwann cells and satellite cells in spinal ganglia (Pepose et al., 1981).

At 2 to 3 weeks post-inoculation susceptible chickens enter into a late cytolytic infection. Lymphocytes are the target of this infection, which leads to a permanent immunosuppression involving both humoral and cell-mediated immunity. During this time, cells of epithelial origin become infected, and feather follicle epithelium starts producing cell-free infectious virus (Calnek et al., 1970). Focal necrosis and intranuclear inclusions can occur in the kidney, pancreas, blood vessels, peripheral nerves, and central nervous system (Payne, 1992).

The final stage of MDV pathogenesis is malignant transformation and neoplastic

disease. Massive lymphomas involving almost all visceral organs, skin, muscles and nerves develop 4 to 6 weeks post-inoculation and, occasionally as early as 2 weeks post-inoculation (Calnek, 1985). The composition of lymphomas is complex since they contain transformed T-cells, inflammatory cells, and immunologically active cells (Calnek, 1985). MDV infection at this stage has also been associated with atherosclerosis in the coronary arteries, aorta, and aortic branches (Fabricant et al., 1978).

## 1.6 Molecular biology of Marek's disease virus

Molecular biological characterization of MDV has lagged behind other herpesviruses. Understanding the molecular biology of MDV has been hindered for three main reasons: 1) MDV is a highly cell-associated virus, 2) MDV DNA has a buoyant density similar to that of chicken DNA (Lee et al., 1971), and 3) there has been no sustainable continuous cell line identified which supports MDV replication. Consequently, the isolation of cell-free virus particles and pure viral DNA is difficult and the ability to test gene function is limited.

### 1.6.1 MDV genome structure

The genome of MDV is a linear, double-stranded DNA of approximately 160-180 kilobase pairs (kb) which is similar in size to Epstein-Barr virus (EBV) DNA but larger than herpes simplex virus (HSV) DNA (Lee et al., 1971). The molecular weight of MDV DNA is approximately 120 x $10^6$ Daltons, whereas that of HVT and serotype 2 MDV DNA is 103 x $10^6$ Daltons or approximately 150 kb (Hirai et al., 1979). Differences in

sedimentation coefficients between sucrose and alkaline gradients has implied the presence of single strand gaps or nicks in MDV DNA similar to that of other herpesviruses (Wilkie, 1973; Lee et al., 1971). The buoyant density of MDV DNA in CsCl gradients was determined to be 1.705 g/cm$^3$, which corresponds to a 46% G+C content (Lee et al., 1971).

MDV was originally classified as a gammaherpesvirus based on its biological characteristics, and the lymphotrophic nature of MD, similar to EBV (Roizman et al., 1981). However, the overall genomic structure of MDV and the colinearity and relatedness of MDV genes to those of alphaherpesviruses (varicella-zoster virus, and herpes simplex virus), have led to reclassification of MDV as an alphaherpesvirus (Cebrian et al., 1982; Roizman, 1992; Roizman and Sears, 1991). The genomic structure of MDV consists of unique long ($U_L$) and unique short ($U_S$) regions, with each unique region flanked by terminal repeats ($TR_L$, $TR_S$) and internal inverted repeats ($IR_L$, $IR_S$) (Cebrian et al., 1982; Fukuchi et al., 1984). MDV genomes also contain several sets of direct repeats (DR1 to DR5), scattered throughout the genome. These direct repeat sequences are located mainly within the repeat regions (Hirai, 1988). One of these direct repeats (DR) has been correlated with attenuation of serotype 1 MDV. DR1 is a 132-bp direct repeat sequence located within both $TR_L$ and $IR_L$. This 132-bp direct repeat sequence is amplified from 1-3 copies in virulent MDV to more than 20 copies during serial *in vitro* passage, coincident with virus attenuation and loss of MDV tumorigenicity (Calnek et al., 1981; Silva and Witter, 1985; Fukuchi et al., 1985; Chen and Velicer, 1991; Maotani et al., 1986). DR2 is located within the $U_L$ region and consists of a direct

repeat of 1.4 kb. DR3 is located within $TR_S$ and $IR_L$ and consists of 178 bp (Hayashi et al., 1988). DR3 is amplified more than 50-fold during viral replication in both oncogenic and non-oncogenic MDV strains. DR4 is located within $TR_S$, and $IR_S$ and consists of 200-bp (Hirai et al., 1984). DR5 is a putative terminal direct repeat at the end of MDV DNA molecules and may contain signals for cleavage of MDV replicative forms.

### 1.6.2 MDV restriction maps

Physical maps have been constructed for all three MDV serotypes using different restriction enzymes (Fukuchi, et al., 1984; Igarashi et al., 1987; and Ono et al., 1992). Although the three serotypes of MDV share strong antigenic similarities, their restriction endonuclease patterns are different (Figure 1.1). Based on reassociation kinetics, 5% homology was detected between serotype 1 and serotype 3 MDV DNA (Hirai et al., 1979). Only the *Bam*HI J fragment of HVT hybridized strongly to MDV DNA under high stringency conditions (Hirai et al., 1984). However, up to 70% DNA homology was observed under low stringency (Gibbs et al., 1984). Direct DNA sequencing of several genes in both serotypes (Chen et al., 1992; Ono et al., 1994; Smith et al., 1995) has substantiated the higher homology figures.

### 1.6.3 MDV attenuation

Serial *in vitro* passage of virulent oncogenic MDV results in loss of MDV tumorigenicity (Churchill and Chubb, 1969). Attenuated MDV fails to induce early cytolytic infection, has reduced ability to infect or replicate in lymphocytes, and no

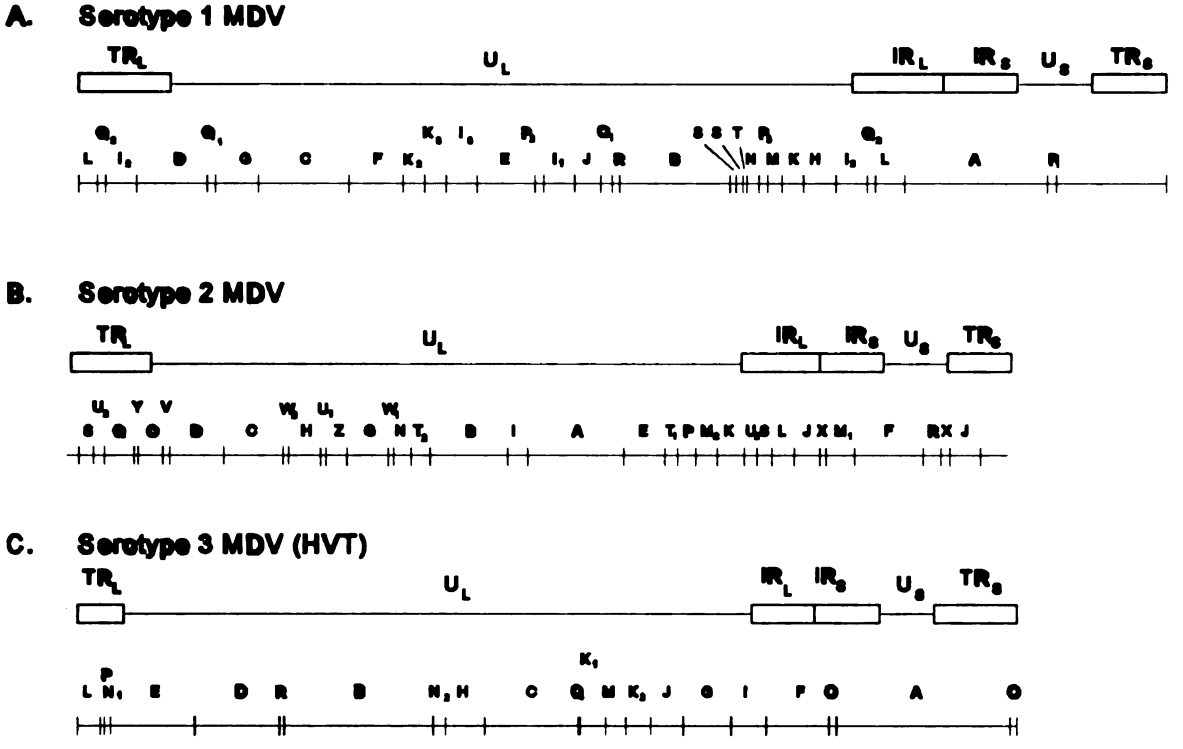


**Figure 1.1** *Bam*HI restriction endonuclease maps of (A). Serotype 1 MDV, (B). Serotype 2 MDV, and (C). Serotype 3 MDV.

longer has the ability to spread by contact (Schat et al., 1985). Attenuated MDV grows faster than wild type MDV in cell culture, contains an altered genomic structure and size, and displays reduced expression of glycoprotein C (gC) when compared to oncogenic strains (Schat et al., 1985; Nazerian, 1980; Wilson et al., 1994).

Attenuation of MDV has been strongly correlated with expansion in the *Bam*HI H and D fragments (present in MDV $TR_L$ and $IR_L$ regions, respectively) (Calnek et al., 1981; Silva and Witter, 1985; Fukuchi et al., 1985; Chen and Velicer, 1991). It was latter discovered that expansion was due to amplification of a 132-bp DR1 sequence (Maotani et al., 1986). Tumor induction studies in susceptible birds suggested that cloned virus populations which exhibit an amplification of the 132 bp repeat region have decreased tumorigenic capability. In contrast, viruses which do not contain amplified *Bam*HI H or D regions efficiently induced tumors in chickens (Fukuchi et al., 1985). Another structural difference between oncogenic and attenuated MDV is a 200 bp deletion in the *Bam*HI L fragment, located in $IR_L$ and $TR_L$ (Wilson and Coussens, 1991). However, *in vivo* studies indicated that the 200 bp deletion of attenuated Md11 did not contribute directly to MDV oncogenicity.

Expression of MDV gC is greatly reduced after serial *in vitro* passage of MDV (Bulow and Biggs, 1975; Churchill et al., 1969; Ikuta et al., 1983; Nazerian, 1980; Silva and Lee 1984; Wilson et al., 1994). Coincident with reduced gC expression, MDV serotypes 2 and 3 lose vaccine efficacy and serotype 1 becomes attenuated with respect to oncogenicity (Calnek and Witter, 1991; Churchill et al., 1969; Silva and Witter, 1985). Reduced gC expression in attenuated strains is not associated with structural changes

within the MDV genome, but rather due to reduced transcriptional efficiency (Wilson et al., 1994). The precise mechanism responsible for decreased transcription of the gC gene in attenuated MDV is not clearly understood at this time.

## 1.7 MDV gene expression

As with other herpesviruses, MDV genes have been classified into three temporal classes, immediate-early (IE or α), early (E or β), or late (L or γ) genes, based on requirements for viral protein synthesis or DNA replication (Honess and Roizman, 1974; Nazerian and Lee, 1974; Schat et al., 1989; Maray et al., 1988).

### 1.7.1 Immediate-early genes

The earliest viral genes expressed, IE genes, are expressed immediately after infection and do not require *de novo* viral protein synthesis. IE gene products activate viral transcription and their transcripts accumulate in the presence of cycloheximide (CHX), a protein synthesis inhibitor. The expression of IE transcripts is affected by enhancer elements. For example, in HSV-1 and probably in MDV, IE gene expression is mediated by a virion-associated transcriptional activator (VP16) ( Boussaha et al., 1996; O'Hare and Goding, 1988). VP16 itself does not bind DNA directly, but interacts with a cellular octamer binding factor (Oct-1) through its cognate recognition sequences, which includes nucleotides found just 5' of a **TAATGARAT** (R is purine) element found in HSV-1 IE gene promoters (O'Hare and Goding, 1988).

MDV IE gene products have been detected in infected cells by CHX reversal

experiments. Four MDV-IE genes have been identified which are clustered primarily in the repeat regions (Schat et al., 1989; Maray et al., 1988),. One such gene MDV-ICP4 has been identified (Anderson et al., 1992) and is a homolog of HSV-1 ICP4. MDV ICP4 is located within the *Bam*HI A fragment.

An MDV specific 14 kDa phosphoprotein (pp14) encoded by a 1.4 or 1.6 kb IE transcript has also been reported (Hong and Coussens, 1994). This protein is MDV serotype 1 specific, and is transcribed as a spliced transcript from *Bam*HI H and $I_2$ fragments. Two other MDV IE transcripts corresponding to gK and ICP27 genes have been reported (Ren et al., 1994). The function and the activity associated with these four IE proteins remains to be determined, but in other herpesviruses IE proteins are usually required for transcription of early genes.

### 1.7.2 Early genes

Early genes become active at approximately 3 hours post-infection and require the activity of IE genes products. Early proteins are required for nucleotide precursor metabolism and viral DNA synthesis (Roizman and Sears, 1991). Some MDV early genes, such as thymidine kinase, DNA polymerase, and DNA binding proteins have been identified by homology to HSV genes (Buckmaster et al., 1988; Sui et al., 1995).

Several MDV specific early genes have been identified in repeats. One such MDV specific early gene product is a 38-kDa phosphoprotein (pp38) that is transcribed from the *Bam*HI H fragment in the leftward direction (Cui et al., 1991; Chen et al., 1992) as a 1.9-kb unspliced transcript (Cui et al., 1991). Since *Bam*HI H is partially located in

the $IR_L$ (Figure 1.1), another gene which encodes a 24 kDa phosphoprotein (pp24) (Zhu et al., 1994) has been found to be transcribed in the rightward direction from the *Bam*HI D fragment (partially located in the $TR_L$, Figure 1.1) (Becker, et al., 1994; Zhu et al., 1994). A third MDV specific gene, *meq*, encodes a protein with high homology to the *fos/jun* family of oncogenes and is transcribed from the *Bam*HI $I_2$ fragment (located in $IR_L$)(Jones et al., 1992). Appearance of early proteins signals onset of viral DNA synthesis, and subsequently induces late gene expression.

### 1.7.3 Late genes

Late gene expression requires both viral protein synthesis and viral DNA replication (Wagner, 1991). While early genes encode proteins required for viral DNA replication, late genes encode structural proteins required for virion, capsid, tegument, and envelop assembly (Roizman and Sears, 1991). In HSV, eight glycoproteins, gB, gC, gD, gE, gG, gH, gI, and gJ have been identified. HSV glycoproteins are involved in virus attachment, penetration and cell-to-cell fusion (Roizman and Sears, 1991).

MDV contains several homologs of these HSV glycoproteins; gB (B-antigen) (Ross et al., 1989), gC (A-antigen) (Coussens and Velicer, 1988; Isfort, et al., 1987), gD and gI (US6 and US7, respectively) (Ross et al., 1991; Brunovskis and Velicer, 1995), gE (US8) (Brunovskis and Velicer, 1995), gH (Scott et al., 1993), gK (Ren et al., 1994), and gL (Yoshida et al., 1994). Among these glycoproteins, gB and gC have been studied at the antigenic level. The gene encoding MDV gB has been mapped to the *Bam*HI $K_3$ and $I_3$ regions and its gene product is processed into a family of proteins known as gp100,

gp60, and gp49 (Chen and Velicer, 1991; Ross et al., 1989). Antibodies against MDV gB can neutralize MDV in cell culture and a recombinant fowl pox virus (FPV) expressing the MDV gB homolog provides 100% protection against Marek's disease in vaccinated chickens (Nazerian et al., 1992).

### 1.8 Status of Marek's disease virus genomes

The status of MDV genomes differ between productively and latently infected cells. It has been reported that in latently infected cells episomal forms of viral DNA, exist exclusively (Tanaka et al., 1978; Hirai et al., 1981). Whereas Hirai et al. (1986), reported the coexistence of episomal and integrated copies. Wilson and Coussens, 1991, using pulsed field electrophoresis, reported that MDV genomes are mostly linear in CEF productively infected with low and high passage Md11. In contrast, recent evidence suggests that MDV genomes in six MDV lymphoblastoid cell lines and MDV tumors are integrated into cellular chromosomes, but episomal forms also existed (Delecluse and Hammerschmidt, 1993; Delecluse et al., 1993). MDV integration sites appear random, but telomeres of large and mid-sized chromosomes appear to be preferential targets (Delecluse and Hammerschmidt, 1993). Based on these observations and the fact that EBV integrates in a number of Burkitt's lymphoma cell lines, MDV DNA integration has been hypothesized to play a major role in cell transformation, presumably by altering cellular gene expression at the site of integration.

The DNA of several oncogenic viruses are methylated in non-producer cell lines, but not in productively infected cells (Doerfler, 1981). The MDV genomes present in

MSB-1 and RP-1 lymphoblastoid cell lines have been shown to be methylated at sites within the repeat regions (Kanamori et al., 1987). This was the first report describing methylation of MDV genomes in MDV lymphoblastoid cell lines and presents a possible explanation for the limited MDV gene expression in these cell lines. Hypermethylation of MDV DNA during latent infection may be a mechanism by which MDV regulates transcription. Treatment of MSB-1 cells with 5-azacytidine resulted in hypomethylation of viral DNA, and increased mRNA transcription from the *Bam*HI H region of the genome (Hayashi et al., 1994 and 1995). Therefore, in transformed cells the low level of transcription may be due to the hypomethylation of the viral genomes.

