# Genome-wide analysis of Marek's disease virus proteins and their role in modulating the innate immune response in chickens

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**A thesis submitted in fulfilment of requirements for the degree of Doctor of Philosophy**

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## Declaration

I hereby declare that this thesis is of my own composition, and that it contains no material previously submitted for the award of any other degree. The work reported in this thesis has been executed by myself, except where due acknowledgement is made in the text.

# Abstract

Marek's disease virus (MDV), the causative agent of Marek's disease in chicken, is an important oncogenic avian pathogen which leads to world-wide economic losses in the poultry industry. It targets the chicken's immune system by initially causing a lytic infection in B-lymphocytes in lymphoid organs (spleen, bursa of Fabricius and thymus), followed by a latent infection of T-lymphocytes, which may lead to tumour formation. Despite the presence of well-established vaccination programs against MDV, it is still a major concern for the poultry industry due to the emergence of more virulent strains. As MDV is also considered an excellent model for herpesvirus-induced oncogenicity and immunosuppression, a better understanding of its pathogenesis, including the functional roles of individual MDV proteins, is of both biomedical as well as economical importance.

All open reading frames (ORFs) of the CVI988 vaccine strain and the RB1B virulent strain were PCR-amplified from BAC DNA and cloned into the pDONR 207 entry vector by recombinatorial cloning (Gateway® system). Subsequently, all ORFs were subcloned into the yeast-two-hybrid (Y2H) vectors pGBKT7–DEST (bait) and pGADT7-DEST (prey), as well as other expression vectors. The Y2H bait and prey vector clone collections were transformed into the yeast strains AH109 and Y187, respectively. More than 140 ORFs, or ORF fragments, were analysed against each other in a comprehensive Y2H assay. Of > 20.000 interactions tested, 435 positive interactions between 115 ORFs were observed. Several of these interactions have previously been reported in other species of herpesvirus indicating that they may be conserved within the family. A subset of the positive interactions were confirmed using co-immunoprecipitation and LUMIER pull-down assays as a second independent assay.

In the second part of the project all MDV proteins were tested for their ability to inhibit the chicken interferon-alpha (chIFN-α)-induced immune response. In functional luciferase reporter assay with a chicken Mx1 promoter containing an

interferon stimulated responsive element (ISRE), four MDV-encoded chIFN-α inhibitors were identified, including UL12, UL26, UL50, and Meq, the main MDV oncoprotein. Both isoforms Meq and L-Meq derived from the oncogenic and the non-oncogenic vaccine strain, respectively, similarly inhibited the interferon response in a dose-dependent way, and Meq deletion mutants revealed that the C-terminal, proline-rich transactivating domain is not required for this inhibitory effect. In transient transfection experiments, Meq induced a dose-dependent proteasomal degradation of the chicken interferon regulatory factor 7 (chIRF7), which is required for chIFN-α- induced activation of ISRE. Over-expression of chIRF7 lead to a dose-dependent degradation of Meq and its accumulation in the cytoplasm, suggesting that proteasomal degradation of both Meq and chIRF-7 is linked. Consistent with these findings, MDV deletion mutant lacking both copies of the Meq gene was more sensitive to chIFN-α treatment compared to wild-type virus.

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# Abbreviations

| | |
|---|---|
| **°C** | Degrees Celcius |
| **µg** | Microgram |
| **µl** | Microlitre |
| **µM** | Micromolar |
| **2'5'OAS** | 2'5'oligoadenylate synthase |
| **aa** | Amino acids |
| **Ab** | Antibody |
| **ACAT** | Aortic cholesteryle ester synthetic activity |
| **ACE** | Angiotensin converting enzyme |
| **AD** | Active domain |
| **AI** | Avian Influenza |
| **BAC** | Bacterial artificial chromosome |
| **BD** | Binding domain |
| **BoHV-1** | Bovine herpesvirus-1 |
| **bp** | Base pair |
| **BR1** | Basic region 1 |
| **BR2** | Basic region 2 |
| **bZIP** | Basic leucine zipper |
| **CASTing** | Cyclic amplification of selected targets |
| **CD** | Cell determinant |
| **cDNA** | Complementary DNA |
| **CE** | Cholesteryle ester |
| **CEF** | Chicken embryo fibroblast |
| **CESCs** | Chicken embryo skin cells |
| **ChIFN1** | Chicken interferon 1 |
| **ChIFN2** | Chicken interferon 2 |
| **chIFN-α** | Chicken interferon-alpha |
| **chIRF3** | Chicken interferon regulatory factor 3 |
| **chIRF7** | Chicken interferon regulatory factor 7 |
| **chTR** | Chicken telomerase |
| **CHX** | Cycloheximide |
| **$CO_2$** | Carbon dioxide |
| **Co-IP** | Co-Immunoprecipitation |
| **CR** | Conserved region |
| **CRE** | Cyclic AMP response element |
| **CtBp** | C-terminal binding protein |
| **CTL** | Cytotoxic lymphocyte |
| **DBD** | DNA binding domain |
| **DEF** | Duck embryo fibroblasts |
| **DEV** | Duck enteritis virus |
| **DMSO** | Dimethyl sulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **dpi** | Days post infection |
| **dsRNA** | Double stranded RNA |
| **dUTPase** | Deoxyuridine-triphosphatase |
| **E** | Experimental infection |

| | |
|---|---|
| **EARCS** | Ellipsoid associated reticulum cells |
| **EBV** | Epstein-Barr virus |
| **ED** | Embryo day |
| **ELISA** | Enzyme linked-immunosorbent assay |
| **ER** | Endoplasmic reticulum |
| **FCS** | Foetal calf serum |
| **FFE** | Feather follicle epithelium |
| **FHV** | Feline herpesvirus |
| **FPL** | Feather pulp lesions |
| **FRET** | Förster resonance energy transfer |
| **g** | Gram |
| **GHV** | Gallid herpesvirus |
| **HA** | Hemagglutinin |
| **HMGCoA** | 3-hydroxy-3-methylglutaryl-coenzyme A |
| **HPAI** | High pathogenic avian influenza |
| **hr** | Hour |
| **HRP** | Horseradish peroxidase |
| **HSV** | Herpes simplex virus |
| **HVT** | Herpesvirus of turkey |
| **i.m** | Intra muscular |
| **IAD** | IRF-association domain |
| **IBD** | Infectious bursal disease |
| **IBDV** | Infectious bursal disease virus |
| **ICP** | Infected cell protein |
| **ICS** | Interferon consensus sequence |
| **IE** | Immediate early |
| **IFNAR** | Interferon-alpha receptor |
| **IFN-α** | Interferon-alpha |
| **IFN-ß** | Interferon-beta |
| **IFN-γ** | Interferon-gamma |
| **IFN-δ** | Interferon-delta |
| **IFN-λ** | Interferon-lambda |
| **IFN-ω** | Interferon-omega |
| **Ig** | Immunoglobulin |
| **IL** | Interleukin |
| **ILT** | Infectious laryngotracheitis |
| **iNOS** | Inducible nitric oxide synthase |
| **IRF** | Interferon regulatory factor |
| **IRFs** | Interferon regulatory factors |
| **IRL** | Internal repeat long |
| **IRS** | Internal repeat short |
| **ISGF3** | Interferon stimulated genes factor 3 |
| **ISRE** | Interferon response elements |
| **kbp** | Kilo-base pair |
| **kDa** | Kilo Dalton |
| **KSHV** | Kaposi's sarcoma associated herpesvirus |
| **L** | Litre |
| **LATs** | Latency associated transcripts |
| **LCA** | Lymphocyte aggregates |
| **LUMIER** | Luminescence-based mammalian interactome mapping |
| **mAbs** | Monoclonal antibodies |