## 1.9 Latency

One of the most distinguish properties of herpesviruses is their ability to establish life-long latent infections in their natural hosts (Rock, 1993). Latency has been defined clinically as the persistence of a virus in a host in the absence of overt, productive viral infection. This virus/host relationship was initially described *in vivo* in the presence of a host's immune response. For most herpesviruses, these interactions cannot be studied *in vitro*, in the absence of a host's systemic influence.

### 1.9.1 Herpesvirus latency

Members of the herpesvirus family can be classified either as neurotropic or lymphotropic, depending on the tissue type in which the virus establishes a latent infection (Garcia-Blanco and Cullen, 1991). The precise mechanism by which

herpesviruses establish, maintain, and reactivate from a latent state are not known. Alpha herpesvirus latency occurs primarily in neurons. Because neurons are nondividing cells these herpesviruses do not need to replicate in order to maintain a latent state (Baichwal and Sugden, 1988). Betaherpesvirus can establish a latent infection in kidney, secretory glands, lymphoreticular cells, and other tissues. Whereas gammaherpesvirus latency is confined to lymphoid tissues (Roizman, 1991). Latent viruses must guarantee that copies of their genome are transferred to daughter cells of dividing lymphocytes, even though their genomes may not be integrated into the host chromosome.

Studies of the replication cycles of various herpesviruses suggest that latent infections may result from an absence of host factors critical for the expression of viral early gene products. Therefore, activation of these host factors in response to extracellular stimuli can induce expression of these viral proteins and lead to reactivation of latent infection to a lytic infection (Garcia-Blanco and Cullen, 1991). Latent infection in most herpesviruses results in the transcription of a very restricted portion of the viral genome. HSV establishes latent infections in neurons of sensory ganglia. A family of transcripts, latency associated transcripts (LATs), which map antisense to an IE gene (ICP0) and accumulate during latency, have been identified in nerve ganglia (Feldman, 1991; Spivack and Fraster, 1987; Stevens et al., 1987). In pseudorabies virus, which establishes latency in trigeminal ganglia of swine (Beran et al., 1980; Gutekust, 1979), LATs which map antisense to the ICP4 homolog have been reported (Priola et al., 1990).

### 1.9.2 MDV latency

Although MDV genomic structure is similar to that of alpha herpesviruses, latent infection of MDV occurs primarily in lymphocytes, which is similar to the gammaherpesviruses (Payne, 1985). As noted earlier, MDV infection switches from a lytic infection of B-lymphocytes to a latent infection of T-lymphocytes at about one week post-infection, and persists in the host for its lifetime.

MDV transformed lymphoblastoid cells are immortalized cell lines which are latently infected with MDV. These cell lines are usually capable of transferring MDV to CEF or DEF *in vitro* and to susceptible chickens *in vivo* (Schat et al., 1985). MDV transformed cell lines are divided into producer and non-producer cell lines, based upon virus recovery and viral antigen expression. Producer cell lines are further divided into expression and non-expression cell lines, based on the proportion of cells which express viral antigens. Expression cell lines, such as MSB-1, contain cells expressing antigens which can be detected by immunofluorescence antibody (IIFA) assays. However, non-expression cell lines, such as MKT-1, contain no or few cells expressing viral antigens. Treatment of producer cell lines with 5-iodo-2-deoxyuridine (IudR) induces a higher level of viral antigen expression. Producer cell lines can transfer MDV infection to CEF and DEF *in vitro* and to susceptible chickens *in vivo*. In non-producer cell lines, such as RP-1, viral antigens are not detectable and virus can not be rescued by co-cultivation.

In MDV lymphoblastoid cell lines, expression of a limited set of viral genes has been detected (Maray et al., 1988; Sugaya et al., 1990). Transcriptional activity is limited

to approximately 20% of the MDV genome, primarily the repeat regions and adjacent sequences. However, different numbers of transcripts have been reported by several groups using different MDV lymphoblastoid cell lines. Maray et al. (1988) reported 29 transcripts in MSB-1 cells (an expression cell line). Whereas, Schat et al., (1989) reported 4 and 7 transcripts in HP1 (non-producer cell line) and CU41 (non-expression cell line), respectively. Most transcripts found in MDV lymphoblastoid cells are from IE genes (Silver et al., 1979; Schat et al., 1989), suggesting that IE genes could have a significant role in the maintenance of latency.

Consistent with transcripts identified in Northern blot analysis, four gene products have been recently identified in MDV lymphoblastoid cell lines. A 38 kDa phosphoprotein (pp38) was first identified as one of three viral proteins (40, 38, and 24 kDa) detected by monoclonal antibody against a λgt11 fusion protein (Silva and Lee, 1984). The gene encoding pp38 is located in the *Bam*HI H fragment and is transcribed in a leftward direction (Cui et al., 1991; Chen et al., 1992). The pp38 gene was identified as a 1.9-kb transcript, and found to be transcribed as an E gene (Chen et al., 1992). The pp38 gene product is localized in the cytoplasm of serotype 1 (oncogenic and attenuated) infected cells (Silva and Lee, 1984; Cui et al., 1991; Chen et al., 1992), MDV lymphoblastoid cell lines prior to (Chen et al., 1992) and following IudR treatment (Nakajima et al., 1987), MDV OU2 cell lines (Abujoub and Coussens, 1995), and tumor lesions of MD affected chickens (Nakajima et al., 1987). Even though pp38 cannot be detected in MDV serotype 2 (Nakajima et al., 1987) or serotype 3 (Nakajima et al., 1987; Chen et al., 1992) infected cells, a pp38 homolog has been identified in MDV serotype 2

(Ono et al., 1994) and 3 (Smith et al., 1995). It is possible that the transforming ability of serotype 1 MDV, may be attributed to a difference between the three MDV serotype pp38 genes. Another gene which encodes a 24 kDa phosphoprotein (pp24) ( Zhu et al., 1994) has been found to be transcribed in the leftward direction from *Bam*HI D fragment (located in the $TR_L$). The N-terminal 65 amino acids of pp38 and pp24 were found to be identical (Becker, et al., 1994; Zhu et al., 1994), but the function of these two genes has not been determined.

Another MDV phosphoprotein, pp14, was identified and mapped to the *Bam*HI H and $I_2$ fragments, within the $IR_L$ region of MDV genome (Hong and Coussens, 1994). pp14 is transcribed as 1.6 kb spliced transcripts with IE kinetics. Similar to pp38, pp14 is expressed in the MDV lymphoblastoid cell line, MSB-1, serotype 1 (oncogenic and attenuated) infected cells (Hong and Coussens, 1994, Hong et al., 1995), and in MDV OU2 cell lines (Abujoub and Coussens, 1995). The pp14 gene product is localized to the cytoplasm, and appears to be serotype 1 specific (Hong et al., 1995). Detailed mapping studies and sequence analysis has revealed that pp38 and pp14 share a promoter-enhancer region (Cui et al., 1991). Organization of this region suggests that pp38 and pp14 form a divergent transcription unit (Hong and Coussens, 1994).

A fourth gene, designated *meq*, encodes a protein with high homology to the *fos/jun* family of oncogenes and is transcribed from the *Bam*HI-$I_2$ and *Eco*RI Q fragments (Jones et al., 1992). *meq* encodes a 362 amino acid polypeptide and contains a basic region and a leucine zipper domain. Using antisera raised against a synthetic peptide corresponding to the leucine zipper region of *meq*, a 40 kDa protein was detected in the

RP4 MDV lymphoblastoid cell line (Jones et al., 1992). Recently, Qian et al., (1995) reported that *meq* behaves like a transcriptional activator when fused to the GAL4 DNA binding domain.

As mentioned earlier, LATs which map antisense to IE genes have been described for many herpesviruses. Recently, a series of mRNAs mapping antisense to the MDV ICP4 gene (MDV LATs) have been detected in various MDV lymphoblastoid cell lines (MSB-1, RPL1, and MKT-1 cells) (Cantello et al., 1994; Li et al., 1994; Mckie et al., 1995). LATs are a family of transcripts that may represent processing products of a 10-kb RNA (Cantello et al., 1994). LAT transcripts appear to be expressed at higher levels in lymphoblastoid cell lines (MSB-1 and RPL1) and are down regulated in lytically infected cells (Li et al., 1994). It has been postulated that antisense ICP4 transcripts may regulate expression of MDV genes, and are therefore important for maintenance of the latent state. MDV LAT expression in MSB-1 and RPL1 is relatively high compared with ICP4 expression (Cantello et al., 1994; Li et al., 1994). However, upon virus reactivation induced by adding IudR to MSB-1 cells, steady state ICP4 RNA levels increased and MDV LATs expression decreased (Cantello, et al., 1994). MDV LATs expression was not detected early in lytic infection, but MDV LAT expression was relatively high at later times during lytic infection (140 hours post-infection) when CEF cells were older and possibly depleted of some necessary cellular factors required for lytic infection (Cantello et al., 1994). Although it is possible that MDV LATs play a role in maintenance of latency, definitive evidence is still lacking.

## 1.10 Objectives

Two major difficulties in working with MDV are the strongly cell associated nature of the virus and lack of a sustainable cell culture system amenable to productive (lytic) infections. Primary CEF and DEF cells are permissive for MDV replication. However, these cultures have a finite life span (approximately 3 weeks), thus requiring passage of infected primary cells onto an uninfected cell monolayer to propagate MDV and to obtain sufficient quantities of virus with which to work. Such conditions also preclude establishment of one-step growth experiments for effective temporal gene regulation studies. The finite life span of CEF and DEF also make positive selection in mutagenesis studies difficult. The main objective of this study is to develop a sustainable chick cell line system, which supports MDV growth and replication. Development of a continuous cell line which will support MDV replication would alleviate many of the difficulties associated with MDV experimentation and vaccine production.

# Chapter 2

## Development of a sustainable chick cell line infected with Marek's disease virus

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## 2.1 Abstract

Marek's disease virus (MDV), is a highly infectious and cell associated avian herpesvirus. Fully productive infections with MDV are restricted to feather follicle epithelium of afflicted birds. In culture, MDV infection of primary chick and duck embryo fibroblast (CEF and DEF, respectively) cells is semi-productive. Passage of MDV and production of MDV vaccines is limited to these primary cell-associated systems. The finite life span of primary avian cell cultures has hampered efforts to use positive selection in generation of recombinant MDV and complicates studies of temporal gene regulation.

In this report, we describe continuous chick fibroblast cell lines (MDV OU2.2 and MDV OU2.1) which support MDV replication. Southern blot and PCR analyses demonstrate that these cell lines harbor MDV DNA. Western blot analyses indicate that MDV OU2.2 cells express at least a limited set of viral proteins, pp38 and pp14, similar to that seen in MDV lymphoblastoid cells. Presence of distinct plaques in confluent MDV OU2.2 cell monolayers is consistent with cytolytic semi-productive infection, similar to that observed in primary CEF. MDV OU2.2 cells are capable of transferring MDV infection to primary CEF cultures and inducing clinical signs of Marek's disease (MD) in susceptible birds. MDV OU2.2 cells have maintained a MDV positive phenotype for over 16 months of active culture. Southern blot hybridization of MDV OU2.2 cell DNA reveals a distinct expansion of the MDV *Bam*HI H fragment in a subset

of viral genomes following long-term cultivation.

## 2.2 INTRODUCTION

Marek's disease (MD) is a highly contagious lymphoproliferative disease of chickens, characterized by lymphocytic infiltration in visceral organs, muscles, and peripheral nerves. The etiological agent of MD, an avian herpesvirus called Marek's disease virus (MDV), is highly infectious and cell associated (Calnek and Witter, 1991). MDV replicates in a productive restrictive manner in B-lymphocytes and cells growing in tissue culture. Production of fully enveloped virus is restricted to feather follicle epithelium of infected birds (Witter et al., 1972; Calnek et al., 1970). MDV rapidly establishes a latent infection in T-lymphocytes, ultimately leading to malignant transformation and neoplastic disease (Shek et al., 1983). However, the precise relationship between latency and transformation in MDV infected T-lymphocytes is unknown. Akiyama et al. (1973) first succeeded in establishing a T-lymphoblastoid cell line from MD-infected chickens. Since then, more than 80 cell lines have been produced from MD lymphomas (Akiyama et al., 1974; Powell et al., 1974; Calnek et al., 1978; Payne et al., 1981; Nazarian and Witter, 1975). Although suitable for some studies, these cell lines are many passages removed from the original event(s) leading to transformation.

Evidence suggests that viral genomes in MD-lymphoblastoid cell lines are predominately integrated into cellular chromosomes, but episomal forms also exist (Delecluse and Hammerschmidt, 1993). Analysis of viral transcription in transformed

lymphoblastoid cell lines has revealed variable but limited transcriptional activity confined to approximately 20% of the viral genome (Maray et al., 1988). MDV-specific transcripts in transformed lymphoblastoid cells are primarily derived from within long and short region terminal repeats ($TR_L$, and $TR_S$, respectively) and internal repeats ($IR_L$, and $IR_S$, respectively). Little transcriptional activity is detected within either the long unique ($U_L$) or short unique ($U_S$) regions (Sugaya et al., 1990). MDV can be rescued from some lymphoblastoid cell lines by co-cultivation with primary or secondary chicken and duck embryo fibroblasts (CEF and DEF, respectively), which support the lytic cycle of MDV *in vitro* (Schat et al., 1989). In addition, some lymphoblastoid cell lines will induce MD upon injection into susceptible birds (Akiyama et al., 1973; Nazarian et al., 1977).

Two major difficulties in working with MDV are the strongly cell associated nature of the virus and lack a sustainable cell culture system amenable to productive (lytic) infections. Primary CEF and DEF are permissive for MDV replication. However, these cultures have a finite life span (approximately 3 weeks), Thus necessitating passage of infected primary cells onto an uninfected cell monolayer to propagate MDV and to obtain sufficient quantities of virus with which to work. Such conditions also preclude establishment of one-step growth experiments for effective temporal gene regulation studies. The finite life span of CEF and DEF also make positive selection in mutagenesis studies difficult. Development of a continuous cell line which will support MDV replication would alleviate many of the difficulties associated with MDV experimentation and vaccine production.

In this report, we detail establishment and characterization of continuous chick fibroblast cell lines (MDV OU2.1 and MDV OU2.2), stably infected with MDV strain Md11 at passage level 15. MDV OU2.1 and MDV OU2.2 cells grow continuously in culture and, once confluent, display plaques characteristic of MDV infection. MDV OU2.1 and MDV OU2.2 cells can be used to transfer infection to CEF, and produce classic symptoms of MD in susceptible birds. MDV OU2.2 cells have remained viable and continue to produce MDV after cryogenic storage and continuous culture for over 16 months.

## 2.3 Materials and Methods

### 2.3.1 Cells and Virus

Preparation, propagation, and infection of CEF cells with MDV were performed as described previously (Glaubiger et al., 1983; Coussens et al., 1988). The very virulent MDV strain Md11 was used in this study at cell culture passage level 15 (Md11p15). CHCC-OU2 cells (Ogura and Fujiwara, 1987) were obtained from Dr. Donald Salter, Avian Disease and Oncology Laboratories (ADOL), U.S. department of Agriculture (USDA), East Lansing, Michigan, and were cultured in Leibovitz L15-McCoy 5A (LM) media supplemented with 10% fetal bovine serum (FBS) and 2% tryptose phosphate broth (TPB) at 37 °C in a humidified atmosphere containing 5% $CO_2$.

CHCC-OU2 cells were infected with MDV strain Md11p15 by combining $5.0X10^7$ CHCC-OU2 cells with $2.0X10^7$ Md11p15 infected CEF prior to plating on 150 mm culture dishes in LM medium supplemented with 4% calf serum (CS). Co-

cultivation of CHCC-OU2 cells with Md11 infected CEF cells was continued for four passages. Cells from each of these passages have been preserved at -135 °C in freezing media (LM media supplemented with 20% CS and 10 % dimethyl sulfoxide (DMSO). At four passages post-infection, numerous plaques (approximately 100 plaques per 150mm culture dish), characteristic of MDV infections in CEF cells, were observed. Two of these plaques were isolated using sterile cloning cylinders. Cylinders were placed on top of individual plaques, cells were trypsinized and aspirated from the cloning cylinders. Aspirated cells were transferred to 35 mm culture dishes containing LM media supplemented with 4% CS for expansion. During expansion, cells were not allowed to become confluent and media was changed every 48 to 72 hours. Expanded clones were designated MDV OU2.1 and MDV OU2.2.

### 2.3.2 Preparation of cellular DNA, Southern blot analysis, and PCR

Total cellular DNA was extracted from uninfected and MDV-infected CHCC-OU2 cells by standard methods (Sambrook et al., 1989). Restriction enzymes (Boehringer Mannheim Biochemicals, Indianapolis, IN) were used according to the manufacturers recommendation. DNA was digested, electrophoresed through 0.8% agarose gels and transferred to Hybond-N or Zeta-probe nylon membranes (Amersham Corp., Arlington Heights, IL. and Bio-Rad Corp., Hercules, CA, Respectively) by Southern blotting (Southern, 1975). Probes were non-radioactively labeled (Digoxigenin-11-dUTP) using a random primer labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Total cellular DNA was also used as template for PCR amplification of MDV specific sequences. Primers used and expected fragment sizes are indicated in Table 1. Briefly, 300 ng of total cellular DNA was combined with 25 mM each dNTP (dATP, dCTP, dGTP, and dTTP), 20 μM of each appropriate primer pair, 10 μl of 10X PCR reaction buffer (Perkin Elmer Cetus, Norwalk, Connecticut), and 2.5 Units Taq polymerase (Perkin Elmer Cetus, Norwalk, Connecticut). PCR reactions were performed using a GeneAmp 9600 thermal cycler (Perkin Elmer Cetus, Norwalk, Connecticut) as follows: 35 cycles of 95 °C for 20 sec, 56 °C for 20 sec., and 72 °C for 30 sec. Two controls, one without DNA and one with uninfected CHCC-OU2 DNA were included in each experiment. High molecular weight DNA isolated from uninfected CEF and CEF infected with Md11p16 were used as controls for specific amplification. PCR products were purified using the Wizard PCR prep kit (Promega Inc., Madison, Wisconsin) as recommended by the manufacturer) and analyzed on 1.2% agarose gels.