| | |
|---|---|
| **mCMV** | Murine cytomegalovirus |
| **MD** | Marek's disease |
| **MDV** | Marek's disease virus |
| **mg** | Milligram |
| **MHC** | Major histocompatibility complex |
| **miRNA** | MicroRNA |
| **ml** | Millilitre |
| **mM** | Millimolar |
| **MOI** | Multiplicity of infection |
| **mRNA** | Messenger ribonucleic acid |
| **MyD88** | Myeloid differentiation primary-response protein 88 |
| **NDV** | Newcastle disease virus |
| **NEAA** | Non-essential amino acids |
| **NF-kB** | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| **ng** | Nanogram |
| **NK** | Natural killer |
| **NLS** | Nuclear localisation signal |
| **nm** | Nanometre |
| **NoLS** | Nucleolar localisation signal |
| **Nsp** | Non-structural protein |
| **o/n** | Overnight |
| **OBP** | Origin binding protein |
| **OD** | Optical density |
| **ORF** | Open reading frame |
| **PAGE** | Polyacrylamide gel electrophoresis |
| **PBS** | Phosphate- Buffered Saline |
| **PCR** | Polymerase chain reaction |
| **PFU** | Plaque forming units |
| **pi** | Post infection |
| **PKR** | Protein kinase RNA |
| **PM** | Post mortem |
| **pmol** | Picomolar |
| **PND** | Persistent neurological disease |
| **PrV** | Pseudorabies virus |
| **PSHV-1** | Psittacid herpesvirus 1 |
| **QM7** | Quail muscle 7 |
| **QP** | Q promoter |
| **QRT-PCR** | Quantitative real time-PCR |
| **REV** | Reticuloendotheliosis virus |
| **RLU** | Relative luciferase units |
| **RNA** | Ribonucleic acid |
| **rpm** | Revolutions per minutes |
| **RT** | Room temperature |
| **s/c** | Subcutaneously |
| **SDS** | Sodium dodecyl sulfate |
| **SEB** | Sample extraction buffer |
| **shRNA** | Short hairpin RNA |
| **siRNA** | Small interfering RNA |
| **SNP** | Single nucleotide polymorphism |
| **SPF** | Specific pathogen free |
| **ssRNA** | Single stranded RNA |

| | |
|---|---|
| **STAT** | Signal transducers and activator of transcription |
| **TBST** | Tris buffered saline plus Tween 20 detergent |
| **TCR** | T cell receptor |
| **TGN** | Trans-Golgi network |
| **Th-1** | T helper-1 |
| **Th-2** | T helper-1 |
| **TLR** | Toll-like receptor |
| **TNF** | Tumour necrosis factor |
| **TP** | Transient paralysis |
| **TRAF** | Tumour necrosis factor receptor-associated factor |
| **TRAIL** | Tumour necrosis factor-related apoptosis-inducing ligand |
| **TRE** | Tetradecanoylphorbol acetate response element |
| **TRIF** | TIR-domain-containing adaptor protein inducing IFN-ß |
| **TRL** | Terminal repeat long |
| **TRS** | Terminal repeat short |
| **U** | Unit |
| **UAS** | Up-stream activation sequence |
| **UK** | United Kingdom |
| **UL** | Unique long |
| **US** | Unique short |
| **USA** | United States of America |
| **V** | Volt |
| **VEGF** | Vascular endothelial growth factor gene |
| **vIL8** | Viral interleukin 8 |
| **vMDV** | Virulent Marek's disease virus |
| **VP** | Viral protein |
| **vTR** | Viral telomerase |
| **vvMDV** | Very virulent Marek's disease virus |
| **VZV** | Varicella zoster virus |
| **W** | Week |
| **Y2H** | Yeast-two-hybrid |

# Introduction

## 1.1 Herpesviridae

Herpesviruses are important pathogens associated with a wide range of diseases in mammals and birds. The family Herpesviridae is divided into the Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae subfamilies. Each of these is further subclassified into several genera containing many species (Table 1.1). Marek's disease virus (MDV), which is the focus of this thesis, belongs to the genera *Mardivirus* in the Alphaherpesvirinae.

**Table 1.1: Summary of the taxonomic structure of the family Herpesviridae**

| Subfamily | Genus | Examples from the species |
|---|---|---|
| **Alphaherpesvirinae** | *Simplexvirus* | *Human herpesvirus 1* |
| | *Varicellovirus* | *Human herpesvirus 3* |
| | *Mardivirus* | *Gallid herpesvirus 2, Gallid herpesvirus 3, Meleagrid herpesvirus 1* |
| | *Iltovirus* | *Gallid herpesvirus 1* |
| **Betaherpesvirinae** | *Cytomegalovirus* | *Human herpesvirus 5* |
| | *Muromegalovirus* | *Murid herpesvirus 1* |
| | *Roseolovirus* | *Human herpesvirus 6* |
| **Gammaherpesvirinae** | *Lymphocryptovirus* | *Human herpesvirus 4* |
| | *Rhadinovirus* | *Saimiriine herpesvirus 2* |
| | *Ictalurid herpes-like viruses* | *Ictalurid herpesvirus 1* |

***Table 1.1 modified from (Roizman and Pellett, 2001; Davison, 2010)***

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

### 1.2.1 Discovery of MDV

Marek's disease virus was first described by Dr Josef Marek in 1907 as a disease that causes paralysis in the legs and wings of adult chickens. The case was restricted to the nervous system so was named as polyneuritis gallinarum or neuritis interstitialis. Similar cases were observed and reported in the USA and the Netherlands in 1921 and 1924 (cited in (Davison and Nair, 2004)). The disease was restricted to the nervous system and was named as fowl paralysis or range paralysis. Thereafter, Pappenheimer and colleagues observed that lesions were not only restricted to the nervous system, but lymphatic tumours in different visceral organs were also involved, and consequently the disease was renamed as neurolymphomatosis gallinarum (pappenheimer, 1926). By 1956 and 1957, two outbreaks of visceral lymphomatosis were reported in broiler age chickens in the USA (Cover, 1957). The gross pathology of these cases appeared on the spleens, livers, ovaries and musculature. This new form of Marek's disease was associated with higher mortality in younger age chickens. Thus, it was named acute Marek's disease while the former one was named classical Marek's disease. Until 1961, it was thought that the disease belonged to the avian leukosis complex, and then it was considered to be a separate disease. In 1967, an electron microscopy examination of infected tissue cultures revealed that the causative agent of Marek's disease is a herpesvirus (Churchill and Biggs, 1967). In the same study, the cell-associated nature and the cytopathic effect of the virus on the kidney chicken culture were identified. After a few years, the cell-free form of the virus was found in lysed materials from the feather follicle epithelium of infected birds (FFE) (Calnek et al., 1970). After many years, new forms and syndromes of MDV started to appear around the world, such as the transient paralysis and the acute cytolytic form. In the USA, Witter and colleagues isolated variant strains from MDV called Md-5 and Md-11 (Witter et al., 1980). These variant strains were characterised by a higher rate of early mortality at a young age as a result of acute cytolytic infection. In Europe, the presence of the acute cytolytic form of Marek's disease was confirmed in 1997, and virulent strains of

MDV, characterised by early mortality due to severe cytolytic infection, were isolated from the UK, Germany and Spain (Kross et al., 1998; Barrow and Venugopal, 1999). The emergence of new virulent strains of MDV has been explained by failure of the existing vaccines to provide the birds with adequate protection against MDV (vaccine breaks) (see Section 1.8.2). Currently, MDV is classified into 4 pathotypes (Witter, 1997), and many laboratories around the world are studying the development of new MDV vaccines and analysing the genomic contents of the virus.

### 1.2.2 Taxonomy of MDV

MDV belongs to the genus *Mardivirus* of the family Herpesviridae. Herpesvirdae, a class of DNA viruses, are classified into three subfamilies: Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae. Each subfamily is further subclassified into several genera containing many species, causing diseases in humans and livestock (Table 1.1). Many species of herpesviruses can infect birds, six of these species are classified as alphaherpesviruses and are fully sequenced: *Gallid herpesvirus II*, *Gallid herpes virus III* and *Meleagrid herpesvirus 1* (Genus *Mardivirus*), *Gallid herpesvirus I* and *Psittacid herpesvirus 1* (Genus *Iltovirus*) and *Duck herpesvirus 1* (Genus unassigned yet). *Gallid herpesvirus II*, *Gallid herpes virus III* and *Meleagrid herpesvirus 1* are serologically-associated viruses and cause Marek's disease virus-1 (MDV-1), Marek's disease virus-2 (MDV-2) and herpesvirus of turkey (HVT), respectively. While *Gallid herpesvirus I* causes infectious laryngotracheitis (ILT) in chickens, and *Psittacid herpesvirus 1* causes Pacheco's disease virus in parrot. While *Duck herpesvirus 1* causes Duck enteritis virus (DEV) in waterfowl.