### 2.3.3 Western immunoblot analysis

Cultured cells were collected and sonicated using a Sonifier cell disrupter model 350 (Branson Ultrasonic Corporation, Danbury, Connecticut). Proteins (20 μg) from each cell type were separated on 12.5% polyacrylamide/1% SDS gels. Separated proteins were electrophoretically transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk and probed with antibodies to MDV proteins: pp14 (Hong and Coussens, 1994), and pp38 (Cui et al. 1991). Immune complexes were detected by incubation with a donkey anti-mouse or anti-rabbit immunoglobulin conjugated with

horseradish peroxidase. Detection was performed using an ECL Western blot kit (Amersham Corp., Arlington Heights, IL) according to the manufacturer's recommendations and exposed to X-ray film. Protein sizes were estimated by comparison to prestained protein molecular weight standards (Bio-Rad, Richmond, Ca.) electrophoresed on the same gel.

### 2.3.4 Inoculation of chickens with cells and virus

*In vivo* experiments were performed using specific pathogen free chickens (line $15I_5$ X $7_1$), obtained from the Avian Disease and Oncology Laboratory, US Department of Agriculture, East Lansing, Michigan. Three groups of chicks each at one day of age, were inoculated intraperitonealy with (1) uninfected CHCC-OU2 cells, (2)1000 plaque forming units (PFU) of Md11p16 in CEF, and (3) 1000 PFU of MDV infected OU2 cells (MDV OU2.2). The first and second groups served as controls (negative and positive, respectively).

Birds were euthanized and necropsied upon signs of morbidity. Blood was collected for isolation of peripheral blood lymphocytes and co-cultivation with CEF as an assay for viable virus. Kidney, spleen, and liver were harvested for DNA isolation and histological evaluation. For histological examination, kidney tissues were fixed in 4% paraformaldehyde and processed using a Fisher model 166 Histomatic Tissue Processor (Fisher Scientific. Pittsburgh, PA). Five micron tissue sections were subsequently stained with hematoxylin and eosin. Total tissue-specific DNA was isolated and used as template for PCR amplification of MDV sequences employing primer sets detailed in

Table 1.

## 2.4 RESULTS

### 2.4.1 Infection of CHCC-OU2 cells with Md11p15

Although CHCC-OU2 cells are chemically immortalized, they are not malignantly transformed, maintain contact inhibition, and exhibit many morphological features of normal chick fibroblasts. These properties led us to reason that CHCC-OU2 cells might be susceptible to infection by MDV. To test this hypothesis CHCC-OU2 cells were co-cultivated with Md11p15 infected CEF cells. Cytopathic effect (CPE), characterized by formation of spherical cells loosely attached to the substratum, was first observed on co-cultivation cell monolayers at two weeks post-infection. The majority of these regions were characterized as "microplaques", consisting of relatively small clusters of rounded cells. CPE was slow in developing and expanding. Fully developed plaques consisting of syncytia and extended regions of rounded, loosely attached, cells were not visible until four weeks post-infection (Figure 2.1). By comparison, a typical CEF monolayer infected with MDV strain Md11 will develop readily visible plaques in 5-7 days post-infection with complete destruction of the monolayer within 10-14 days. After four weeks of co-cultivation, cells were cryogenically preserved at -135 °C for two weeks. Cell cultures were re-established from frozen cells by combining infected (Md11p15/OU2) and uninfected CHCC-OU2 cells. Plaques consistent with MDV infection were not observed until cells reached confluence, approximately 14 days post-plating.

### 2.4.2 Detection of MDV DNA in infected CHCC-OU2 cells

Polymerase chain reaction (PCR) was used as an initial assay for presence of MDV DNA in infected CHCC-OU2 cells. Three hundred ng of total DNA from Md11p15/OU2 and uninfected CHCC-OU2 cells was used as template for PCR amplification with several primer pairs corresponding to various MDV genes, as described in Materials and Methods. Bands of appropriate sizes (Table 2.1) were obtained in reactions with Md11p15/OU2 DNA but not from uninfected OU2 DNA templates (Figure 2.2). Reactions containing DNA isolated from uninfected CEF and Md11 infected CEF were used as negative and positive controls, respectively (data not shown).

Results of PCR analyses suggested that MDV DNA was present in infected CHCC-OU2 cultures. Although unlikely, given our extended culture conditions, PCR analysis could have detected MDV DNA from residual Md11p15 infected CEF cells. In addition, PCR analyses do not provide critical information on integrity of MDV DNA in Md11p15/OU2 cells. To address these concerns, MDV DNA in infected CHCC-OU2 cells was analyzed by Southern blot hybridization using a cocktail of MDV *Bam*HI fragments (B, F, H, and $I_2$) as a probe. Total genomic DNA isolated from Md11p16 infected CEF and Md11p15/OU2 cells contained MDV specific fragments corresponding to *Bam*HI fragments B, F, H and $I_2$. As expected, similar fragments were not detected in DNA isolated from uninfected CEF or CHCC-OU2 cells (Figure 2.3 Panel A).

Continuous passage of oncogenic MDV strains in primary cell culture leads to attenuation of the virus with a concomitant heterogeneous expansion of repeat regions

within *Bam*HI fragments H and D. To determine if expansion of *Bam*HI-H and -D occurred in MDV DNA isolated from highly passaged MDV OU2.2 cells, Southern blot hybridization was performed on DNA isolated from OU2.2 cells which had been in continuous culture for almost 16 months (passage 12). Using *Bam*HI fragment H as probe, both *Bam*HI-H and -D fragments are clearly visible in DNA isolated from Md11p16 infected CEF cells (Figure 2.3 Panel B). In high passage Md11p86 infected cell DNA, *Bam*HI fragment H is highly heterogenous, consistent with published reports (Silva and Witter, 1985; Fukuchi et al., 1985 ). Expansion of *Bam*H I D is also evident in DNA isolated from CEF infected with high passage Md11(Figure 2.3 Panel B), although expansion of *Bam*H I D appears to be more homogeneous. DNA isolated from MDV OU2.2 cells at passage 12 contained discrete *Bam*HI-H and -D bands (Figure 2.3 Panel B), similar to those observed in low passage (oncogenic) Md11p16. A distinct band at 5.8 kb, visible in DNA isolated from MDV OU2.2 cells, may be due to specific expansion of the *Bam*H I H fragment (Figure 2.3 Panel B). No expansion of *Bam*HI D is evident in this sample. These results indicate that MDV DNA in MDV OU2.2 cells is relatively stable, at least through passage 12 and over many months of continuous culture.

### 2.4.3 Establishment of infected cell lines

Although our culture conditions and freeze-thaw cycles should have eliminated most of the original CEF cells used for establishing infection, it was possible that residual CEF cells were contributing to MDV specific DNA detected in our Md11p15/OU2 cultures. To address this concern, isolation and expansion of individual

plaques from infected CHCC-OU2 cultures was initiated. Two Md11p15/OU2 cell lines (MDV OU2.1 and MDV OU2.2) were established by plaque isolation and expansion as described in Materials and Methods. Despite arising from distinct plaques, both cell lines exhibited initial growth characteristics indistinguishable from uninfected CHCC-OU2 cells. Plaques characteristic of MDV infection were only observed in MDV OU2.1 and MDV OU2.2 cell cultures after confluence had been reached at 10 to 14 days post-plating.

To confirm infectious virus could be rescued from MDV OU2.1 and MDV OU2.2 cultures, cells from each isolate were used as inoculum to infect CEF cells by combining $1x10^6$ MDV OU2.1 or MDV OU2.2 with $5x10^7$ secondary CEF. Numerous ($>10^3$) plaques, consistent with MDV infection of CEF cells were visible within 5 days post co-cultivation. In contrast, no plaques were evident in control plates containing $1x10^6$ uninfected CHCC-OU2 cells and $5x10^7$ CEF cells (data not shown). In subsequent studies, the yield of virus from MDV OU2.2 cells has remained at $10^3$ to $10^4$ PFU/$10^6$ cells as determined by transfer to CEF monolayers.

### 2.4.4 Detection of viral proteins expressed in MDV OU2.2 cells

To verify that MDV OU2.2 cells indeed supported replication and growth of MDV, detection of MDV proteins was initiated. Monoclonal antibody H19.47 (Cui, et al., 1990) against MDV pp38 (generous gift from Dr. Lucy Lee, USDA-ADOL, East Lansing, Michigan), specifically recognized polypeptides of approximately 24, 38, and 41 kDa in extracts from CEF cells infected with Md11p15, consistent with previous reports

(Cui et al., 1990; Zhu et al., 1994 ). A 38 kDa protein was also identified in extracts from MDV OU2.2 cells (Figure 2.4 Panel A). Although proteins of 24 and 41 kDa were not detected in MDV OU2.2 cell extracts, a protein with an apparent size of 84 kDa was specifically recognized by monoclonal antibody H19.47 in these extracts (Figure 2.4 Panel A). The origin of this larger protein is, at present, unknown. Polyclonal antisera to pp14, an MDV-specific immediate-early phosphoprotein (Hong and Coussens, 1994) also reacted with an appropriately sized polypeptide in extracts from confluent MDV OU2.2 cells and Md11p16 infected CEF cells but not in uninfected cell extracts (Figure 2.4 Panel B). A polyclonal antisera to β-actin was used to ensure similar amounts of protein were analyzed in each lane (Figure 2.4 Panel C). Taken together, results of PCR amplification, Southern hybridization, and Western blot analysis indicated that MDV OU2.2 cells represent a continuous anchorage dependent cell line which harbors MDV and is permissive for semi-productive infection.

### 2.4.5 MDV OU2.2 cells induce MD in susceptible chickens

Marek's disease may be experimentally induced by injection of MDV infected cells into susceptible birds. MDV lymphoblastoid cell lines such as MSB-1 are also able to induce MD in susceptible birds following intraperitoneal injection. To determine if MDV OU2.2 cells could be used in a similar manner, line $15I_5$ X $7_1$ chickens were inoculated with uninfected CHCC-OU2 cells, MDV OU2.2 cells, or Md11p16 infected CEF cells at one day of age. Chickens injected with either Md11p16 infected CEF or with MDV OU2.2 cells developed classical signs of MD (reduced growth and paralysis of

neck, wings, and legs) and had to be euthanized at 10 days post-infection. In contrast, negative control chickens injected with uninfected CHCC-OU2 cells showed no clinical signs of MD, even at 12 weeks of age.

To confirm presence of MDV in infected birds, peripheral blood lymphocytes (PBLs) were isolated from blood collected at various times post-inoculation and seeded onto secondary CEF. Plaques consistent with MDV infection were observed on CEF monolayers at 4 days post-culture on plates seeded with PBLs isolated from birds injected with Md11p16 infected CEF or MDV OU2.2 cells. No plaques were observed on plates of CEF cells mixed with PBLs obtained from control birds injected with uninfected CHCC-OU2 cells.

To further confirm replication of MDV in infected birds, total cellular DNA isolated from infected bird kidneys was used as template for PCR amplification using primers specific for the MDV pp38 gene. Consistent with the presence of MDV DNA an 850 bp fragment was amplified using DNA isolated from kidneys of birds injected with Md11p16 infected CEF or MDV OU2.2 cells. In contrast, similar bands were not detected when DNA from birds injected with CHCC-OU2 cells was used as template (Figure 2.5).

Histological evaluation revealed lymphocytic infiltration and early, active lymphomas in various tissues, including kidney, from birds injected with CEF/Md11p16 and MDV OU2.2 (Figures 2.6 Panel C B, respectively), whereas CHCC-OU2 inoculated birds showed no signs of MD at the microscopic level (Figure 2.6 Panel A). Taken together, these results clearly demonstrate that MDV OU2.2 cells contain MDV and that

the virus may be transferred to birds via intraperitoneal injection.

## 2.5 Discussion

One of the major difficulties in working with MDV is lack of a sustainable cell culture system for virus growth and selection. Although primary CEF and DEF are permissive for MDV replication, primary cultures are characterized by slow growth and limited life span. These factors necessitate continual passage of infected cells onto uninfected cells in order to obtain sufficient quantities of virus with which to work. In addition, CEF and DEF must be prepared on a regular basis from 10 or 11 day old chick embryos, adding significantly to the expense and difficulty of culturing MDV. These same factors add significantly to the expense of producing MDV vaccines.

The CHCC-OU2 cell line is an immortalized fibroblastic cell line derived from chick embryo cells (Ogura and Fujiwara, 1987). CHCC-OU2 are not oncogenic, based on the fact that CHCC-OU2 cells failed to produce tumors when injected into syngeneic chickens (Ogura and Fujiwara, 1987). In addition CHCC-OU2 are virus free and susceptible to avian retrovirus infection (avian sarcoma viruses of subgroups A, B, and C). Newcastle disease virus also replicates well in CHCC-OU2 cell cultures (Ogura and Fujiwara, 1987).

In this report, we provide evidence that CHCC-OU2 cells may be suitable as a sustainable cell culture system for replication and study of MDV. The initial phase of CHCC-OU2 infection with MDV, strain Md11p15, was slow and characterized by a low number of visible plaques. Fully developed plaques were first observed in confluent

cultures at four weeks post-infection. At this time, plaques were clearly visible and quite abundant (approximately 100 plaques/150 mm tissue culture plate). In subsequent passages, plaques were visible every 10-14 days in culture. Following long-term cultivation of clonal MDV infected CHCC-OU2 cells (MDV OU2.2), virus yield has remained constant at $10^3$ to $10^4$ PFU/$10^6$ cells, as determined by transfer to CEF monolayers.

Southern blot and PCR analyses confirmed that clonal cell lines, MDV OU2.1 and MDV OU2.2, indeed harbor MDV DNA. Fragments detected by Southern blot hybridization in DNA from MDV OU2.2 cells were similar in size to those detected in Md11p15 infected CEF, indicating that no gross structural rearrangements had occurred during initial infection. In addition, fragments detected in MDV OU2.2 cell DNA represent diverse regions of the MDV genome, including the unique long (*Bam*HI B and F), terminal repeat long (*Bam*HI D), and internal repeat long (*Bam*HI H and $I_2$) segments. Intensity of these fragments suggests that MDV OU2.2 cells allow MDV DNA replication, as it is highly unlikely the observed amount of DNA would arise from residual CEF cells used to establish initial infections. Subsequent Southern blot hybridization using an MDV *Bam*HI H fragment specific probe indicated that, while no expansion of *Bam*HI fragment D had occurred, a specific expansion of *Bam*HI H had occurred in highly passaged MDV OU2.2 cells (passage 12, 16 months in culture). The nature of this expansion is, at present unknown, but may represent three copies of a previously characterized 132 bp region within *Bam*HI H (Maotani et al., 1986) .

Western blot analyses demonstrated that MDV OU2.2 cells express at least a

limited set of viral proteins, pp38 and pp14. Proteins related to pp38 (pp24 and pp41), which are readily detected by monoclonal antibody H19.47 in extracts from Md11p15 infected CEF, were not observed in extracts from MDV OU2.2 cells. A larger polypeptide of 84 kDa was, however, specifically recognized by monoclonal antibody H19.47 in MDV OU2.2 cell extracts. The origin of this protein is, at present, unknown.

Despite numerous attempts, we were unable to detect MDV glycoproteins gC, gB, gE, and gI by western blot analyses. Each of the particular antisera employed was able to detect the respective protein in Md11p15 infected CEF cell extracts. Thus, results of western blot analyses are consistent with MDV existing in MDV OU2.2 cells as a latent infection, similar to that seen in MDV lymphoblastoid cells. However, presence of distinct plaques in MDV OU2.2 cell monolayers is not consistent with latent infection as this would imply cytolytic activity related to MDV infection. Additional experiments designed to further characterize the state of MDV in MDV OU2.2 cells are currently in progress.

*In vivo* experiments clearly demonstrate that MDV OU2.2 cells are capable of transferring MDV infection to CEF monolayer cultures and inducing clinical signs of MD in susceptible birds. Birds injected with either MDV OU2.2 or Md11p16 infected CEF developed clinical signs of MD, characterized by a marked decrease in growth rate and paralysis of legs, wings, and neck. There was little or no difference in virulence observed in groups inoculated with Md11p15 infected CEF or MDV OU2.2 cells. PCR analysis of tissues, including kidney and spleen, demonstrated that MDV was present in remote tissues of birds injected with MDV OU2.2 cells. In addition, PBLs isolated from birds

injected with MDV OU2.2 cells were able to transfer infection to CEF monolayers. In contrast, no evidence of tumor formation or viremia was observed in birds inoculated with uninfected CHCC-OU2 cells.