Infectious laryngotracheitis (ILT) is an important respiratory disease in chickens. It was identified as a respiratory syndrome affecting chickens in 1925 by May and colleagues, as been reviewed in (Cover, 1996). The viral cause of the problem was identified in 1930 by Beach (Beach, 1930). The virus usually infects birds via the respiratory route. The symptoms of the disease vary according to the virulence of the infective strain from mild respiratory signs to haemorrhagic inflammatory lesions in the upper respiratory tract. Similar to other herpesviruses, ILT infection is divided into two phases: the acute phase and the latent phase, the latter is established only in the trigeminal gangalion (Bagust, 1986; Williams et al., 1992). Neither neutralising antibodies, nor local antibodies, are able to protect chickens from ILT infection (Fahey and York, 1990). Only cell-mediated immunity has this protective role. Two live viral vaccines are available for ILT control. The first one is a virulent strain, and it is applied via cloacal route. The second one is a live attenuated strain, and it is applied via the respiratory route.

Duck enteritis virus (DEV) is an important infectious disease, occurring amongst duck, geese and swans. The oral and cloacal routes are considered the main route for virus transmission. The disease is associated with septicaemia, as well as vascular damage and haemorrhagic patches in the digestive tract, and annular bands in the small intestine. In the late stage, the lesions in the gastrointestinal tract turn to yellowish necrotic diphtheritic membrane (Pattison et al., 2008). A live attenuated vaccine is used for prevention of the disease and repeated annually.

Marek's disease virus-2 (MDV-2) is a naturally non-oncogenic virus belonging to *Gallid herpesvirus III* species. SB-1, MDV-2 strain, can induce early protection against MDV challenges after a few days of SB-1 inoculation (Schat and Calnek, 1978b; Schat and Calnek, 1978a). Therefore, it was used as vaccine for MDV prevention and will be discussed in detail in Section 1.8.

Herpesvirus of turkey (HVT) is a non-pathogenic herpesvirus isolated from turkey (Witter et al., 1970; Witter, 1972). It is non-pathogenic in chickens, but it can induce cross protection against MDV-1 infection. Because of this, HVT has been used as a potent vaccine against MDV, either alone or in combination with other non-virulent or attenuated strains (Geerligs et al., 1999) and will be discussed in detail in Section 1.8.

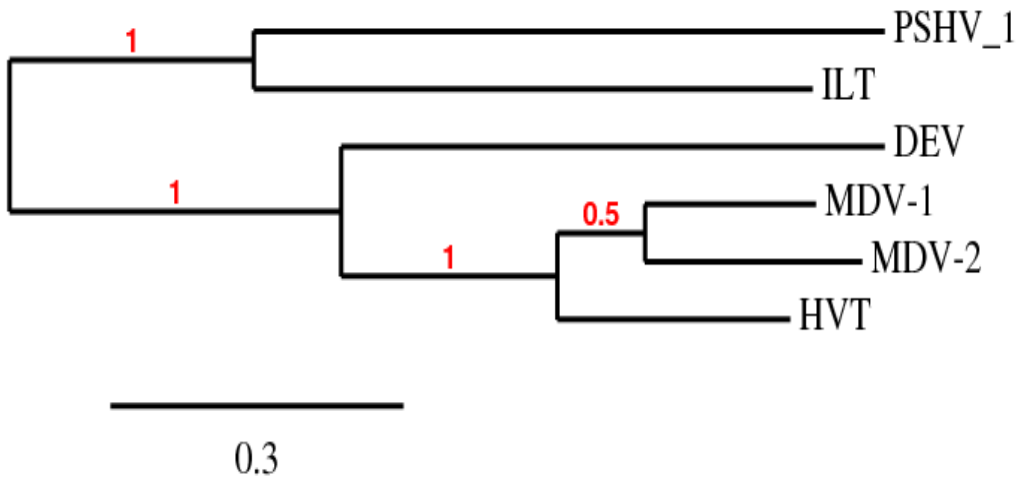


**Figure 1.2.2: Phylogeny of the avian alpha-herpesviruses.** The phylogenetic tree represents the relationship between the conserved large polymerase gene (UL30) of the most popular avian alpha-herpesviruses; Marek's disease virus-1, Marek's disease virus-2, herpesvirus of turkey, infectious laryngotracheitis, Pacheco's disease virus and duck enteritis virus. The tree was created using the Phylogeny.fr web site (Dereeper et al., 2008).

### 1.2.3 MDV morphology

Marek's disease virus is a double stranded DNA virus. Electron microscopy has revealed that MDV is morphologically similar to other herpesviruses (Ahmed and Schidlovsky, 1968; Nazerian and Burmester, 1968; Nazerian, 1974). It is composed of an inner electron dense cylindrical core surrounded by the virus genome (Figure 1.2.3), an icosahedral nucleocapsid with 256 capsomeres, and an outer envelope. In addition, an amorphous layer known as a tegument lies between the nucleocapsid and the surrounding envelope. The mature enveloped virus displays glycoprotein spikes on its surface.

A recent electron microscopy study of chicken embryo skin cells (CESCs) infected with eGFP-recombinant MDV revealed the following: the hexagonal MDV nucleocapsid has a diameter of approximately 100 nm (Denesvre et al., 2007). The primary enveloped virus was detected in the perinuclear space of the infected cells 130-160 nm, and it is composed of nucleocapsid surrounded by dense envelope. The mature enveloped virus is 153-274 nm in diameter and contains a dense tegument layer beneath the envelope. The size of the mature virion was estimated in an earlier study on infected feather follicle epithelium (FFE) to be 273-400 nm (Calnek et al., 1970). This variation may be attributed to the difference in the tissues examined or the sample preparation techniques.

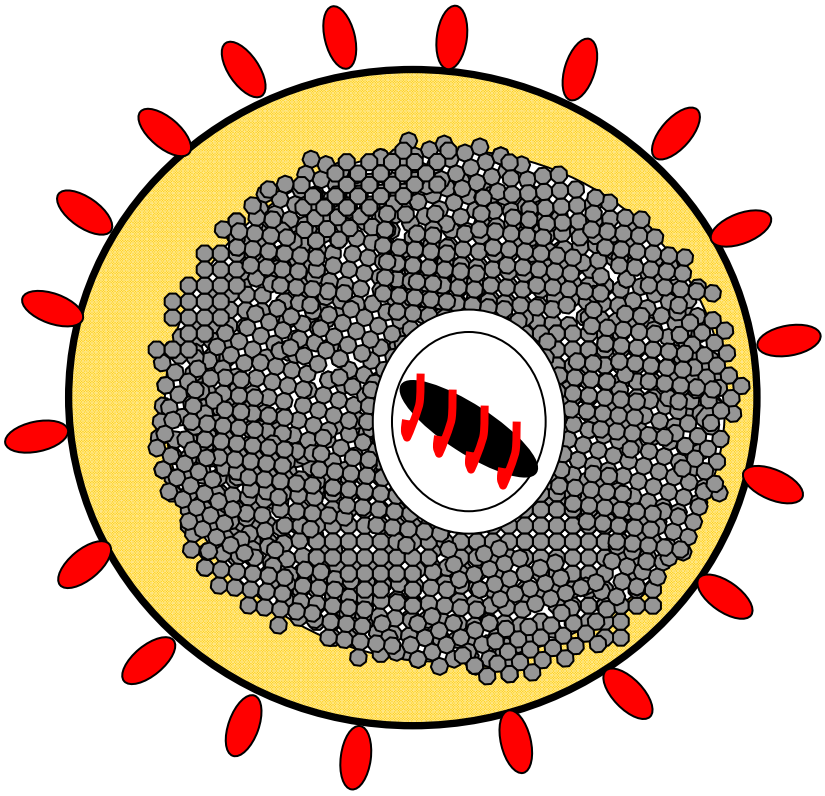

**Figure 1.2.3: The morphology of herpesvirus.** MDV DNA is coiled around this dense structure within the hexagonal capsid. An amorphous tegument layer separates the nucleocapsid and the envelope. The envelope carries glycoprotein spikes on its surface.

## 1.3 MDV replication

It is believed that the mechanism of herpesvirus replication is conserved throughout the entire family. While most of our current knowledge comes from research on herpes simplex virus (HSV) (Roizman and Knipe, 2001), it is believed that other herpesviruses follow similar pathways. Infection starts when the herpesvirus virion attaches to the host cell through the envelope glycoproteins (Figure 1.3). The viral envelope fuses to the plasma membrane in a pH-dependent manner, releasing the capsid into the cytoplasm. The capsid travels to the nuclear membrane where the viral DNA is released. The linear genome enters the nucleus and circularises. Once in the nucleus, the viral genes are transcribed by cellular RNA polymerase II. In

herpesviruses, viral gene expression is tightly regulated and divided into 3 kinetic classes of expression. In HSV, the tegument protein VP16 transactivates transcription of five immediate-early (IE or alpha) genes, which generally encode transcriptional activators. These IE proteins then initiate transcription of the early (E or beta) genes. These gene products are enzymes needed to increase the pool of nucleotides and for viral replication. In addition to proteins directly involved in DNA replication, late (L or gamma) genes are transcribed for production of viral structural proteins. After transcription in the nucleus, all mRNA transcripts are translated into proteins in the cytoplasm. Subsequently, the proteins can be transported to the nucleus, stay in the cytoplasm, or become a part of the membrane bilayer. Capsid proteins assemble in the nucleus to form empty capsids. Full-length viral DNA is packaged into these capsids to form nucleocapsids. The progeny nucleocapsids exit from the nucleus by budding at the inner nuclear membrane into the perinuclear space. The process of virus egress and envelopment is still controversial and it could be through one of three probable pathways. The first pathway is the perinuclear enveloped virions transport inside vesicles in secretary pathway to the Golgi complex and exocytosis at the cell surface. The second pathway is perinuclear enveloped virions de-envelope at the outer leaflet of perinuclear membrane and secondary envelopment occurs in the trans-Golgi area, then the virions released at the cell surface acquiring their glycoproteins. The third pathway is depending on virus egression from the nucleus to the cytoplasm directly through the dilation of nuclear pores.

Electron microscopy showed the stages of virus development in FFE at 12 to 32 dpi (Gilka and Spencer, 1993). The nucleocapsids were shown inside the nuclei as coiled threads, either naked or partially covered by a membrane. Primary vesicles, containing granular materials and some fibrils, were formed inside the cytoplasm. The nucleocapsids bud into these primary vesicles and a dense material formed between the naked nucleocapsid and the vesicle membrane, probably virus tegument. The vesicles containing the virus were named as cytoplasmic vesicular inclusions. Secondary vesicles were formed from the membranes of these vesicular inclusions.

During the cytolysis of the FFE, the vesicular inclusions lost their membranes due to its fragility, and may be fused together. This is resulted in the formation of proteinaceous fluid containing complete and incomplete virus particles and cell debris.

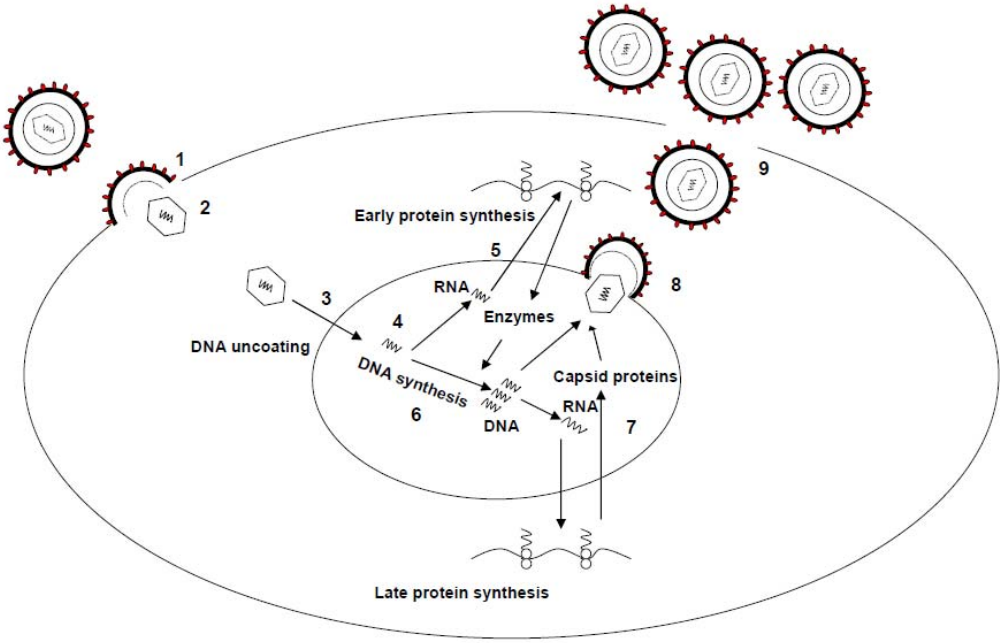


**Figure 1.3: Herpesvirus replication.** The herpesvirus virion fuses with the cell membranes for the host cell through the envelope glycoproteins[1]. The capsid is released into the cytoplasm[2] and travels along the cytoskeleton to the nuclear pores[3], where the viral DNA is released[4]. In the nucleus, the immediate-early and early viral genes are transcribed to form transcriptional regulators and enzymes required for DNA replication[5]. After the formation of the new DNA progeny[6], late (L or gamma) genes are transcribed for the production of viral structural proteins such as capsid proteins[7]. Full-length viral DNA is packaged into these capsids to form nucleocapsids. The progeny nucleocapsids exit from the nucleus by budding at the inner nuclear membrane into the perinuclear space[8]. Thereafter, the virus egresses and envelopment occurs, in order to form and release complete virus particles[9].

## 1.4 MDV classification and nomenclature

Marek's disease has many different synonyms, such as neuritis, polyneuritis, neurolymphomatosis gallinarum and range paralysis. Based on its virulence in chickens, its ability to induce T-cell lymphomas and its antigenic properties there are three MDV serotypes: MDV-1 (e.g. RB-1B, Md5, GA & CVI988 strains), MDV-2 (e.g. HPRS24 strain) and MDV-3, which is also called herpesvirus of turkeys (HVT) (e.g. FC126 strain). MDV-1 can be further divided into four pathotypes: (i) mild, (ii) virulent, (iii) very virulent and (iv) very virulent + (Witter, 1997; Calnek et al., 1998; Witter and Schat, 2003). According to the most recent nomenclature, MDV-1 is classified as *Gallid herpesvirus II* (GHV II), MDV-2 as *Gallid herpesvirus III* (GHV III), and HVT is classified as *Meleagrid herpes virus 1*.

## 1.5 MDV pathogenesis

### 1.5.1 Pathogenesis and cellular tropism of MDV

Since MDV is mainly lymphotropic virus, the affinity of MDV for certain types of lymphocyte is mainly correlated with the stage of virus infection (Figure 1.5.1). Infection with MDV can be divided into four stages (Calnek, 1986; Calnek, 2001): the first stage is the lytic infection of bursa-derived B-lymphocytes between 3 and 6 days post-infection (dpi). The second stage starts with the infection of thymus-derived T-lymphocytes. Later, the latent infection is established between 5 and 10 dpi in T-lymphocytes, then the virus is reactivated from the latency between 14 and 21 dpi, and in the fourth stage lymphomas may develop in different organs of infected birds (Calnek, 1986; Calnek, 2001).