Although adding little to our characterization of the state of MDV in MDV OU2.2 cells, results of *in vivo* experiments clearly demonstrate that MDV OU2 cells may be used to establish infections in susceptible birds, a quality of considerable importance for MDV vaccine development and production of MDV mutants by positive selection. Importantly, we have recently succeeded in developing cell lines harboring a turkey herpesvirus (HVT strain FC126) and serotype 2 MDV (strain SB1) (Reilly J. D., Abujoub A.A., and Coussens P.M., unpublished observations). Characterization of these important cell lines is currently in progress.

## 2.6 Acknowledgments

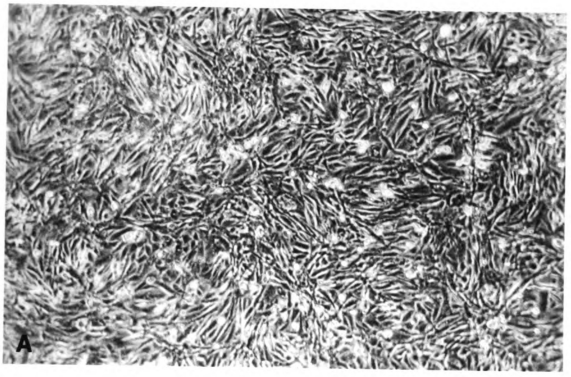
We thank Ron Southwick and Jean Robertson for excellent technical assistance and assistance with computer graphics. We also thank Dr. L.F. Lee for monoclonal antibody H19.47, Donald Salter for providing CHCC-OU2 cells, and Sheila Abner for assistance with histological examination of kidney sections. This work is supported by Research Excellence Funds from the State of Michigan, by grants 94-37204-0935 and 95-37204-2118 awarded to PM Coussens under the Competitive Research Grant program administered by U.S. Department of Agriculture, and by the Michigan Agriculture Experiment Station.

**Table 2.1** Sequence of MDV-specific oligonucleotide primers used in PCR amplification.

| Primer Sequences* | Locus | Expected Size (kb) |
|---|---|---|
| GTAGTGAAATCTATACCTGGG<br>GTGTCTAGAGAGGGAAGATATGTAGAGGGTTAC | gC gene promoter | 0.3 |
| ATGGAATTCGAAGCAGAACAC<br>CTCCAGATTCCACCTCCCCAGA | pp38 gene | 0.85 |
| TGCTAATTGTGGCTCC<br>GGTGCTTCCATCTCGGC | ICP4 gene | 0.9 |
| GATCTAGACGTTTCTGCCTCCGGAGTC<br>GCAAGCTTCAACATCTTCAAATAGCCGCAC | US3 gene promoter | 0.6 |
| GTCTAGACGCGATAGCGAGTTGTTGGACC<br>GGAAGCTTTATTAAGGGAGATTCTACCC | ICP4 gene promoter | 1.1 |
| GTGAAAGAGTGAACGGGAAG<br>CGTCAAAGCGATAATAGGC | *Bam*HI L fragment | 1.20 |
| CCGGGGATCCCGAAATGTCGTTAGAACATC<br>CGGGGTCGACTAAGGCAAATAGGCACGC | UL54 gene | 1.1 |

* Primer sequences are written as 5' to 3', left to right. In each case, the upper primer represents the upstream sequence while the lower primer represents the downstream sequence.

**Figure 2.1** Monolayer of uninfected CHCC-OU2 cells display a cobblestone appearance, similar to that seen with primary CEF and DEF (Panel A). At four weeks post-infection (by co-cultivation as described in Materials and Methods), numerous plaques consistent with MDV infection were observed on monolayers of Md11p15/OU2 cells (Panel B).


A

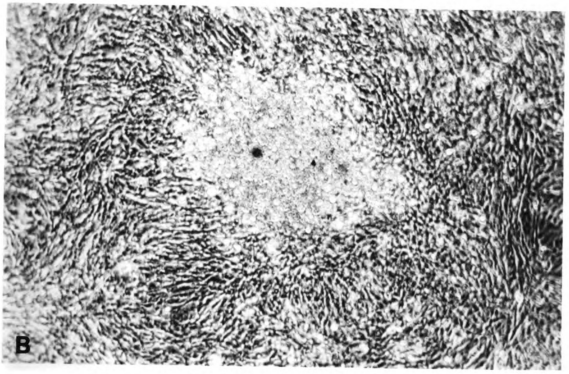

B

**Figure 2.2** PCR amplification of MDV-specific sequences. PCR amplification was carried out on DNA isolated from Md11 infected CHCC-OU2 cells (lanes 2-6). MDV-specific oligonucleotide primers (Table 1) were used to amplify particular MDV sequences, as detailed in Table 1. Negative control (lane 1), ICP4 gene promoter sequences (lane 2), 900 bp region of ICP4 coding sequence (lane 3), 1200 bp of *Bam*HI L fragment (lane 4), gC gene promoter sequences (lane 5), US3 gene promoter sequences (lane 6), and UL54 gene sequences (lane 7). Positions of selected bands from a 1kb ladder marker (Life Technologies, Inc., Gaithersburg, Md.) are indicated on the left.

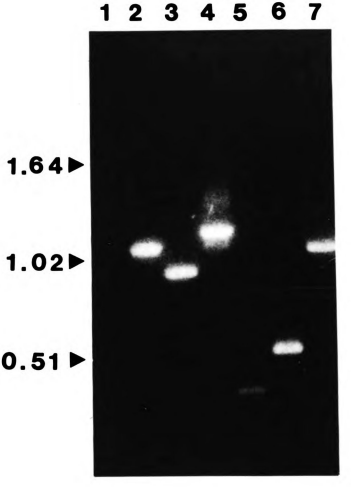
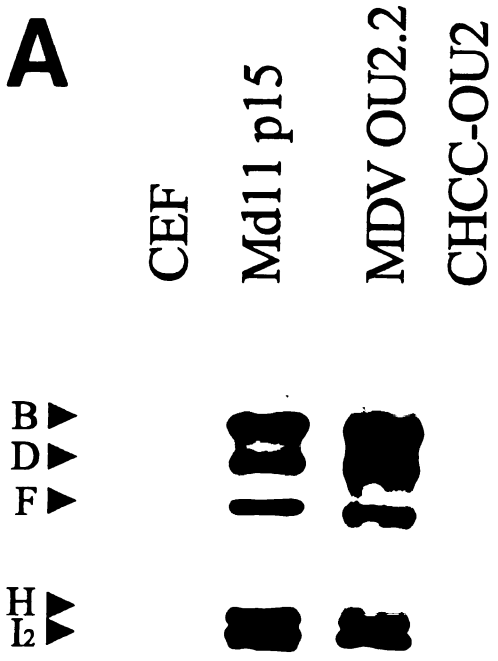

1 2 3 4 5 6 7
1.64
1.02
0.51

**Figure 2.3** Detection of viral DNA in infected CHCC-OU2 cells. DNA was extracted from cells, digested with *Bam*HI, electrophoresed on 0.8% agarose gels, transferred to Hybond-N or Zeta-Probe Nylon membranes, hybridized to non-radioactive probes under high stringency conditions, and autoradiographed as described in Materials and Methods. **Panel A:** DNA was extracted from MDV infected CHCC-OU2 cells at passage level four. Cloned MDV DNA *Bam*HI fragments B, D, F, H, and $I_2$ (Fukuchi et al., 1984) were used as probe. Locations of each fragment were determined by comparison to a DNA size standard (lambda DNA digested with *Hind*III) and are indicated by an arrowhead to the left. **Panel B:** DNA was isolated from MDV OU2.2 cells following 16 months of continuous cultivation. Expansion of direct repeat units within terminal repeats and internal repeats flanking the MDV unique long region was examined in highly passaged MDV OU2.2 cells using a MDV *Bam*HI H fragment probe. Locations of each fragment were determined by comparison to a DNA size standard (1 kb ladder, Life Technologies, Gaithersburg, MD). Fragment sizes are indicated to the right.


A
CEF
Md11 p15
MDV OU2.2
CHCC-OU2
B
D
F
H
I2


B
CEF
CHCC-OU2
Md11p15
MDV OU2.2p12
Md11p96
D
H
11.5
5.8
5.4

**Figure 2.4** Western blot analysis for detection of specific MDV proteins. Cell lysates from uninfected CEF (CEF), Md11p15 infected CEF (Md11p15), uninfected CHCC-OU2 cells (CHCC-OU2), and MDV OU2.2 cells (MDV OU2.2) were resolved on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes, followed by immunodetection using specific antisera as described in Materials and Methods. In each Panel, positions of molecular size markers (in Kilodaltons), are indicated. **Panel A:** detection of MDV-specific protein pp38 using a monoclonal antibody (generously provided by Dr. Lucy Lee, USDA-ADOL, East Lansing, MI). **Panel B:** Detection of MDV-specific protein pp14 using polyclonal antisera generated against pp14 fusion proteins (Hong and Coussens, 1991). **Panel C:** Protein loading in each lane was verified by detection of β-actin using a commercial antisera (Santa Cruz Biotechnology, Santa Cruz, CA).

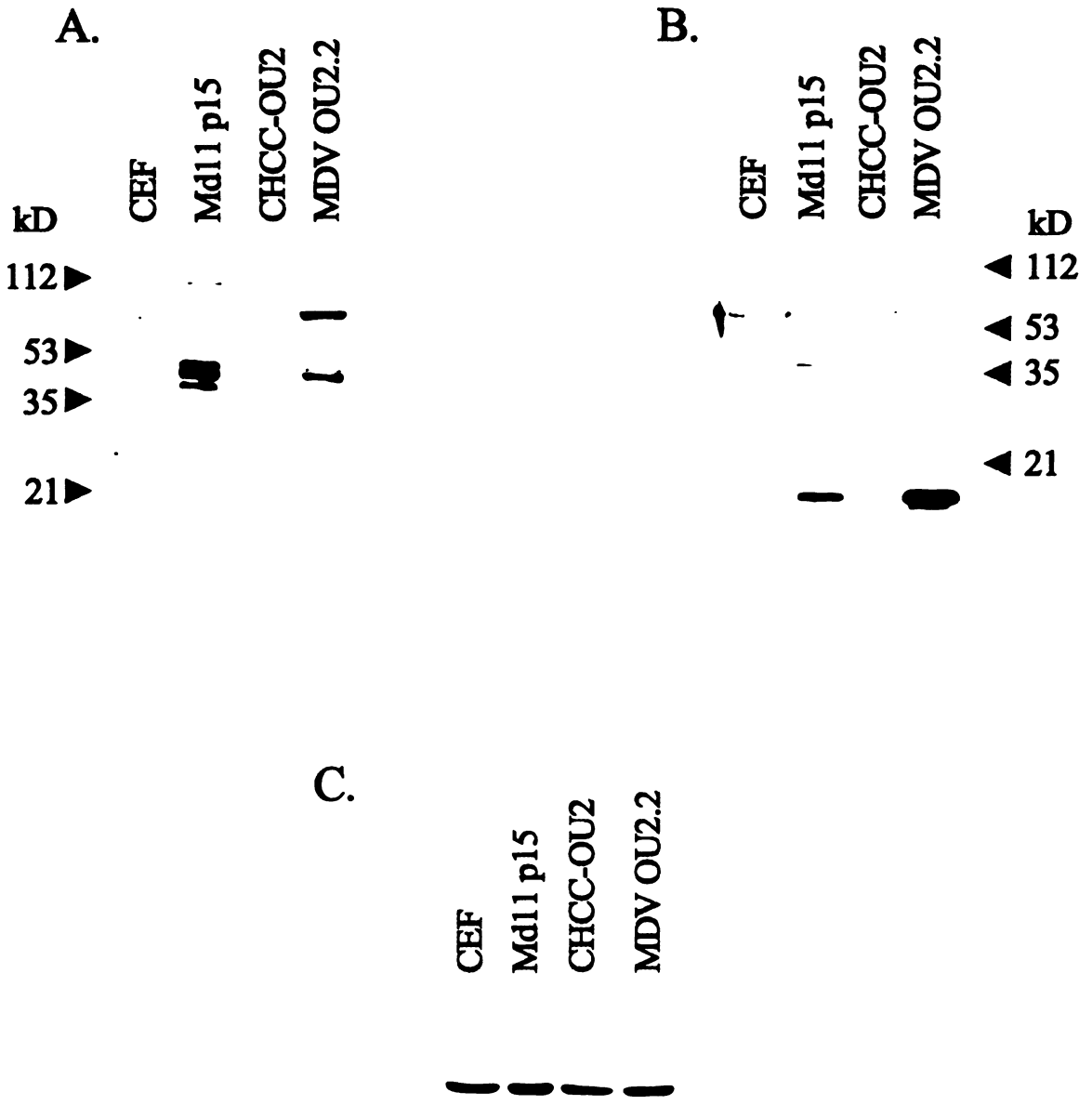

A.
CEF
Md11 p15
CHCC-OU2
MDV OU2.2
kD
112
53
35
21
B.
CEF
Md11 p15
CHCC-OU2
MDV OU2.2
kD
112
53
35
21
C.
CEF
Md11 p15
CHCC-OU2
MDV OU2.2

**Figure 2.5** PCR amplification of 850 bp pp38 gene segment. PCR amplification was carried out on DNA isolated from kidneys of birds injected with CHCC-OU2 (lanes 2 and 3), MDV OU2.2 (lanes 4-6), and Md11p16 infected CEF (lanes 7 and 8). Negative control (lane 1) included all reaction components except template DNA. Additional controls included DNA isolated from uninfected CEF (lane 9), and DNA isolated from Md11p16 infected CEF as positive control (lane 10). In each case (except negative control), 300 ng DNA was used as template for PCR amplification using pp38 specific primers (Table 1). PCR products were analyzed on 1.2% agarose gels containing 10ug/ml ethidium bromide. Fragment sizes were determined relative to DNA size standards (1 Kb Ladder, Life Technologies, Gaithersburg MD).

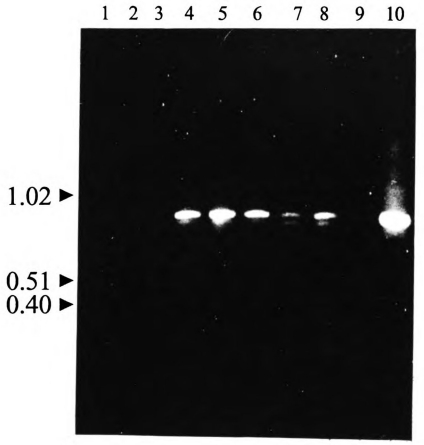
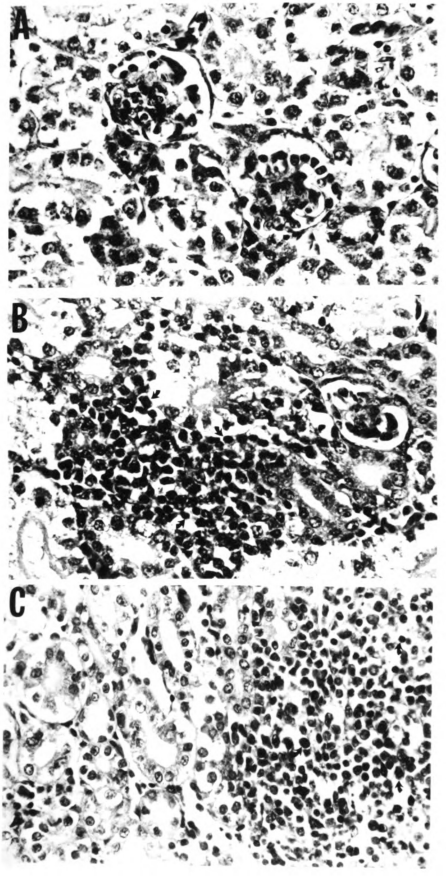

1 2 3 4 5 6 7 8 9 10
1.02
0.51
0.40

**Figure 2.6** Histological examination of kidney tissues. Tissues were prepared as described in Materials and Methods. Examination of tissues from birds inoculated with MDV OU2.2 cells (**Panel B**), and Md11p15 infected CEF cells (**Panel C**) by light microscopy revealed lymphocytic infiltration (representative lymphocytes are indicated by arrows) characteristic of early MDV-induced lymphoma development. In contrast, little or no infiltration of lymphocytes was observed in tissue sections from control birds inoculated with uninfected CHCC-OU2 cells (**Panel A**).


A
B
C

## Chapter 3

# Evidence that Marek's disease virus exists in a latent state in a sustainable fibroblast cell line

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## 3.1 Abstract

Previously, we reported the development of two fibroblastic cell lines (MDV OU2.1 and OU2.2) infected with Marek's disease virus (MDV). The two cell lines, in non-confluent continuous cultures, displayed characteristics consistent with MDV existing in a latent state. However, presence of distinct plaques in confluent cell monolayers and the ability to transfer cytolytic infection to susceptible birds and primary chick embryo fibroblasts, suggest that, if latent, the virus is easily reactivated from MDV OU2 cell lines.

In this report, we present evidence which supports the hypothesis that MDV genomes in MDV OU2 cells are latent. PCR analyses and *in vivo* experiments demonstrate that CHCC-OU2 cells stabilize MDV so that serial *in vitro* passage does not attenuate the virus. Following more than two years of active culture, MDV genomes in MDV OU2 cells are still oncogenic, similar to that seen in MDV-lymphoblastoid cell lines. Expansion of the 132 bp repeat within MDV *Bam*HI fragments H and D, typical of highly passaged serotype 1 MDV has not been observed beyond two copies in MDV OU2 cells. Indirect immunofluorescence assays clearly demonstrate that MDV OU2 cells do not express glycoproteins B and I when sparse. However, upon reaching confluence these proteins are expressed in readily detectable amounts. Using RT-PCR we also demonstrate that MDV latency associated transcripts (LATs), which are antisense to ICP4 transcripts and have been associated with latent MDV infection, are expressed in sparse

MDV OU2 cells. MDV LAT expression is down regulated when MDV OU2 cells become confluent.