**a. Virus entry**

MDV infects birds through the respiratory route via inhalation of cell-free virus in the feather dander (Calnek et al., 1970). The macrophage cells present in the lung act as transporters for MDV from the respiratory tract to different organs (Calnek, 2001; Barrow et al., 2003). They carry MDV through the blood stream to the lymphoid organs, especially the spleen, in order to start the virus infection cycle. There is a

possibility that MDV is transported from the blood to the spleen via capillaries located in the peri-ellipsoid lymphocyte sheath of the spleen (Jeurissen et al., 1989a; Jeurissen et al., 1989b). Once MDV passes through this sheath, it is transferred to the adjacent ellipsoid associated reticulum cells (EARCS) then to the B-lymphocytes (Jeurissen et al., 1989a; Jeurissen et al., 1989b). EARCS are non-lymphoid antigen presenting cells, located in the B-cell region of the spleen.

**b. Early cytolytic infection**

After MDV particles are delivered to the lymphoid organs, the virus undergoes cytolytic infection in these organs 4-6 dpi (Calnek, 1986; Calnek, 2001; Witter and Schat, 2003; Davison and Nair, 2004). B-cells derived from the bursa, thymus and spleen are considered the main target cells for the lytic MDV infection (Shek et al., 1983; Baigent et al., 1996; Baigent et al., 1998; Witter and Schat, 2003). Staining of spleen lymphocytes isolated from infected birds during the cytolytic stage with monoclonal antibodies (mAbs) for both PP38 MDV specific Ag and different lymphocytes markers confirmed that B-cells are the main cells for MDV lytic infection.

Additionally, these experiments showed that T-cells could also act as target cells for lytic infection, but to a much lesser extent than B-cells. A small population of $CD4^+$ and $TCR\alpha\beta1^+$ $CD8^+$ T-cells were detected in PP38-positively stained spleen cells during the early stage of infection (Baigent et al., 1996; Baigent et al., 1998).

Immunocytochemistry was also used to investigate whether MDV could infect macrophage cells *in vivo*. Surprisingly, spleen macrophages from infected birds were lytically infected with MDV (Barrow et al., 2003). Furthermore, confocal microscopy analysis of the infected macrophages showed the expression of MDV proteins from three kinetic classes (ICP4, PP38 and gB).

Usually, lytic infection appears as a necrotic infection in the lymphoid organs, especially in reticular cells and lymphocytes. Moreover, it causes hyperplasia of the

reticular cells which leads to an enlargement in the spleen. Thereafter, thymus and spleen atrophy can occur (Witter and Schat, 2003). Microarray analysis of MDV infected spleens showed that 79 proteins from MDV have transcriptional activity during lytic infection (Heidari et al., 2008b). In these 79 proteins, there were: vIL8, PP38, vLIPase, US3 and UL49.5, all of which play critical roles in virus pathogenesis.

### c. Latent Infection

Herpesviruses, in general, are characterised by their ability to establish latent infection inside host cells. The cells harbouring latent virus contain the viral genomes as a closed circular molecule and only a small subset of viral genes are expressed (Roizman and Knipe, 2001). MDV also has this ability to remain latent inside the host cells. After the establishment of lytic infection in B-lymphocytes, MDV becomes latent inside T-lymphocytes. This mainly starts at 6-7 dpi (Witter and Schat, 2003; Davison and Nair, 2004). The latent stage of MDV infection could be subclassified into 3 phases: establishment, maintenance and then reactivation from latency (Davison and Nair, 2004).

The ability of MDV to establish latency was initially suggested by Pepose and colleagues. They suggested that the MDV-induced neural lesions in nerves and dorsal ganglia were initiated by the establishment of virus latency. Furthermore, they showed that latency can be established in non-neuronal mononuclear cells which infiltrate nerves and ganglia (Pepose et al., 1981). Later, it was revealed $CD4^+$ bearing T-cells are the major cell population targeted by MDV during latent infection (Calnek et al., 1984; Morimura et al., 1998; Osterrieder et al., 2006). Usually, the MDV genome is present during latency in a methylated state. The former was confirmed when isolated SB-1 DNA from avian lymphoid leukosis transformed derived cell line had the restriction enzyme pattern of 5′ CPG 3′ methylation in dinucleotides regions (Fynan et al., 1993).

Indeed, cell-mediated immunity is required for the establishment and maintenance of MDV latency. Induction of an immunosuppressed state in the infected birds by cyclosporine, betamethasone or thymectomy resulted in prolonged early and late cytolytic stages (Buscaglia et al., 1988). On the other hand, the induction of immunosuppression in MDV infected birds by infectious bursal disease virus (IBDV) and Reticuloendotheliosis virus (REV) resulted in the reduction of both cytolytic and latent infection (Buscaglia et al., 1989). This inconsistent result may be due to either IBD or REV killing MDV target cells, making the cells more resistant to MDV infection or by causing cytokine induction (Buscaglia et al., 1989).

Reactivation from latency is associated with a significant increase in expression of the lytic protein PP38 (Baigent et al., 1996; Baigent et al., 1998; Parcells et al., 2003). Parcells and colleagues presented a model for the mechanism of virus maintenance and reactivation from latency (Parcells et al., 2003). This model suggests that Meq homodimers (Meq-Meq complex formation) bind to the origin of replication. This binding results in the inhibition of the expression of PP38 gene products, and therefore maintenance of the latency state. Indefinite changes in the cellular environment could result in splitting of this homodimer and the formation of Meq heterodimer with any other (basic leucine zipper) bZIP proteins in the cells. At this point, the expression of PP38 gene product starts again and the infection is reactivated.

Recently, microarray analysis of MDV gene expression in the spleen from infected birds has been established. Meq, 23KDa and RLORF5 proteins were the only proteins which showed a marked increase during the latent infection stage (Heidari et al., 2008b).

#### d. Late cytolytic Infection and productive infection in feather follicles

After the 2nd or 3rd week of infection, a restricted productive infection appears in different epithelial tissues (Calnek, 1986). It has been suggested that latently infected cells carry the virus to different organs such as proventriculus, oesophagus, kidney,

adrenal gland, thymus and bursa of Fabricius (Calnek, 1986; Witter and Schat, 2003; Davison and Nair, 2004). This infection results in necrotic-inflammatory lesions in the affected organ, as well as infiltration with mononuclear lymphocytes. The feather follicles epithelium (FFE) is also affected by the second lytic infection as virus particles could be detected in the skin at 12 dpi (Gilka and Spencer, 1993). Interestingly, the FFE is considered the only tissue which can support fully productive infection and disseminate complete virus particles. Earlier, electron microscopy of lysed material from FFE of MDV infected bird showed complete enveloped virus particles (Calnek et al., 1970). Moreover, skin biopsies taken from MDV infected birds at different time points revealed two types of lesions in the perifollicular area associated with MDV infection. The first one is the inflammatory-associated lesion and the other one is the tumour-associated lesion. The inflammatory-associated lesion was characterised by the presence of small lymphocyte aggregates (LCA), distributed in the connective tissue of the perifollicular dermis (Cho et al., 1996).

The genetic background of the birds can affect the presence of the virus in the feather follicles. Though the MDV genome load was lower in MDV resistant birds, compared with MDV susceptible birds at 21 dpi (Abdul-Careem et al., 2009b). MDV shedding from FFE is associated with the stimulation of the cell-mediated immune response at the site of virus release. The cell-mediated immune response is represented in increasing in the infiltration of T-cells, especially $CD8^+$, in the feather pulp area. In addition, the level of interferon-gamma and interferon–alpha secretion increased (Abdul-Careem et al., 2008c).

**e. Transformation**

The transformation in MDV is defined as the neoplastic changes of the latently infected lymphocytes to lymphoblastoid tumour cells (Davison and Nair, 2004). Three to four weeks post infection, latently infected lymphocytes migrate into different visceral organs and peripheral nerves, where they differentiate to form lymphoma. $CD4^+$ $CD8^-$ T-helper cells are the main target cells for natural MDV

transformation (Schat et al., 1991; Burgess and Davison, 2002). The phenotypes of MDV transformed cells were investigated in two groups of cell lines (Schat et al., 1991). The first group was derived from a naturally occurring lymphoma and the second group was derived from local MDV-induced lesions. The former cell lines were $CD3^+$, mainly $CD4^+$ and either $TCR2^+$ or $TCR3^+$. While, the latter cell lines derived from MDV induced lesions were also $CD3^+$ and either $TCR2^+$ or $TCR3^+$. Interestingly, the latter cell lines have only 21% $CD4^+$ $CD8^-$ cells and the rest were $CD4^-$ $CD8^+$. This confusing result could be due to the difference in the conditions of MDV tumour formation in each group.

A feature of transformed MDV infected cells is the high expression level of AV37, an extracellular MDV Ag. The immunocytochemistry revealed that the transformed cells in MDV naturally occurring lymphoma are mainly a population of $CD4^+$ T helper cells which are either $TCR2^+$ or $TCR3^+$, $MHCI^{hi}$, $MHCII^{hi}$, $PP38^-$, $gB^-$ and $AV37^{hi}$ (Burgess and Davison, 2002). These $AV37^{hi}$ cells fulfil the neoplastic transformation criteria as follows: they have a high proliferation rate, support latent MDV infection, a low death rate and are very frequent cells in MDV lymphoma. Also, $AV37^{hi}$ lymphoma cells need to escape the immune response in order to survive as a tumour cell. Thus, $AV37^{hi}$ cells escape the immune system via the down-regulation of CD28 at the mRNA level (Burgess and Davison, 2002). Later, AV37 was recognised as CD30 and the MDV Lymphoma was recognised as a $CD30^{hi}$ lymphoma (Burgess et al., 2004).

$AV37^+$ $CD4^+$ cell ($CD30^+$ $CD4^+$) infiltration usually appears in genetically susceptible as well as genetically resistant breeds. However, after the cytolytic stage the fate of these cells changes according to host genetic susceptibility (Burgess et al., 2001). Hence, cell infiltration appeared 5 dpi in the genetically susceptible breeds and 7 dpi in the genetically resistant breeds. Thereafter, $CD4^+$ T lymphoid cell infiltration increased in the genetically susceptible lines and decreased in the genetically resistant breed, until it became rare and very hard to detect at 35 dpi.

Meq is an MDV protein which is able to transform Rat-2, NIH3T3 and DF1 cell lines (Liu et al., 1998; Levy et al., 2005; Ajithdoss et al., 2009). Interestingly, $CD30^{hi}$ transformed cells contain the Meq oncogene, and the presence of Meq is positively correlated with the expression of $CD30^{+}$ (Ross et al., 1997). Furthermore, Meq forms heterodimers with the cellular protein c-Jun, and this is important for tumour formation and growth (Levy et al., 2005).

MicroRNAs (miRNAs) are small RNAs that act as post-transcriptional regulators of protein expression, and they have been implicated in many different biological processes, including oncogenesis and cell regulation. Studies on the role of miRNAs in MDV oncogenesis and transformation have found an up-regulation of miR-221 and miR-222 in MSB-1 cell line (Lambeth et al., 2009a). These miRNAs target the degradation of chicken $P27^{kip1}$, a cell cycle regulatory protein. It is possible that MDV uses a miRNA-mediated mechanism to transform infected cells.

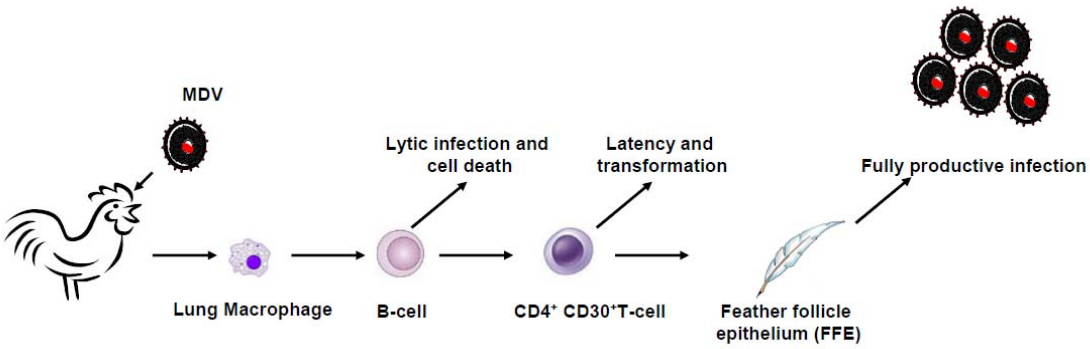


**Figure 1.5.1: Pathogenesis and cellular tropism of MDV.** MDV infects birds through the respiratory route. Thereafter, the virus infects macrophages, which distribute the virus to the lymphoid organs. Lytic infection is established in B-lymphocytes (3-6 dpi) followed by latent infection in T-lymphocytes (7-14 dpi). Later, the virus is reactivated from latency and spreads to the external environment through FFE.

### 1.5.2 MDV Transmission

MDV is an airborne disease, transmitted by direct and indirect contact (Witter and Schat, 2003). Birds are usually infected at a very young age via the inhalation of cell-free virus, which is present in the feather follicle dander, desquamated skin epithelium and poultry house dust (Calnek et al., 1970; Carrozza et al., 1973; Gilka and Spencer, 1993). In addition, the virus is highly endemic in poultry houses and can live at room temperature for several months (Carrozza et al., 1973; Witter and Schat, 2003). Therefore, early vaccination is extremely important to prevent early infection and subsequently leads to a reduction in virus transmission rate. Many studies were established to identify MDV proteins responsible for the horizontal transmission of the virus, and US2, UL13 and gC are considered good candidates. Hence, MDV lacking these genes is not able to spread from infected to susceptible chickens (Jarosinski et al., 2007b). However, the mechanism behind the role of these genes in virus transmission remains inconclusive.

## 1.6 Clinical disease and pathology

Marek's disease is a very important lymphotropic avian disease which causes great worldwide economic losses in the poultry industry. Additionally, study of Marek's disease virus (MDV) has contributed significantly to our understanding knowledge of herpesvirus-associated oncogenicity. The main host of MDV is the chicken. However, quails, turkey and pheasants can also be infected (Witter and Schat, 2003). In addition, there is evidence of its isolation from white-fronted geese in Japan and the Far East region of Russia (Murata et al., 2007). MDV is highly cell-associated and is only cell-free in the feather follicles. As described in Section 1.2.1, it was first described by Marek in 1907; thereafter many forms of the disease started to be revealed. MDV is a multi-phasic disease composed mainly of early cytolysis and late transformation (see Section 1.5). The disease has many forms including classical MDV, lymphomatosis, ocular form, skin form, early mortality syndrome, acute cytolytic infection, transient paralysis and atherosclerosis (Table 1.6). MDV clinical signs vary greatly according to the stage of infection and the virulence of the virus.

### 1.6.1 Classical MDV (Fowl paralysis)

Fowl paralysis, the classical MDV, is the original form of MDV which is characterised by both neuropathy in peripheral nerves and lymphomatosis in visceral organs, particularly the ovaries (Biggs and Payne, 1967). The disease appears in the form of asymmetrical paralysis called spastic paralysis (Figure 1.6d). Thereafter, leg paralysis develops to complete paralysis and death. Sometimes the vagus nerve is involved and results in dilation of the crop (pendulous crop) and gasping (Witter and Schat, 2003). Paralysis is usually associated with the gross enlargement of the peripheral nerves, which usually appears 3-4 weeks after infection in experimentally-challenged susceptible birds (Biggs and Payne, 1967).

### 1.6.2 Lymphomatosis (Visceral lymphomas)

The affected birds with MDV-induced lymphomatosis have no particular clinical signs. However, there are a few external clinical signs, which range from general depression to totally comatose. Visceral lymphomas occur in the ovaries, lung, heart, mesentery, kidney, liver, spleen, bursa, thymus, adrenal gland, pancreas, proventriculus, iris, skeletal muscle and skin in the presence or absence of gross nerve lesions (Payne and Rennie, 1976; Witter and Schat, 2003; Davison and Nair, 2004). The gross pathology of MDV lymphomas is reviewed in details in (Witter and Schat, 2003). The general shape of an MDV lymphoma appears as an enlargement of the organ with diffuse or nodular white or grey discoloration (Figure 1.6b). The ovary has a distinct cauliflower appearance as the normal foliated shape is replaced with a cauliflower-shaped mass. Thickness and firmness is seen in the proventriculus wall plus paleness in the heart and occasionally nodular lymphomas appear on the myocardium. Diffuse or nodular enlargement is seen on the liver with firm texture and sometimes granular appearance. Histologically, interfollicular lymphomatous proliferation in the bursa of Fabricius, lymphomatus proliferation in the thymus medulla, as well as lymphoproliferative foci in the spleen are usually present in chickens suffering from MDV lymphomatosis (Payne and Rennie, 1976).