## 3.2 Introduction

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by Marek's disease virus (MDV). MDV is an avian herpesvirus, which is highly infectious and cell associated (Calnek and Witter, 1991). In infected birds, a lytic cycle of MDV replication takes place in differentiating epithelial cells and bursal lymphocytes. Infectious viruses are produced only in feather follicle epithelium and are shed with feather dander and dust (Witter et al., 1972; Calnek et al., 1970). Following an initial burst of lytic infection, T-lymphocytes become latently infected with MDV. Subsequently, chickens infected with virulent, oncogenic (serotype 1) strains of MDV develop aggressive T-cell lymphomas (Calnek and Witter, 1991). Evidence accumulated to date suggests that latent infection of T-lymphocytes is a prerequisite to malignant transformation and neoplastic disease (Shek et al., 1983).

Although MDV cannot produce fully infectious particles (enveloped virus) in tissue culture, cytopathic infections can be produced in monolayers of primary or secondary chicken and duck embryo fibroblasts (CEF and DEF, respectively) as well as chick kidney cells (CKC). CEF, CKC and DEF support the lytic cycle of MDV *in vitro* (Schat et al., 1989). In addition, more than 90 lymphoblastoid cell lines have been isolated and established from MD tumors (Schat et al., 1991; Akiyama and Kato, 1974; Powell et al., 1974; Calnek et al., 1978; Payne et al., 1981; Nazarian and Witter, 1975). MDV transformed lymphoblastoid cells are immortalized cell lines which are latently

infected with MDV and usually capable of transferring MDV to CEF or DEF *in vitro* and to susceptible chickens *in vivo*.

In MDV lymphoblastoid cell lines a limited set of viral genes have been found to be transcribed (Maray et al., 1988; Sugaya et al., 1990). Transcriptional activity is limited to approximately 20% of the MDV genome, primarily in repeat regions and adjacent sequences. Most transcripts found in MDV lymphoblastoid cells are derived from immediate early (IE) genes (Silver et al., 1979; Schat et al., 1989), suggesting that IE genes could have a significant role in maintenance of latency. Latency associated transcripts (LATs) which map antisense to IE genes have been described for many herpesviruses. Transcripts antisense to ICP4 have been reported for pseudorabies virus and have been postulated to be involved in latency (Priola, et al., 1990). In the case of herpes simplex virus, LATs map antisense to the ICP0 gene (Feldman, 1991). In MDV, LATs which map antisense to ICP4 have been detected (Li et al., 1994, Cantello et al., 1994, Mckie et al., 1995). These LAT transcripts appear to be expressed at higher levels in lymphoblastoid cell lines (MSB-1 and RPL1) and are down regulated in lytically infected cells (Li et al., 1994). Although it is possible that MDV LATs play a role in maintenance of latency, definitive evidence is still lacking.

MDV can be rescued from some lymphoblastoid cell lines by co-cultivation with primary or secondary CEF and DEF (Schat et al., 1989). In addition, some lymphoblastoid cell lines will induce MD upon injection into susceptible birds (Akiyama et al., 1973, Nazarian et al., 1977). Reactivation of cytolytic infection, however, varies with each cell line. MDV lymphoblastoid cells contain multiple copies of MDV genomes

(Akiyama and Kato, 1974; Ross et al., 1981; Rziha and Bauer, 1982; Schat et al., 1989). Recent evidence suggests that some MDV genomes, in MDV lymphoblastoid cell lines, are integrated into cellular chromosomes, but episomal forms also exist (Delecluse and Hammerschmidt, 1993; Delecluse et al., 1993).

Serial *in vitro* passage of virulent oncogenic MDV results in loss of MDV tumorigenicity (Churchill and Chubb, 1969). Attenuation of MDV was strongly correlated with an expansion in two regions (*Bam*HI-H and -D), present in MDV long terminal repeat ($TR_L$) and long inverted repeat ($IR_L$) regions, respectively (Silva and Witter, 1985; Fukuchi et al., 1985; Chen and Velicer, 1991). It was latter discovered that expansion was due to amplification of a 132-bp direct repeat (DR) sequence found within MDV *Bam*HI-H and -D fragments, (Maotani et al., 1986). Tumor induction studies in susceptible birds suggest that cloned virus populations which exhibit an amplification of the 132 bp repeat region have decreased tumorigenic capability. In contrast, viruses which do not contain amplified *Bam*HI -H or -D regions efficiently induce tumors in chickens (Fukuchi et al., 1985).

Recently we reported development of fibroblastic cell lines (MDV OU2.1 and OU2.2) infected with serotype 1 MDV, strain Md11 (Abujoub and Coussens, 1995). MDV OU2 cell lines are similar to certain lymphoblastoid cell lines that MDV infection can be transferred to primary and secondary CEF monolayer culture and MD induced in susceptible birds (Abujoub and Coussens, 1995). However, MDV OU2 cell lines, unlike lymphoblastoid cell lines, are not intrinsically tumorigenic.

Latency in many viruses results from lack of host factors critical for the

expression of viral IE gene products (Garcia-Blanco and Cullen, 1991). Reactivation of these viruses from the latent state is not completely understood, but could be due to activation of specific cellular factors in response to an external stimuli. Stimulation will activate viral regulatory proteins and lead to a state of lytic infection. The exact mechanism involved in the switch from latent to lytic infection is not clearly understood for many herpes viruses, including MDV. The MDV OU2 cell system may represent an ideal *in vitro* system to study factors involved in the switch between lytic and latent herpesvirus infections.

As a first step in examining the status of MDV in the MDV OU2 cell lines, we report here that MDV exists in a continuous latent state in MDV OU2 cell lines and virus lytic cycle is activated upon confluence. Virus produced from MDV OU2 cells remained virulent and oncogenic after more than 30 *in vitro* passages. MDV cultivated in CHCC-OU2 cells appears to be stabilized unlike MDV cultivated on CEF cells. After more than two years of continuous culture, only a minor expansion of an unstable 132 bp DR sequence (het region) was detected in MDV OU2 cells.

## 3.3 Materials and methods

### 3.3.1 Cells and Virus

Preparation, propagation, and infection of CEF cells with MDV were performed as described previously (Glaubiger et al., 1983; Coussens and Velicer, 1988). The very virulent MDV strain Md11 was used at cell culture passage levels 15, 28, 35, 48 and 86 (Md11p15, Md11p28, Md11p35, Md11p48, and Md11p86 respectively). CHCC-OU2

cells (Ogura and Fujiwara, 1987), MDV OU2.2, and MDV OU2.1 cells were maintained as described previously (Abujoub and Coussens, 1995). MDV OU2 cells were used at cell culture passages 12 to 34 (MDV OU2.1p12 to p34 and MDV OU2.2p12 to p34) for PCR, and RT-PCR analyses . MDV OU2.1 and OU2.2 cells are passaged every 1-2 weeks prior to formation of confluent monolayers.

### 3.3.2 Preparation of cellular DNA, and Polymerase chain reaction (PCR)

Total cellular DNA was extracted from CEF, CEF/Md11, MSB-1, CHCC-OU2, and MDV OU2 cells by standard methods (Sambrook et al., 1989). Total cellular DNA was used as a template for PCR amplification of the 132-bp DR sequences. The upstream primer (5'-TGCGATGAAAGTGCTATGGAGG-3') anneals 3 bp 5' to the 132-bp DR sequences, while the downstream primer (5'-GAGAATCCCTATGAGAAAGCGC-3') anneals 6 bp from the 3' end of the 132-bp DR sequences. PCR using these two primers amplifies a 317 bp fragment when two copies of the DR sequence are present (Silva, 1992). PCR conditions were slightly modified from those suggested by Silva, (1992). Briefly, 200 nanogram of total cellular DNA was mixed with 20 mM of each dNTP , 20 μM of each oligonucleotide primer pair, 10 μl 10 X PCR reaction buffer (GIBCO BRL, Gaithersburg, MD), 1.5 mM $MgCl_2$, and 1.0 U *Taq* polymerase (GIBCO BRL). PCR reactions were performed using a GeneAmp 9600 thermal cycler (Perkin Elmer Cetus, Norwalk, CT). Following an initial denaturing step at 95 °C for 5 minutes, DNA was amplified during 25 cycles of 95 °C for 30 seconds, 67 °C for 30 seconds, and 72 °C for 30 seconds. PCR reactions were completed by a final elongation step at 72 °C for 10

minutes. A negative control with CHCC-OU2 DNA was included in each PCR reaction. Amplification of an 850 bp fragment of the pp38 gene was performed as previously described (Abujoub and Coussens, 1995). PCR products were analyzed on 6% polyacrylamide gels, stained with ethidium bromide, and photographed under ultraviolet light. Sizes of amplified fragments were determined by comparison to a 1 kb DNA ladder marker (GIBCO BRL).

### 3.3.3 RNA isolation and Reverse Transcriptase PCR (RT-PCR)

Total RNA was isolated from CHCC-OU2, MDV OU2.2p31, Md11p35, and MSB-1 cells, using the Trizole reagent (GIBCO BRL) according to the manufacturer's recommendation. Prior to use, RNA was treated with 10 units RNase-free RQ1 DNAase (Promega, Madison, WI) for 30 min at 37 °C. Coupled cDNA synthesis and PCR amplification of extracted RNAs was carried out in two steps. First, cDNA synthesis was performed in 250 mM Tris-HCl (pH 8.3), 375 mM KCL, 15 mM $MgCl_2$, 100 mM dithiothreitol, and 10 mM each dATP, dCTP, dGTP, and dTTP. First strand synthesis mixture also contained 0.5 U RNasin (Promega), 200 U Superscript II Reverse Transcriptase (GIBCO BRL), 1 μm of oligonucleotide corresponding to nt 411-435 (complementary) of M49 cDNA clone of MDV (Li et al., 1994) (5'-CGTCGGACATGTTTCCAGATCGCC-3'), and 15 μg of total cellular RNA. Reactions were incubated at 42 °C for 60 min, followed by enzyme inactivation for 15 min at 70 °C. Second, the cDNA synthesized in the first step was used as a template for PCR reaction as follows: 10% of the first strand reaction was amplified in 2.0 mM Tris-HCl (pH 8.4),

5.0 mM KCL , 1.5 mM (final concentration) , 20 mM of each dNTP, and 10 μm each of oligonucleotide corresponding to nt 98-122 of M49 cDNA clone of MDV (5'-CGTTGGACGGCTCGGCGGACTTGGG-3') and nt 411-435 (complementary) (Li et al., 1994). PCR reactions were performed using a GeneAmp 2400 thermal cycler (Perkin Elmer Cetus). Following an initial denaturing step at 95 °C for 5 min, DNA was amplified during 30 cycles of 95 °C for 45 seconds, 62 °C for 45 seconds, and 72 °C for 45 seconds. PCR reactions were completed by a final elongation step at 72 °C for 15 minutes. A negative control without template and a positive control with Md11p35 DNA was included in each RT-PCR reaction.

### 3.3.4 Inoculation of chickens with cells and virus

*In vivo* experiments were performed using specific pathogen-free chickens (SPF), obtained from SPAFAS (Chicago, IL). Chicks were divided into three groups of 5 chicks per group at 1 day of age, and groups inoculated intraperitoneally with either: (A) $2.0x10^6$ uninfected CHCC-OU2 cells, (B) 2000 plaque forming units (PFU) of MDV OU2.2p8 cells, or (C)2000 PFU of MDV OU2.2p23.

Birds were euthanized and necropsied upon severe signs of morbidity. Blood was collected, peripheral blood leukocytes (PBLs) were isolated as described (Tardef and McQueen, 1993) and co-cultivated with secondary CEF cells as an assay for production of viable virus. Various tissues, including heart, liver, kidney, and spleen were frozen for subsequent DNA isolation. Total tissue-specific (Kidney) DNA was isolated and used as template for PCR amplification of the 132 bp DR sequence, and for an 850 bp region of

MDV pp38 gene sequences.

### 3.3.5 Indirect immunofluorescence antibody (IIFA) staining

Uninfected CEF, CEF infected with Md11p15, CHCC-OU2, and MDV OU2.2 cells, grown on glass cover slips in 35 mm tissue culture plates, were used for indirect immunofluorescence labeling according to standard protocols ( Harlow and Lane, 1988; Hong et al., 1995). CEF and CHCC-OU2 cells were processed three days post-plating on cover slips. CEF infected with Md11p15 were processed one day after displaying plaques characteristic of MDV infection. MDV OU2.2 were processed either when they were 60-80% confluent (sparse) or five days after forming a confluent monolayer (confluent). Cells were washed with cold PBS, fixed and permeablized with 2 ml of ice cold acetone:methanol (1:1) for 3 minutes at room temperature with gentle agitation. Following three washes with cold PBS, cells were preincubated with 3% BSA in PBS for one hour at room temperature. H19.47 anti-pp38 (Cui et al., 1990) and IAN86 anti-MDV/gB (Silva, and Lee, 1984) monoclonal antibodies ( generously provided by Dr. Lucy Lee, USDA-ADOL) were added to the cells at a 1:40 dilution in PBS and incubated for one hour at room temperature. Anti-pp14 (Hong and Coussens, 1994) and anti-MDV/gI polyclonal antibodies (generously provided by Dr. Lee Velicer, Department of Microbiology, Michigan State University) were added to cells in a 1:20 dilution in PBS and incubated for one hour at room temperature. After extensive washing with PBS, sheep anti-mouse IgG (whole molecule) conjugated with fluorescein-5'-isothiocyanate (FITC) (Sigma) was used as secondary antibody for cells incubated with either anti-pp38

or anti-MDV/gB as primary antibodies. Goat anti-rabbit IgG ([H+L] affinity purified) conjugated with R-phycoerythrin (PE) (Vector Laboratories Inc., Burlingame, CA) was used as a secondary antibody for cells incubated with anti-pp14 antibody. Goat anti-rabbit IgG (whole molecule) conjugated with FITC (Sigma, St. Louis, MO) was used as a secondary antibody for cells incubated with anti-MDV/gI as a primary antibody. All secondary antibodies were diluted 1:20 in PBS. After extensive washing with PBS, Slow-Fade (Molecular Probes, Eugene, OR) was added prior to mounting to glass slides to minimize quenching of fluorescence. Cells were photographed on an Olympus BH-2 fluorescence microscope.

## 3.4 Results

### 3.4.1 MDV is stably maintained in subconfluent MDV OU2 cells

Recently we reported development of two fibroblastic cell lines (MDV OU2.2 and OU2.1) capable of supporting replication and growth of MDV (Abujoub and Coussens, 1995). MDV OU2 cells cannot be visibly distinguished from uninfected parental CHCC-OU2 cells when grown at a subconfluent level ( Figure 3.1, panels A, and B). Subconfluent MDV OU2.2 cells, do not display any signs of lytic MDV infection and exhibit a doubling time only slightly shorter than parental cells. Within 5 days of becoming confluent, monolayers of MDV OU2 cells display plaques similar in appearance to those observed in MDV infected primary or secondary CEF cells (Figure 3.1, panel C, and D). Thus MDV OU2 cells have been viable in cell culture for more than two years and display plaques only when allowed to reach confluence.

Within MDV OU2 cells, MDV may exist in a latent state and is reactivated when cells become confluent. Alternatively, cytolytic infection of CHCC-OU2 cells by MDV may be offset by cellular growth in sparse cultures. In this case, plaques become visible only when contact inhibition decreases cellular growth. To distinguish between these possibilities, the nature of MDV infection in MDV OU2 cells was examined.

### 3.4.2 Detection of viral proteins by IIFA staining

MDV OU2 cells express pp38 and pp14 in quantities sufficient to be detected by western blot analysis (Abujoub and Coussens, 1995). However, expression of other MDV antigens, particularly those of structural glycoproteins, was not detectable by western blot analysis (Abujoub and Coussens, 1995). These results are consistent with MDV existing in MDV OU2 cells in a latent state, similar to MDV lymphoblastoid cells. Rapid and widespread plaque formation in confluent cultures, however, is consistent with a fully lytic MDV infection. Therefore, we compared MDV antigen expression by IIFA staining of sparse and confluent MDV OU2 cells. Consistent with previous results, monoclonal antibody H19.47 (Cui et al., 1990) against MDV pp38 (generous gift from Dr. Lucy Lee, USDA-ADOL) detected an abundant cytoplasmic protein (pp38) in Md11p15/CEF, sparse MDV OU2.2p16 (Figure 3.2 panels B, and D respectively), and confluent MDV OU2.2p16 cells (data not shown). Also consistent with previous results, polyclonal antisera to pp14 (Hong and Coussens, 1994) identified a cytoplasmic protein (pp14) in Md11p15/CEF, sparse MDV OU2.2p16 (Figure 3.3 panels B, and D respectively), and confluent MDV OU2.2p16 cells (data not shown). Uninfected CEF

and CHCC-OU2 cells were used as negative controls (panels A and C in Figures 3.2, and 3.3). In contrast, anti-MDV/gB (Silva and Lee, 1984) monoclonal antibody IAN 86 ( generously provided by Dr. Lucy Lee, USDA-ADOL) detected a protein consistent with MDV gB in confluent MDV OU2.2p16, but not in sparse MDV OU2.2p16 cells (Figure 3.4 panels D, and B respectively). Similarly anti-MDV/gI (Brunovskis, and Velicer, 1995) polyclonal antibodies detected a protein consistent with MDV gI in confluent MDV OU2.2p16, but not in sparse cells (Figure 3.5 panels, D and B respectively). A similar protein was not detected in either sparse or confluent CHCC-OU2 cells (panels A, and C in Fig. 4, and 5). Uninfected CEF and Md11p15/CEF cells were used as negative and positive controls, respectively (data not shown). Together, results of IIFA staining, western blot analysis (Abujoub and Coussens, 1995), and MDV OU2 cell growth characteristics suggest that fully lytic growth of MDV and corresponding expression of late proteins is initiated only after MDV OU2 cells form a confluent monolayer.