As described in detail in Section 1.5.1, the $CD3^+CD4^+$T-cell population is the main cell type in an MDV lymphoma. The histological changes of MDV visceral lymphomas from 1 to 9 weeks post infection (pi) have been recorded (Cho et al., 1998). The MDV lesions of the visceral organs and nerves were infiltrated with small lymphocytes 1-3 weeks pi, thereafter the lesions were infiltrated with a mixture of lymphoblasts and small lymphocytes 4-6 weeks pi. At 7-9 weeks pi, the visceral organ lesions consisted mainly of lymphoblast cells with some small lymphocytes. Interestingly, the visceral and nerve-lymphoma-affected birds have characteristic cytological changes in the feather pulp lesions (FPL) compared to MDV-infected birds with no lymphomas. These cytological changes consisted mainly of small lymphocyte cells aggregates (LCA) 1-2 week pi (Cho et al., 1998). Thereafter, the number of the lymphoblast cells increased in the aggregates until 7-9 weeks when the aggregates became large lymphoid aggregates of polymorphic lymphoblast cells.

### 1.6.3 Ocular form

In the USA, two flocks of vaccinated replacement pullets >10 weeks old suffered from an outbreak of MDV. The main clinical manifestation of this outbreak was blindness in the infected birds (Ficken et al., 1991). Whilst ocular MDV has been observed in sporadic cases, this was the first outbreak in which it presented as the main clinical symptom. The gross pathology of the eye revealed acute uveitis and corneal oedema (Figure 1.6c). Microscopically, there was mononuclear cell infiltration in the iris, ciliary body, cornea, retina, pectin and optic nerve.

### 1.6.4 Skin form (Skin leukosis)

Skin leukosis is considered another lymphoproliferative form of MDV infection (Figure 1.6a), and lesions usually appear 4 weeks post infection (pi) (Cho et al., 1996; Witter and Schat, 2003). Macroscopically, the lesion extends from the dermis to the subcutaneous layer until it reaches the skeletal muscle. A few weeks later (7-9 weeks pi) the lesions appear as nodular skin lesions on many areas of the skin which fuse together and increase in size (Cho et al., 1996; Cho et al., 1997). The presence

of feathers is not mandatory for the development of MDV induced skin tumours (Heidari et al., 2007). The lesions are usually more abundant in the bird dorsocervical tract, followed by ventrocervical, abdominal, sternal, outer crural and inner crural tracts (Cho et al., 1996; Cho et al., 1997).

Microscopically, MDV skin lesions have two distinct patterns in the perifollicular area: a tumour-associated pattern and a non-tumour-associated pattern (Cho et al., 1996). The tumour-associated pattern is classified into two types according to the cellular composition of LCA: the progressive and regressive types. The progressive type starts with an accumulation of small LCA in the perifollicular area 1 week pi. These small LCA develop to large LCA containing a mixture of small lymphocytes and lymphoblast cells 4-6 week pi. Later on, at 7-9 weeks pi, the LCA cells accumulate together and the lymphoblast cells become the major cell population. The muscles adjacent to the skin lesions are infiltrated with proliferated fibroblasts and the connective tissue in between contain small LCA (Cho et al., 1996). The regressive type is characterised by gradual regression of the LCA after 4 weeks pi, which is localised to the connective tissue of the perifollicular dermis. No lymphoblast cells are detected in the regressive type. Cho and colleagues classified the MDV-induced skin leukosis into five types (A, B, C, D and E) depending on the size and the cellular composition of LCA, where type A contains small LCA of small lymphocytes. Thereafter, the lesions progress and lymphoblast cells increase until the small lymphocytes are completely replaced with lymphoblasts in type E (Cho et al., 1997).

### 1.6.5 Early mortality syndrome and acute cytolytic infection

This form was reported earlier when Witter and colleagues isolated two variant strains of MDV: Md5 and Md11. The clinical disease of the early mortality syndrome varies according to bird susceptibility. Hence, it is characterised by acute cytolytic infection and high rates of early morality in the susceptible birds. However, it can induce lymphoma in HVT vaccinated birds and genetically resistant birds (Witter et al., 1980). Thereafter, these variant strains were classified as very virulent

MDV strains (vvMDV) (Witter, 1983). The acute cytolytic infection of these strains is characterised by marked atrophy in the bursa and thymus and higher spleen necrosis than other MDV strains (Witter et al., 1980; Witter, 1983). Whilst it induces a high rate of visceral lymphoma in both genetically resistant and susceptible birds, it induces fewer neural lymphomas in the genetically resistant birds than the vMDV strains does (Witter, 1983). Other two vvMDV strains, MS1 and MS2, were isolated in Japan, and they also caused early mortality syndrome in P-2 genetically susceptible chickens and vaccinated with HVT (Imai et al., 1992). These two strains are viscerotropic and neurotropic and differ from Md5 strain, as they produced gross lesions in visceral organs and nerves at a similar rate. In Europe, early mortality syndrome was recorded when C12/130, MR36 and MR48 MDV isolates induced high mortality during the cytolytic stage associated with lymphoid atrophy (Barrow and Venugopal, 1999).

### 1.6.6 Transient paralysis

MDV infection of the central nervous system (CNS) usually results in cases of general flaccid paralysis called transient paralysis (TP). It was believed that TP is one form of temporary flaccid paralysis which disappears after 2-4 days (Kenzy et al., 1973). Then, Gimeno and colleagues classified and described 4 forms of MDV-induced transient paralysis, which appeared in experimentally-infected birds: classical-transient paralysis, acute-transient paralysis, persistent neurological disease and late paralysis (Gimeno et al., 1999). Birds suffering from classical-transient paralysis (TP) have different degrees of paralysis 9-11 dpi, from which they completely recover by 12-14 dpi. This classical form is mainly caused by infection with the virulent MDV pathotype, whilst acute-TP is caused by the very virulent and very virulent + MDV pathotypes. Signs of acute paralysis appear 9-10 dpi, often with signs of flaccid paralysis in the neck and limbs, and birds die 24-48 hr. In persistent neurological disease (PND), birds recover from the paralysis, but other neurological signs, such as ataxia and torticollis, appear and persist. The late paralysis (LP) form is similar to the acute-TP form, but it appears later at 21 dpi. Both PND and LP occur in chickens that have survived infection with very virulent or very virulent + MDV

strains. Brain and cerebellum lesions are usually associated with MDV transient paralysis signs. Such lesions appear in the brain and cerebellum 6-8 dpi with vasculitis, perivascular oedema, microgliosis and mononuclear perivascular cuffing (Gimeno et al., 1999; Witter et al., 1999).