### 3.4.3 MDV LATs are expressed in sparse MDV OU2 cells

The pattern of MDV gene expression in sparse MDV OU2 cell lines is similar to that observed in MDV transformed lymphoblastoid cell lines. Restricted expression of MDV genomes in these cells has led to the conclusion that MDV exists in a latent state in most lymphoblastoid cells isolated from MDV tumors. Recently, a series of mRNA's mapping antisense to the MDV ICP4 gene (MDV LATs) has been detected in various MDV lymphoblastoid cell lines (MSB-1, RPL1, and MKT-1 cells) (Cantello et al., 1994; Li et al., 1994; Mckie et al., 1995). It is postulated that antisense ICP4 transcripts may

regulate expression of MDV genes and are therefore important for maintenance of the latent state. We used RT-PCR to determine if antisense ICP4 mRNA's could be detected in MDV OU2 cell lines under sparse and confluent conditions. Total cellular RNA isolated from CHCC-OU2 cells, MDV-OU2 cells (sparse and confluent), MSB-1 cells, and Md11p35/CEF cultures was used for RT-PCR to detect expression of MDV LATs. A 335 bp fragment corresponding to the 5' end of M49 cDNA which maps antisense to the MDV ICP4 gene (Li et al., 1994) was detected in RNA isolated from sparse MDV OU2 cells, MSB-1 cells, and occasionally in confluent MDV OU2 cells, but was not detected in RNA isolated from lytically infected Md11p35/CEF or uninfected CHCC-OU2 cells (Figure 3.6 panel A). A fragment of approximately 230 bp amplified during RT-PCR of MSB-1 cells RNA may represent an alternatively spliced variant of MDV LATs (Figure 3.6 panel A, lane 6).

To verify that products amplified in RT-PCR reactions were indeed antisense to the MDV ICP4 homolog gene, PCR products were transferred to a Zeta-probe membrane and probed with DNA corresponding to the MDV ICP4 gene. Hybridization of radioactively labeled MDV ICP4 probes to RT-PCR products from reaction with MDV OU2 and MSB-1 RNA confirmed that these products were located within the ICP4 gene. No hybridization was detected in lanes containing RT-PCR reactions with CHCC-OU2 or Md11p35 RNA (data not shown). As a control for DNA contamination, no hybridization was detected when RNA templates were used for PCR without prior addition of Superscript II Reverse Transcriptase (data not shown). There was no hybridization of ICP4-specific probe to the 230 bp band in LAT-specific or ICP4-specific RT-PCR

reactions with MSB-1 RNA(Figure 3.6 panel A, lane 6), or to the 175 bp band in Md11p35/CEF and MSB-1 (Figure 3.6 panel B, lanes 5 and 8), suggesting these bands represented non-specific amplification products. Expression of MDV LATs in MDV OU2 cells combined with MDV protein expression patterns strongly suggest that MDV is in a latent state in these cells. Down regulation of MDV LATs in confluent MDV OU2 cells is associated with an increase in ICP4 gene expression as deduced from RT-PCR results (Figure 3.6 panel B, compare lanes 6 and 7). MDV ICP4 gene expression in sparse MDV OU2 cells was very low compared to expression in Md11p35 infected CEF cells and confluent MDV OU2 cells (Figure 3.6 panel B lanes 5 and 6). These results are consistent with previous evidence that ICP4 transcripts are predominantly expressed in lytically infected cells and antisense transcripts are predominantly produced in latently infected cells (Cantello et al., 1994; Li et al., 1994).

### 3.4.4 Serial *in vitro* passage does not cause attenuation of MDV genomes in MDV OU2 cells

Serial in vitro passage of serotype 1 MDV in CEF or CKC cells is usually associated with an increase in copy number of a 132-bp direct repeat (DR) within the *Bam*HI H and D fragments (Maotani et al., 1986). Expansion of the 132-bp DR region is correlated with attenuation and loss of oncogenicity (Fukuchi et al., 1985). However, expansion is limited in latently infected MDV lymphoblastoid cells and oncogenicity is preserved through extended *in vitro* cultivation. Therefore, we examined MDV genomes from MDV OU2 cells at different passage levels for expansion of the 132-bp DR region.

A PCR assay developed by Silva (1992) was used to estimate the number of

132-bp DR sequences present in MDV genomes following serial *in vitro* passage of MDV OU2 cells and various control infections. Total DNA from CHCC-OU2, Md11p15/CEF, Md11p28/CEF, Md11p48/CEF, Md11p86/CEF, MDV OU2.2p12, MDV OU2.2p23, and MDV OU2.2p28 cells was used as template for PCR amplification with a primer set flanking the 132-bp DR sequence (het region). In all reactions, except the CHCC-OU2 negative control, it was possible to detect a 185-bp amplified fragment corresponding to one copy of the 132-bp DR (Figure 3.7 panel A). A 317-bp fragment corresponding to two copies of the 132-bp DR was predominant in reactions containing pathogenic Md11p15/CEF DNA as well as in DNA isolated from MDV OU2.2p12 and p23 (Figure 3.7 panel A lanes 3, 4, and 5). Although the 317-bp fragment was present in DNA amplified from cells infected with Md11p86, the predominant PCR products were distributed over a range of bands representing between four and seven copies of the 132-bp DR sequence (Figure 3.7 panel A lane 6).

Results of PCR analyses indicated that the 132-bp DR sequence in MDV DNA was not significantly expanded following extended serial *in vitro* passage in MDV OU2 cells. In contrast, and as expected, the 132-bp DR region was expanded following serial *in vitro* passage in secondary CEF cells. Therefore similar to lymphoblastoid cell lines, MDV OU2 cells appear to stabilize MDV DNA. Despite many years in culture, lymphoblastoid cell lines are still capable of inducing MD in susceptible birds. Upon PCR analysis, 3-5 copies of the 132-bp DR sequences (data not shown) were detected in MDV genomes from MSB-1 cells. To further verify stabilization of MDV het region DNA in MDV OU2 cells, an equal passage variant of Md11 was generated in CEF cells.

Due to a switch in passage numbering upon transfer to the CHCC-OU2 culture system, Md11p48/CEF represents an equal number of passages as MDV OU2.2p28 cells. A comparison of equal passage MDV in MDV OU2 cells (MDV OU2.2p28) and CEF cells (Md11p48) (Figure 3.7 panel B lanes 3 and 2, respectively) clearly demonstrated that the 132-bp DR sequence is stabilized within MDV OU2 cells (Figure 3.7B lane 3) relative to an equal passage counterpart cultivated on CEF cells (Figure 3.7 panel B lane 2). More than seven copies of the 132-bp DR were detected in PCR amplification (Figure 3.7 panel A lane 6, and panel B lane 2) of DNA from CEF cells infected with Md11p86, and Md11p48 respectively.

### 3.4.5 High passage MDV OU2 cells induce MD in susceptible chickens

Early passages of MDV OU2.2 cells can induce MD in susceptible birds within 3-5 weeks following intraperitoneal injection (Abujoub and Coussens, 1995). Stabilization of het region DNA in MDV OU2 cells suggested that, as with MDV lymphoblastoid cells, MDV pathogenicity should also be preserved in long term cultures of MDV OU2 cells. To confirm that the MDV genomes were not attenuated after extended serial *in vitro* culture in MDV OU2 cells, SPF chickens were inoculated with either MDV OU2.2p8 (low passage) or p23 (high passage) cells at one day of age. As a negative control, chickens were injected with parental CHCC-OU2 cells. Consistent with previous results, chickens injected with MDV OU2.2p8 cells developed classical signs of MD by the end of the fourth week post-inoculation and either died or had to be euthanized by the end of the fifth week due to widespread paralysis. Necropsy revealed

complete bursal atrophy and splenomegaly in all birds inoculated with MDV OU2.2p8. Chickens inoculated with MDV OU2.2 p23 also displayed signs of MD within 3-5 weeks post-inoculation. In addition to severe paralysis, birds inoculated with MDV OU2.2p23 cells displayed severe weight loss when compared to CHCC-OU2 and MDV OU2.2p8 injected birds (data not shown). All birds in this group were euthanized by the end of the fifth week due to signs of severe illness. Upon necropsy, complete bursal atrophy and early signs of tumor formation were observed in livers of dissected birds. In contrast, chickens inoculated with parental CHCC-OU2 cells showed no clinical signs of MD. Control birds inoculated with CHCC-OU2 cells were euthanized at 16 weeks post inoculation. Necropsy revealed no signs of bursal atrophy, splenomegaly, or tumor formation.

To confirm that injected birds harbor MDV genomes, total cellular DNA isolated from kidneys was used as template for PCR amplification using a primer set specific for the 132-bp DR sequence (Silva, 1992) and for amplification of an 850 bp segment of the pp38 gene (Abujoub and Coussens, 1995). No PCR products were detected when DNA isolated from two separate control bird kidneys was used as template (Figure 3.8 panel A lanes 2 and 3). In contrast, amplification products consistent with MDV sequences containing two to four copies of the 132-bp DR sequence were amplified from MDV OU2.2p8 inoculated bird kidneys (Figure 3.8 panel A lanes 4 and 5). PCR reactions using DNA isolated from MDV OU2.2p23 injected birds resulted in amplification of products consistent with two to five copies of the 132-bp DR sequence (Figure 3.8 panel A lanes 6 and 7). Md11p15/CEF DNA was used as a positive control for PCR reactions

(Figure 3.8 panel A lane 8). PCR reactions primed with a primer set specific for pp38 gene sequences amplified an 850 bp fragment in reactions containing DNA isolated from kidneys of birds inoculated with MDV OU2.2p8 (Figure 3.8 panel B lanes 4 and 5), or MDV OU2.2p23 (Figure 3.8 panel B lanes 6 and 7). No detectable MDV products were amplified in reactions containing DNA from kidneys of birds inoculated with CHCC-OU2 (Figure 3.8 panels A and B, lanes 2 and 3). The presence of MDV specific PCR products indicated that both MDV OU2.2p8 and MDV OU2.2p23 cells were capable of transferring MDV to susceptible birds.

As an additional test for disseminated viremia in infected birds, blood was collected at the time of euthanization, and peripheral blood leukocytes (PBLs) were isolated from whole blood, washed with PBS, and co-cultivated with secondary CEF cells. CEF monolayers co-cultivated with PBLs from MDV OU2.2p8 and p23 injected birds displayed plaques consistent with MDV infection 3-5 days post-cultivation. In contrast and, as expected, no plaques were observed on CEF monolayers co-cultivated with PBLs from chickens injected with CHCC-OU2 cells. The disseminated viremia is an indication that MDV OU2 cells induced a systematic MDV infection in the inoculated birds and the virus was not localized to the site of inoculation (intraperitoneal cavity).

Results of *in vivo* experiments strongly support our findings that MDV is stabilized in MDV OU2 cells. After more than two years of continuous culture, virus harbored in MDV OU2 cells is still capable of inducing MD in susceptible birds.

## 3.5 Discussion

The MDV strain Md11 infected fibroblastic cell lines MDV OU2.1 and OU2.2 (Abujoub, and Coussens, 1995), are permissive for MDV replication. In contrast with primary CEF and DEF, these cell lines have an unlimited life span, and have been in continuous culture for more than two years under conditions similar to that used for primary fibroblasts. Growth characteristics and appearance of MDV OU2 cells are indistinguishable from the parental CHCC-OU2 immortalized fibroblastic cell line (Ogura and Fujiwara, 1987). MDV OU2 cells display characteristics consistent with MDV genomes existing in a latent state, similar to that observed in MD-lymphoblastoid cell lines. However, the appearance of distinct plaques in confluent cell monolayers is more consistent with a lytic infection (Abujoub, and Coussens, 1995). Thus, MDV OU2 cells appear to "switch" from a latent to lytic infection, co-incident with cells reaching confluence. Both MDV OU2 cell lines are capable of transferring MDV infection to CEF in culture and inducing clinical signs of MD in susceptible birds. Virus yields from these cell lines is comparable to or greater than yields produced by CEF cultures. Thus, CHCC-OU2 and MDV infected derivatives provide an excellent system for cultivation of MDV vaccine viruses, production of MDV mutants (with coordinated expression of essential genes), and a model for herpesvirus latency/reactivation.

The overall aims of this study were to determine the status of MDV genomes in MDV OU2 cells and to examine the effect of *in vitro* passage on stability of MDV genomes and virus pathogenesis within MDV OU2 cells. To achieve our first goal, we used IIFA staining and RT-PCR to examine differential gene expression in sparse versus

confluent MDV OU2 cells. Our results, summarized in Table 3.1, support the hypothesis that MDV is in a latent state in MDV OU2 cells, similar to MDV lymphoblastoid cell lines. MDV pp14 (the product of an IE gene) and pp38 (the product of an early gene) are expressed in both lytically and latently infected cells, whereas glycoproteins (encoded by late genes) are expressed mainly in lytically infected cells. IIFA staining data clearly demonstrate that both pp14 and pp38 are expressed in both sparse and confluent MDV OU2 cells, whereas expression of structural glycoproteins (glycoproteins B and I), occurs only in confluent cell monolayers. This pattern of protein expression is consistent with MDV genomes existing in a latent state in sparse MDV OU2 cells. MDV appears to be reactivated and a full cycle of lytic infection initiated after cells reach confluence.

Cantello et al.,(1994) and Li et al., (1994), separately reported the identification of transcripts (MDV LATs) that map antisense to the ICP4 homolog gene of MDV. MDV LATs are expressed at a significantly higher level in lymphoblastoid cell lines (MSB-1 and RPL1) than in lytically infected cells. Based on these findings, it was speculated that MDV LATs may play a role in maintenance of latency by negatively regulating MDV ICP4 expression (Cantello et al., 1994; Li et al., 1994). Using RT-PCR, we examined the level of expression of MDV LATs in MDV OU2 cells from sparse cultures versus confluent cultures. Expression of MDV LATs in sparse MDV OU2 cells was comparable to that observed in MSB-1 cells. MDV LAT expression is down regulated when cells become confluent and was not detected or detected at a very low levels when cells were allowed to display plaques characteristic of lytic MDV infection. When the level of MDV ICP4 expression was examined, the level of ICP4 expression was inversely

proportional to the level of MDV LAT expression. The similarity between MDV LAT/ICP4 expression patterns in sparse MDV OU2 cells to MSB-1 cells strongly supports our hypothesis that MDV exists in a latent state in MDV OU2 cells in sparse cultures. In addition, our results confirm the hypothesis of Cantello et al., (1994) and Li et al., (1994) that MDV LATs may serve to down regulate expression of MDV ICP4.

The factors responsible for the switch from a latent state in sparse cultures to a lytic infection in confluent monolayers is not understood. Generally, factors important in determining latent or lytic infection cycles in many herpes viruses are cellular activators or repressors which in turn activate or repress viral gene products responsible for determining the pathway of viral infection (Garcia-Blanco and Cullen, 1991). MDV OU2 cells are similar to their parental CHCC-OU2 cells in being contact inhibited. It is possible that changes in cell growth associated with confluence and contact inhibition act to trigger reactivation of MDV leading to expression of the full complement of MDV genes and lytic replication. It is tempting to speculate that such a trigger may be a regulator (repressor) of MDV LAT expression. As in CEF cells, lytic replication of MDV in confluent MDV OU2 cell cultures appears to be of the productive restrictive class with no infectious virions released into the culture fluid (Abujoub and Coussens, unpublished observations)

Serial *in vitro* passage of virulent MDV strains on CEF cells results in attenuation and loss of oncogenicity which is correlated with het region expansion. In this report, we provide evidence that a highly variable MDV genome region is stabilized in MDV OU2 cells, and after over two years of continuous *in vitro* culture MDV from MDV OU2 cells

is still oncogenic. PCR analyses showed that the number of 132-bp DR sequences did not change significantly in MDV genomes after more than 30 *in vitro* passages. Whereas, Md11 passed in CEF culture for the same number of passages (Md11p48/CEF) exhibited significant expansion of the 132 bp DR region. The number of 132-bp DR sequences in oncogenic MDV strains is typically between 2-5 copies, whereas the average number in strains attenuated by *in vitro* cultivation on CEF cells is 3 to greater than seven copies. These results suggest that, as with MDV lymphoblastoid cell lines MDV OU2 cell lines tend to stabilize the MDV genome. However, CHCC-OU2 cells are non-tumorigenic and thus may offer an attractive alternative for production of recombinant MDV which may be unstable in CEF cultures.

Marek's disease can be induced by injection of MDV infected cells into susceptible birds. Tumor incidence can reach 100%, but is dependent on various natural and experimental conditions and factors, for example virus strain and dose, site of injection, age at primary exposure, and the genetic background of the birds (Calnek, and Witter, 1991). Oncogenic serotype 1 MDV infected CEF, DEF, and CKC cells as well as some producer MD-lymphoblastoid cell lines such as MSB-1, CU36, and CU41 are able to induce MD in susceptible birds. Similarly, MDV OU2 cells are capable of inducing MD in susceptible birds. With serial *in vitro* passage, in CEF, DEF, and CKC cells oncogenic MDV strains are rapidly attenuated. Non-oncogenic MDV strains tend to lose potency as vaccines with continuous *in vitro* cultivation. Propagation in lymphoblastoid cells can stabilize viral genomes and preserve the initial character of resident MDV strains. However, Lymphoblastoid cells are oncogenic and have not been found to harbor

common vaccine strains of MDV.

Since, PCR analyses showed that the 132-bp DR region of MDV was stabilized during prolonged cultivation in MDV OU2 cells. It was important to determine if prolonged cultivation of MDV in CHCC-OU2 cells could preserve oncogenicity. Susceptible birds inoculated with MDV OU2.2p23 developed signs of MD including severe weight loss and paralysis similar to those inoculated with MDV OU2.2p8. PCR amplification of DNA isolated from kidneys of infected birds demonstrated systemic presence of MDV and indicated that infectious virions contained between 2-5 copies of the MDV 132-bp DR sequences. Our PCR and *in vivo* data indicates that MDV genomes are stabilized within MDV OU2 cell lines, similar to that seen in lymphoblastoid cell lines. However, unlike lymphoblastoid cell lines, the parental CHCC-OU2 cells are non-oncogenic (Abujoub and Coussens, 1995; Ogura and Fujiwara, 1987). No evidence of tumor formation or any illness in birds inoculated with CHCC-OU2 parental cells up to 16 weeks post-inoculation has been found.

This report shows, for the first time, the presence of latent MDV genomes in a sustainable fibroblast cell line. Establishment and characterization of these cell lines will reduce many of the difficulties associated with MDV experimentation and vaccine production. For example, studying differential gene expression between sparse and confluent MDV OU2 cells will help in the search for key switches in MDV latency. A key difference noted in the present report between latent and lytic MDV OU2 infections is down regulation of MDV LAT expression coincident with culture confluence. Regulation of these transcripts in sparse and confluent MDV OU2 cells is currently under

investigation. Stabilization of MDV genomes within MDV OU2 cell lines will make this system ideal for generation of mutants and recombinant viruses. In addition, propagation of MDV vaccine strains on CHCC-OU2 cells, unlike on CEF, offers the possibility that continuous passage will not offset efficacy of MDV vaccines.

**Table 3.1** Summary of IIFA and RT-PCR results.

| Expressed gene / Cell Type | pp38 | pp14 | gB | gI | ICP4 | LATs |
|---|---|---|---|---|---|---|
| MSB-1 | ND | ND | ND | ND | + | ++ |
| Md11/CEF | ++ | ++ | ++ | ++ | ++ | - |
| Sparse MDV OU2 | ++ | ++ | - | - | +/- | ++ |
| Confluent MDV OU2 | ++ | ++ | ++ | ++ | ++ | +/- |
| CHCC-OU2 | - | - | - | - | - | - |

ND not determined.
+/- low level of expression.
+ Normal level of expression
++ high level of expression.
- not detected.

**Figure 3.1** Subconfluent MDV OU2.2p23 and MDV OU2.1p23 cells display a cobblestone appearance, similar to that seen with uninfected parental CHCC-OU2 cells (Panels A and B respectively). At 5-7 days after cells became confluent, numerous plaques consistent with MDV lytic infection are observed on cultures of MDV OU2.2p23 and MDV OU2.1p23 cells (Panels C and D respectively).

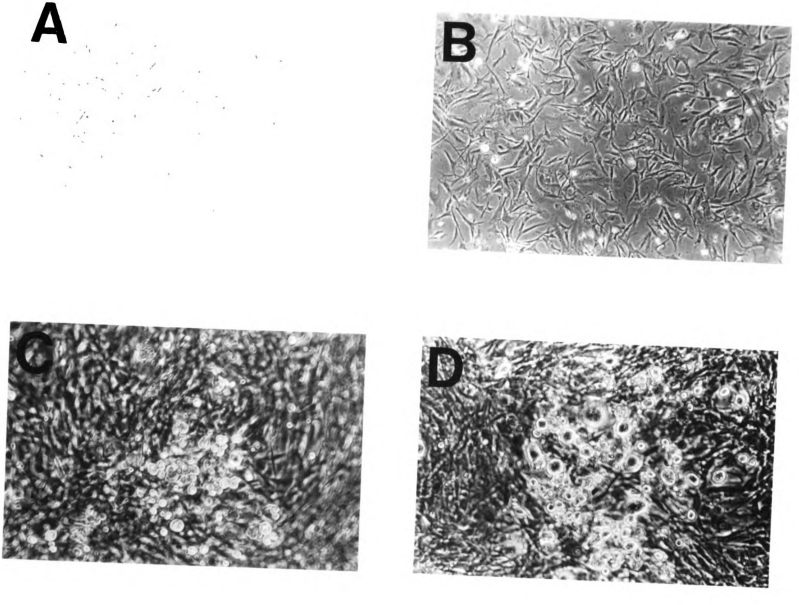

A
B
C
D

**Figure 3.2** IIFA staining with monoclonal antibody H19.47 specific for MDV pp38. Uninfected CEF, Md11p15, CHCC-OU2, and sparse MDV OU2.2 cells were grown on glass cover slips. Cells were fixed and stained as described in Materials and Methods. Sheep anti-mouse IgG conjugated with FITC (Sigma) was used as a secondary antibody. Cells were photographed on an Olympus BH-2 fluorescence microscope with a 40X objective and 3.3X photo eyepiece. **Panel A:** uninfected CEF cells. **Panel B:** Md11p15/CEF cells. **Panel C:** CHCC-OU2 cells. **Panel D:** Sparse MDV OU2.2p16 cells.

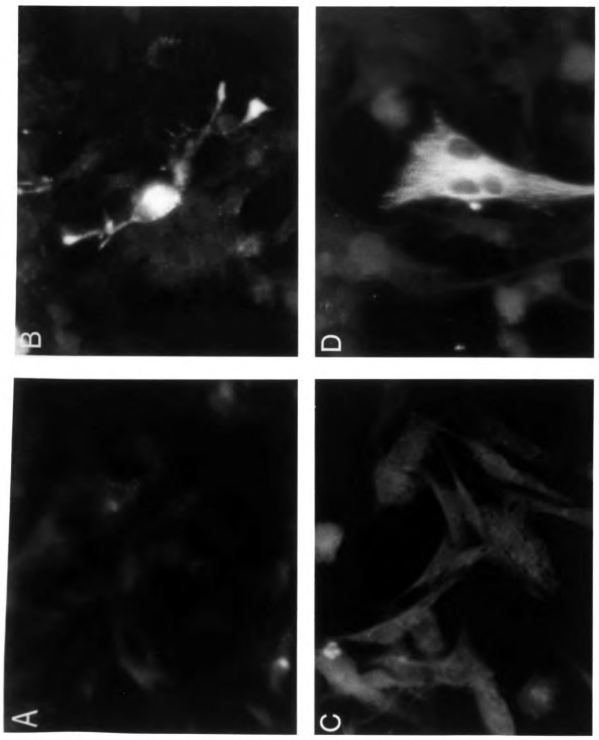

A
B
C
D

**Figure 3.3** IIFA staining with polyclonal antisera against MDV pp14. Uninfected CEF, Md11p15, CHCC-OU2, and sparse MDV OU2.2 cells were grown on glass cover slips. Cells were fixed and stained as described in Materials and Methods. Goat anti-rabbit IgG conjugated with PE (Vector Laboratories Inc., Burlingame, CA) was used as a secondary antibody. Cells were photographed on an Olympus BH-2 fluorescence microscope with a 40X objective and 3.3X photo eyepiece. **Panel A:** uninfected CEF cells. **Panel B:** Md11p15/CEF cells. **Panel C:** CHCC-OU2 cells. **Panel D:** Sparse MDV OU2.2p16 cells.

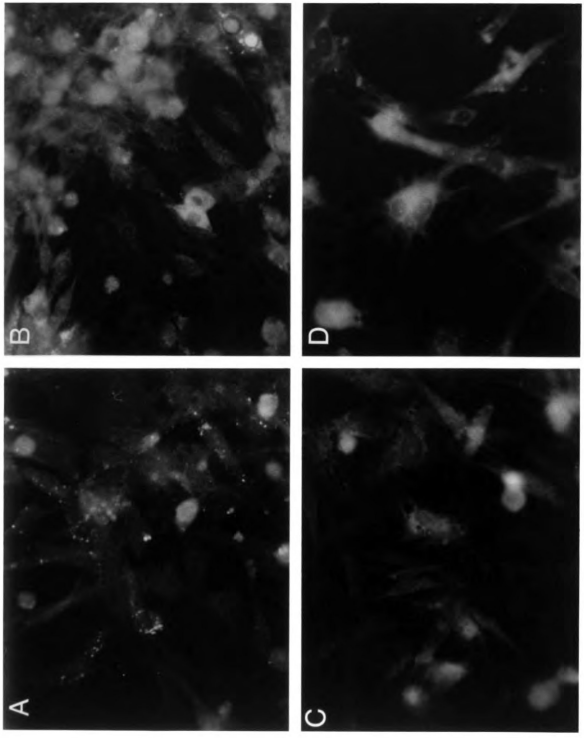

A
B
C
D

**Figure 3.4** IIFA staining with monoclonal antibody IAN86 specific for MDV gB homologue. Sparse CHCC-OU2, sparse MDV OU2.2, confluent CHCC-OU2, and confluent MDV OU2.2 cells were grown on glass cover slips. Cells were fixed and stained as described in Materials and Methods. Sheep anti-mouse IgG conjugated with FITC (Sigma) was used as a secondary antibody. Cells were photographed on an Olympus BH-2 fluorescence microscope with a 40X objective and 3.3X photo eyepiece. **Panel A:** Sparse CHCC-OU2 cells. **Panel B:** Sparse MDV OU2.2 cells. **Panel C:** Confluent CHCC-OU2 cells. **Panel D:** Confluent MDV OU2.2 cells.

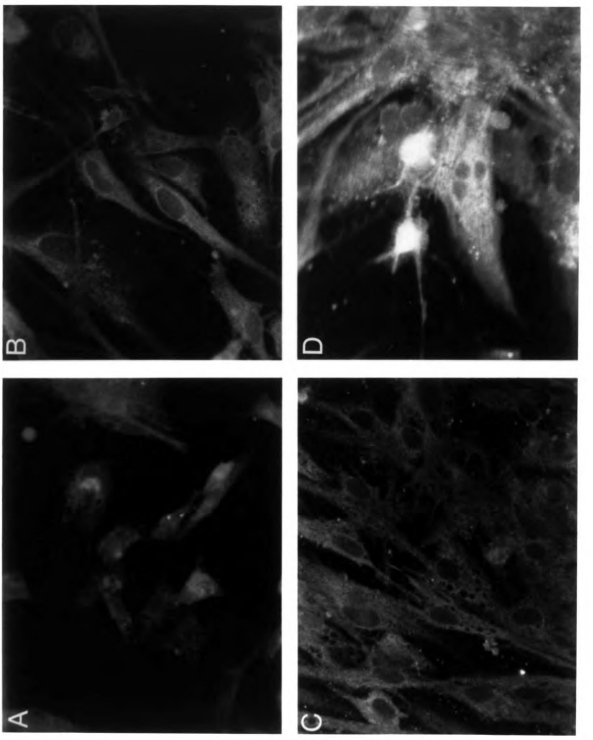

A
B
C
D

**Figure 3.5** IIFA staining with polyclonal antisera against MDV gI homologue. Sparse CHCC-OU2, sparse MDV OU2.2, confluent CHCC-OU2, and confluent MDV OU2.2 cells were grown on glass cover slips. Cells were fixed and stained as described in Materials and Methods. Sheep anti-rabbit IgG conjugated with FITC (Sigma) was used as a secondary antibody. Cells were photographed on an Olympus BH-2 fluorescence microscope with a 40X objective and 3.3X photo eyepiece. **Panel A:** Sparse CHCC-OU2 cells. **Panel B:** Sparse MDV OU2.2 cells. **Panel C:** Confluent CHCC-OU2 cells. **Panel D:** Confluent MDV OU2.2 cells.

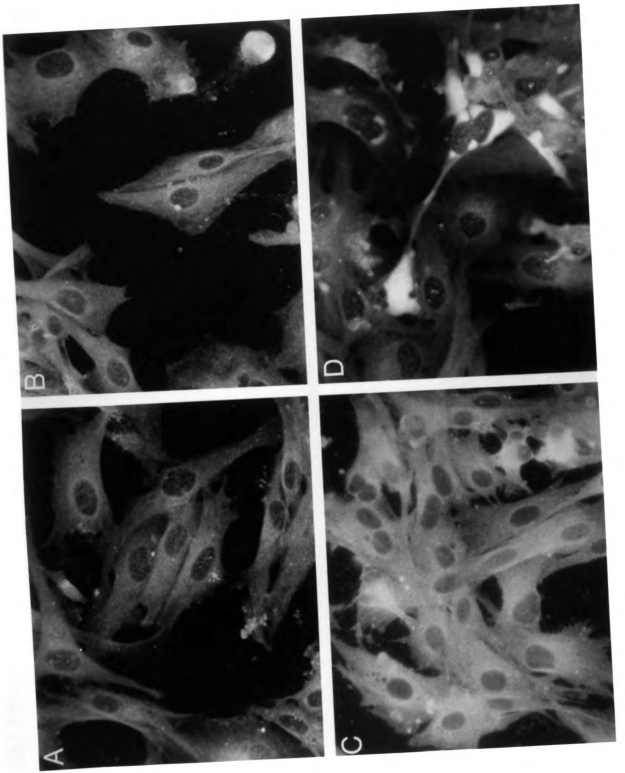
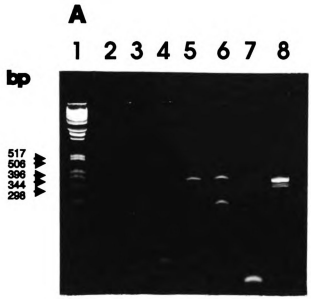

B
D
A
C

**Figure 3.6** RT-PCR amplification of a 335 bp fragment of MDV LATs and ICP4 gene. **Panel A)** LAT specific primers were used to amplify a 335 bp fragment using total RNA isolated from CHCC-OU2 (lane 2), Md11p35/CEF (lane 3), confluent MDV OU2.2p31 (lane 4), sparse MDV OU2.2p31 (lane 5), MSB-1 cells (lane 6). Lanes 7 and 8 are PCR negative and positive control respectively. **Panel B)** Primers specific for the MDV ICP4 transcript were used for RT-PCR amplification of a 300 bp fragment using RNA isolated from CHCC-OU2 (lane 4), Md11p35/CEF (lane 5), confluent MDV OU2.2p31(lane 6), sparse MDV OU2.2p31 (lane 7), MSB-1 cells (lane 8). Lanes 2 and 3, represent PCR negative and positive controls, respectively. A 1 kb DNA ladder marker (GIBCO BRL) (lane 1) was used for size comparison. Numbers at the left indicate approximate sizes of amplified fragments. Smaller size fragments are due to non-specific amplification and were not detected using Southern blot analysis (data not shown)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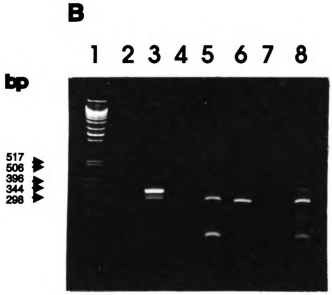
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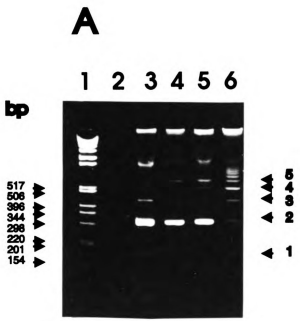
**Figure 3.7** PCR amplification of the 132-bp DR sequence. **Panel A)** DNA isolated from CHCC-OU2 (lane 2), Md11p15/CEF (lane 3), MDV OU2.2p12 (lane 4), MDV OU2.2p23 (lane 5), and Md11p86/CEF (lane 6) was used as template for PCR amplification. A 1 KB ladder marker (GIBCO BRL) (lane 1) was used for size comparison. DNA isolated from CHCC-OU2 cells (lane 2) served as negative control. **Panel B)** DNA isolated from Md11p15/CEF (lane 1), Md11p48/CEF (lane 2), and MDV OU2.2p28 (lane 3) was used as template for PCR amplification of DR sequences. Numbered arrows at the right indicate number of copies of the 132-bp DR sequence. Arrows at the left represent positions of selected bands from the 1 KB ladder marker (GIBCO BRL).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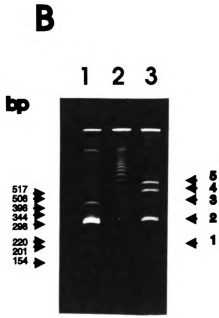
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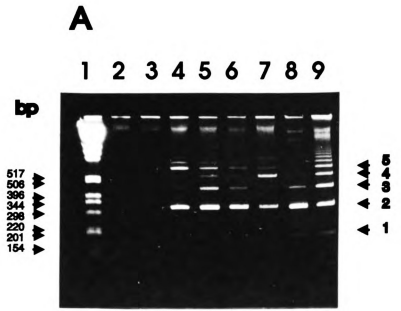
**Figure 3.8** PCR amplification of an 850 bp of MDV pp38 gene. **Panel A)** DNA isolated from Kidneys of birds inoculated with CHCC-OU2 (lanes 2 and 3), MDV OU2.2p8 (lanes 4 and 5), and MDV OU2.2p23 (lanes 6 and 7) was used as template for PCR amplification of the 132 bp DR region as described in Materials and Methods. DNA isolated from Md11p15 and p28/CEF (lanes 8 and 9) served as positive controls for DNA isolation and PCR reaction. **Panel B)** DNA isolated from Kidneys of birds inoculated with CHCC-OU2 (lanes 2 and 3), MDV OU2.2p8 (lanes 4 and 5), and MDV OU2.2p23 (lanes 6 and 7) was used as template for PCR amplification of 850 bp as described in Material and Methods. DNA isolated from Md11p15 and p28/CEF (lanes 8 and 9) served as positive controls for DNA isolation and PCR reaction. A 1 Kb ladder marker (GIBCO BRL) (lane 1) was used for size comparisons.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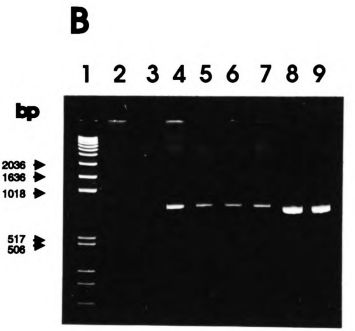
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# Chapter 4

# DISCUSSION

## 4.1 SUMMARY OF RESULTS AND CONCLUSION

The two major difficulties confronting MDV research are, 1) the cell associated nature of the virus, and 2) the lack of a continuous cell line for virus growth and selection. Although primary CEF and DEF cells are permissive for MDV replication, primary cultures are characterized by slow growth and a limited life span. These factors require continual passage of MDV infected cells onto uninfected cells in order to obtain sufficient quantities of MDV for research and vaccine production. In addition, CEF and DEF primary cells must be prepared on a regular basis from 10 or 11 day old chick embryos, adding significantly to the expense and difficulty of studying MDV, and to the expense of producing MDV vaccines.

In this dissertation we have taken the immortalized, virus free, CHCC-OU2 cells and established a sustainable cell culture system for study of MDV. The CHCC-OU2 cell line we used is a fibroblastic cell line that was derived from chemically mutagenized chick embryo cells (Ogura and Fujiwara, 1987). When the CHCC-OU2 cells were infected with Md11p15/CEF, plaques characteristic of MDV infection were observed on the monolayer 3-4 weeks post infection. The plaques were isolated and expanded in order to obtain pure monoclonal cell lines (MDV OU2.1 and MDV OU2.2). To confirm that these clonal cell lines did harbor MDV, DNA was isolated and used for PCR and Southern blot analyses. The results of these analyses clearly demonstrated the presence

of MDV genomes within each MDV OU2 cell line.

To verify that MDV OU2 cells support the replication of MDV, western blot analysis and IIFA staining were used to detect MDV proteins. Western blot analysis clearly demonstrated that a subset of viral proteins (pp38 and pp14) were expressed at a detectable level in the MDV OU2 cell lines, however we were not able to detect expression of structural glycoproteins. These glycoproteins are only expressed in productively (lytically) infected cells, but not in latently infected cells. pp38 and pp14 are expressed in both lytically and latently infected cells (Chen et al., 1992; Hong and Coussens, 1994). The western blot results suggested that MDV persisted in a latent state in MDV OU2 cells. However, the presence of distinct plaques in confluent MDV OU2 cells monolayers implied cytolytic activity associated with MDV infection. Based on these preliminary results we hypothesized that MDV may exists in a latent state within sparse MDV OU2 cells and is reactivated when cells become confluent.

To answer this question, we further analyzed the pattern of protein expression in MDV OU2 cell lines, IIFA staining was used to study differential gene expression in sparse versus confluent MDV OU2 cells. Consistent with the western blot results, MDV pp14 and pp38, were detected as abundant cytoplasmic proteins in both sparse and confluent MDV OU2 cells. In contrast, MDV gB and gI, structural glycoproteins were not detected in sparse MDV OU2 cells, however were abundant in confluent cells. The western blot results, the IIFA staining results, and MDV OU2 cell growth characteristics suggested that productive MDV infection and corresponding expression of late proteins is initiated only after MDV OU2 cells become confluent.

MDV LATs expression is associated with MDV latency, and was examined in sparse versus confluent MDV OU2 cells. Expression of MDV LATs in sparse MDV OU2 cells was comparable to that observed in latently infected MDV lymphoblastoid cell lines (MSB-1, RPL1, and MKT-1). Down regulation of MDV LAT expression when MDV OU2 cells became confluent was associated with an increase in MDV ICP4 expression. Conversely, MDV ICP4 expression was very low in sparse MDV OU2 cells when compared to expression in confluent cells. The correlation between expression of MDV ICP4 and LATs in sparse versus confluent MDV OU2 cells strongly supported our hypothesis that MDV exists in a latent state in MDV OU2 cells in sparse cultures, with similar LAT/ICP4 ratio to that seen in MDV lymphoblastoid cell lines.

MDV lymphoblastoid cell lines are immortalized cell lines, which are considered to be latently infected with MDV, and can transfer MDV to CEF or DEF *in vitro* and to susceptible birds *in vivo* (Schat et al., 1985). Similar to MDV lymphoblastoid cell lines, MDV OU2 cells were capable of transferring MDV infection to CEF monolayer cultures *in vitro* and induced clinical signs of MD in *vivo* which was characterized by a marked decrease in growth rates, blindness, and paralysis of legs, wings, and neck. Necropsy revealed bursal atrophy, splenomegaly, nerve demyelination, and signs of early tumor infiltration of the liver, kidney and spleen. There was no difference in virulence observed in groups inoculated with MDV OU2 cells or Md11p15/CEF. In contrast, birds inoculated with parental CHCC-OU2 showed no clinical signs of illness, no evidence of tumor formation, or viremia for more than 16 weeks after inoculation. PCR analysis of DNA isolated from kidney tissues demonstrated

that MDV was present in remote tissues of birds injected with MDV OU2 cells. As an additional test for disseminated viremia in MDV OU2 infected birds, peripheral blood leukocytes were collected and co-cultivated with secondary CEF cells. CEF monolayers displayed plaques consistent with MDV infection 3-5 days post-cultivation.

Serial *in vitro* passage of serotype 1 MDV in CEF cells is usually associated with an amplification in copy number of the 132-bp DR within the *Bam*HI H and D fragments. Expansion of the 132-bp DR region has been correlated with attenuation and loss of oncogenicity. However, this expansion is limited in latently infected MDV lymphoblastoid cell lines and oncogenicity is preserved through extended *in vitro* cultivation.

A PCR method developed by Silva (1992) to distinguish attenuated from wild type MDV, was used to estimate the copy number of the 132 bp DR. PCR results indicated that the 132 bp DR in MDV OU2 cells was not significantly expanded after extensive serial *in vitro* passage. Three to five copies of the 132-bp DR were detected in MDV genomes from MDV OU2 cells. Whereas MDV of the same passage number in CEF cells, exhibited significant expansion in the 132-bp DR. Thus, similar to lymphoblastoid cell lines, MDV DNA appears to be stabilized in MDV OU2 cells. To confirm that MDV in high passage MDV OU2 cells was still oncogenic and could induce MD, susceptible chickens were inoculated with MDV OU2 cells, which had been in continuous culture for more than two years. These chickens developed signs of MD similar to those injected with low passage MDV OU2 cells. PCR amplification of DNA isolated from kidneys of the infected birds demonstrated both the systemic presence of

MDV and that MDV contained only 3-5 copies of the 132 bp DR. Our *In vivo* data demonstrated that MDV oncogenicity was preserved in MDV OU2 cells after prolonged *in vitro* cultivation.

These MDV OU2 cell lines are the first fibroblastic cell lines that can support MDV replication and does not attenuate MDV. We have also presented evidence that MDV in sparse MDV OU2 cells appear to be latent and that at confluence the MDV infection becomes productive with the appearance of distinct plaques, expression of late structural proteins, down regulation of LATs, and up regulation of ICP4 expression. Therefore, MDV appears to switch from a latent state in sparse MDV OU2 cells to a lytic "productive" infection when confluence is reached.

Establishment and characterization of these cell lines has the potential to eliminate many difficulties associated with MDV experimentation. MDV OU2 cells can now be used for the production of MDV mutants by positive selection. Also a continuous cell line expressing essential MDV genes can be used in knockout experiments. The cell line may also serve as a model for MDV latency and reactivation. In addition, the MDV infected derivatives of CHCC-OU2 provide an excellent system for cultivation of MDV for vaccine use. The same approach described in this dissertation has been successful in developing cell lines that harbor MDV vaccine strains, such as herpesvirus of turkeys (HVT strain FC126), serotype 2 MDV (strain SB-1), and an attenuated serotype 1 MDV (Md11p83).

## 4.2 FUTURE RESEARCH

Development of a sustainable cell culture system for replication and study of MDV provides many new opportunities for MDV research, and is especially useful for MDV vaccine development. These cell lines may be an excellent *in vitro* model to understand maintenance of latency and reactivation for all alphaherpesviruses. However, many questions remain to be answered and future research should focus on studying: 1) transcription patterns in sparse MDV OU2 cells 2) differential gene expression between sparse and confluent MDV OU2 cells, 3) the effect of 5-iodo-2-deoxyuridine and 5-azacytidine on latent cells, 4) the status of viral genomes in MDV OU2 cells, and 5) cell cycle progression and expression of cell specific proteins in sparse versus confluent MDV OU2 cells.

### 4.2.1 Studying transcription patterns in sparse MDV OU2 cells

A limited set of viral genes are usually expressed in latent cells, and the number of transcripts varies between different lymphoblastoid cell lines. In MSB-1 cells (an expression cell line) 29 transcripts have been reported. In CU41 cells (a non-expression cell line), only 7 transcripts have been detected, and in HP1 cells (a non-producer cell line), only 4 transcripts were detected. Evidence presented in this dissertation suggests that MDV OU2 cells are similar to MSB-1 cells. Therefore, it would be important to compare transcription activity in sparse MDV OU2 cells with that in MSB-1 cells. Since transcriptional activity in MSB-1 cells is limited to the repeat regions of MDV genome, northern blot analysis (using DNA probes corresponding to the repeat regions of MDV

genomes), should yield significant information about the transcriptional activity in sparse MDV OU2 cells.

### 4.2.2 Studying differential gene expression between sparse and confluent MDV OU2 cells

A key difference noted between latent and lytic MDV OU2 infections, is down regulation of MDV LAT expression when the cells become confluent. Cellular and/or viral transactivators may play a major role in maintenance and reactivation from latency. The differential display technique (Liang and Pardee, 1992) could be a way to compare mRNAs from CHCC-OU2, sparse MDV OU2 and, confluent MDV OU2 cells. This technique would identify transcripts that have been up regulated or down regulated when the MDV OU2 cells switch from a latent to lytic state. Cloning and identifying these transcripts would help understand the MDV-host interactions that lead to latency and the interactions necessary for reactivation.

### 4.2.3 Studying the effect of 5-iodo-2-deoxyuridine and 5-Azacytidine on sparse MDV OU2 cells

MDV DNA methylation may play a role during latency (Fynan et al., 1993). MDV genomes within MSB-1 and RPL-1 lymphoblastoid cell lines, have been found to be methylated at various sites within the repeat regions (Kanamori et al., 1987). Also, MDV genomes within an avian leukosis virus (ALV) transformed B-cell lines was found to be methylated, and less than 2% of these cells expressed MDV antigens (Fynan et al.,

1993). However when DNA methylation was prevented by 5-azacytidine, MDV replication increased. Similarly, 5-azacytidine treatment of MSB-1 cells resulted in hypomethylation and increased mRNA transcription from the repeat region (Hayashi et al., 1994 and 1995). Since our evidence suggests that MDV is latent in sparse MDV OU2 cells, and lytic in confluent cells, it would be important to compare the methylation of MDV DNA from latent and lytic MDV OU2 cells.

To compare MDV DNA from sparse and confluent MDV OU2 cells, MDV DNA would be digested with *Hpa*II or *Msp*I, and then analyzed by Southern blot hybridization using the *Bam*HI H (transcriptionally active region) and B fragments (transcriptionally in active region) as probes (Hayashi et al., 1994). Both *Hpa*II and *Msp*I recognize and cleave the sequence 5'-CCGG-3'. However, when the internal cytosine is methylated, this site can be cleaved only by *Msp*I but not by *Hpa*II. Thus, the cleavage patterns generated will differentiate methylated 5'-CCGG-3' sites from unmethylated ones. On the other hand, treatment of MDV lymphoblastoid cell lines with IudR induces viral antigen expression but does not affect viral DNA replication (Silver et al., 1979). Would IudR have an effect on sparse MDV OU2 cells? IudR can be added directly to sparse cells and protein expression can be examined by IIFA staining before and after the addition of IudR.

### 4.2.4 Studying the state of viral genomes in MDV OU2 cells

MDV genomes in MDV lymphoblastoid cell lines appear to be primarily integrated into cellular chromosomes (Delecluse and Hammerschmidt, 1993; Delecluse et

al., 1993). MDV DNA in MDV OU2 cells could be linear, episomal or integrated. Many different approaches can be taken to distinguish between these possibilities. The Gardella gel technique (Gardella et al., 1984) allows differentiation between linear and episomal forms of viral DNA, and can be used to indirectly detect MDV integration into cellular chromosomes. However, a more conclusive and direct method is exonuclease V digestion. Exonuclease V digests linear double-stranded DNA but does not cut circular DNA. In addition it also does not digest DNA contains nicks or gaps. Therefore treating MDV DNA from MDV OU2 cells with exonuclease V, followed by Southern hybridization will provide information regarding the state of MDV DNA in MDV OU2 cell lines.

### 4.2.5 Studying cell cycle progression and expression of cell specific proteins

In culture, fibroblasts can be arrested from further cell proliferation and enter a quiescent state. The cells are usually arrested in the $G_0$ phase of the cell cycle, either by contact inhibition or by serum starvation. These cells can resume the cell cycle when the conditions blocking growth are removed. Molecular characterization of the mechanisms regulating cell transition from the quiescent to the growing state, in human fibroblasts, resulted in identification of statin (Wang, 1985). Statin, a 57,000-Dalton nuclear phosphoprotein, is associated with the nuclear envelop, and is only present in nonproliferating fibroblasts (Wang, 1985, Lee et al., 1992). Statin was found to be associated with a 45,000-Dalton serine/threonine kinase (p45 kinase), which was found to be only active in nonproliferating fibroblasts (Lee et al., 1992). When these cells re-

entered the cell cycle, statin rapidly declined and was removed from the nuclear matrix prior to initiation of DNA synthesis (Wang and Lin, 1986), while expression of another nuclear protein, cycline (PCNA), a protein found only in replicating cells, increased in parallel with DNA synthesis (MacDonald-Bravo and Bravo, 1985).

A 57,000-Dalton protein similar to statin was purified from terminally differentiated rat liver hepatocytes (Rlp57) (Sester et al., 1990). Another antigenically related protein, a 49,000-Dalton (pS1) was identified by screening a rat brain λgt11 expression library with a monoclonal statin antibody (Ann et al., 1991). The pS1 mRNA was found to be most abundant in $G_0$ phase of 3T3 mouse fibroblasts, but become significantly reduced in $G_1$ and S phase cells (Ann et al., 1991). All these reports have identified a group of genes that are expressed specifically in nonproliferating cells.

To determine the phase of the cell cycle, where MDV OU2 cells and their parental counter parts are arrested as a results of contact inhibition. Cell cycle phase distribution can be measured by quantitation of DNA by propidium iodide (PI) staining. At the same time, IIFA staining using a monoclonal antibody , S-30, against human fibroblast statin (Wang, 1985), or the polyclonal antibody, raised against Rlp57 (Sester et al., 1990), can be used to search for a statin homolog in the CHCC-OU2 cells and their MDV derivatives. The association between statin and p45 kinase in nonproliferating human fibroblasts, suggests that a similar system may play a role in MDV reactivation from latency by phosphorylating a protein (MDV or cellular encoded) critical for the switch from latent to lytic infection. Identifying and cloning a gene encoding an avian statin homolog, and examining the effect of its expression on MDV gene expression and on

MDV replication on sparse MDV OU2 cells would enable investigation of the role cellular factors has on establishing MDV latency and reactivation. These identified avian genes (statin homolog gene) can be cloned downstream of an inducible promoter and the recombinant transfected into MDV OU2 cells. The effect of the statin homolog on expression of viral antigens and viral replication in confluent versus sparse MDV OU2 cells can be monitored by IIFA staining against late viral proteins and by light microscopy (plaque formation).

Further characterization of the MDV OU2 cell lines will have a major impact on MDV research and specifically on MDV latency and reactivation .

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