### 1.6.7 Atherosclerosis

Atherosclerosis is a pathological case of thickness in the artery walls as a result of cholesterol and other lipid droplets deposition. Infection with MDV is sometimes associated with atherosclerosis in the aorta, coronary arteries and other large arteries. It usually appears in the arteries >1 month pi, and the virus is detected in the middle-smooth muscle cells layer of the affected arteries (Fabricant et al., 1978; Minick et al., 1979; Hajjar et al., 1986; Fabricant and Fabricant, 1999). Macroscopically, MDV-induced atherosclerosis appears as a marked narrowing in the lumen of the affected artery with small focal plaques 1-2mm in diameter (Minick et al., 1979). Microscopically, the arterial lesions are classified into: fatty, proliferative and fatty-proliferative (Fabricant et al., 1978; Minick et al., 1979). Fatty arterial lesions have intra- and extra-cellular lipid accumulation, low cellular proliferation and a small quantity of dense collagen deposition. The proliferative lesions have fibro-cellular thickening of the artery intima, fragmentation of the internal elastic lamina and low level of mononuclear cell infiltration, whilst fatty-proliferative lesions combine both the fatty arterial and proliferative arterial lesions. Hajjar and colleagues have suggested that the aortic deposition of cholesterol and cholesteryle ester (CE) in MDV infected birds is due to alterations in CE metabolism (Hajjar et al., 1986). Briefly, aortic cholesteryle ester synthetic activity (ACAT) was increased in MDV infected bird 50% and 2 folds 4 and 8 months after MDV infection, whilst aortic hydrolytic activity, represented by lysosomal CE hydrolase (ACEH) and neutral CE hydrolase (NCEH) activity, was reduced by 30 and 80% 8 months after MDV infection, respectively. Therefore, the reduction in CE hydrolytic activity and the increase in CE synthetic activity led to an accumulation of CE on the artery wall, which leads to development of atherosclerosis. MDV-induced atherosclerosis can be reduced by treatment of the birds with 3-hydroxy-3-methylglutaryl-coenzyme A

(HMGCoA) or angiotensin converting enzyme (ACE) for 60 days (Lucas et al., 1998).

**Table 1.6: Different disease syndromes of Marek's disease infection in chickens**

| SYNDROMES | LYMPHOPROLIFERATIVE SYNDROMES | | | | LYMPHODEGENERATIVE SYNDROMES | | | TRANSIENT PARALYSIS CNS SYNDROMES | | | | VASCULAR SYNDROMES |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Lymphomas and nerve lesions** | **Fowl paralysis** | **Skin leukosis** | **ocular lesions** | **Early mortality syndromes** | **Acute cytolytic infection** | **Immuno-depression** | **Classical-transient paralysis** | **Acute-transient paralysis** | **Persistent neurological disease** | **Late paralysis** | **Atherosclerosis** |
| **Susceptible age** | 4-90 w | 8-20 w | 4-8 w | >10 w | [E]1-6 day pi | 9-16 day | 9-16 w | 5-12 w | 5-12 w | [E] 5-12 w | [E] 5-12 w | [E]Adult birds |
| **Mortality** | 0-60% | 0-20% | - | - | [E] 0-100% | [E]0-100% | [E]0-100% | Rare | [E]0-100% | Rare | Rare | None |
| **Clinical signs** | Depression, death, paralysis | Paralysis and lameness | Swollen feather follicles | | Increased disease susceptibility | Increased disease susceptibility | Increased disease susceptibility | Flaccid paralysis of neck and wing follow by recovery 24-48 hr pi | Flaccid paralysis of neck and wing follow by death 24-72 hr pi | Persistent ataxia and torticollis | Flaccid paralysis of neck and wing | - |
| **PM** | Lymphoma in visceral organs and peripheral nerves | Enlargement of the peripheral nerves | Swollen feather follicles | | Atrophy in thymus and bursa, large necrotic area in the spleen | Atrophy in thymus and bursa, large necrotic area in the spleen | Atrophy in thymus and bursa, large necrotic area in the spleen | Vasogenic oedema in the cerebellum | Vasogenic oedema in the cerebellum | Vasogenic oedema in the cerebellum | Vasogenic oedema in the cerebellum | - |

***E*** *Experimental infection*
***w*** *week*
***pi*** *post infection*

Table 1.6 adapted from (Gimeno et al., 1999; Witter et al., 1999; Witter and Schat, 2003).

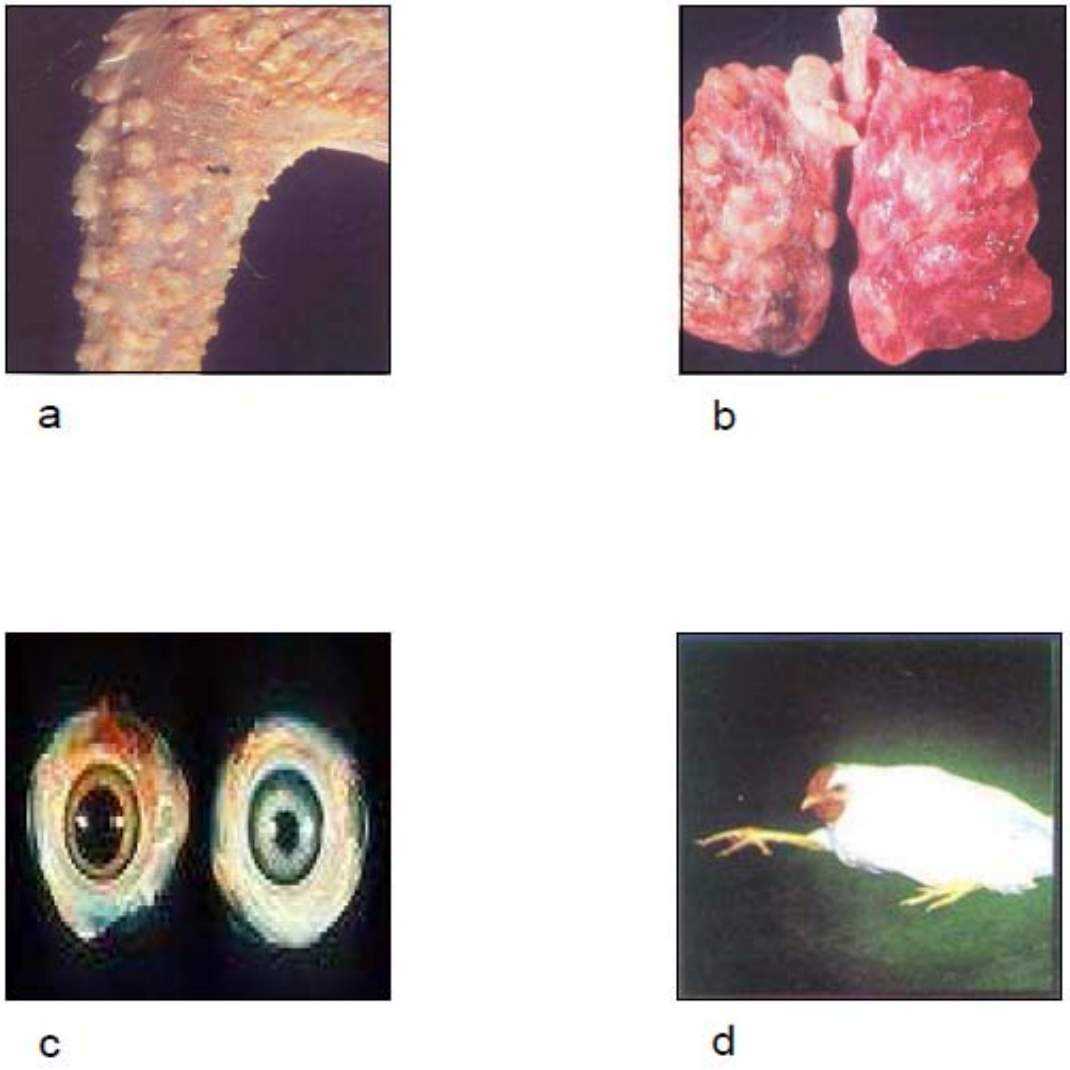


**Figure 1.6: MDV clinical disease and pathology.** (a) A case of skin lymphoma in an MDV infected chicken. (b) A case of lung lymphoma in an MDV infected chicken. (c) A case of ocular lymphoma in the eye of an MDV infected chicken. (d) A case of spastic paralysis (leg paralysis due to a tumour in the sciatic nerve) in an MDV infected chickens (Witter and Schat, 2003).

## 1.7 Immune responses to MDV

MDV is a lymphotropic virus, and its infection is strictly associated with immune cells (B-cells and T-cells). As infection with MDV also results in an increased severity of subsequent secondary infections or a reduction in the post vaccinal immune response, it is considered a major immunosuppressive disease in chickens.

In this section, the general immune response upon MDV infection and/or vaccination will be discussed in detail. Generally, the host immune response following the invasion of any micro organism is divided into the innate and adaptive immune responses. The innate immune response is a non-specific immediate and temporary immune response against a spectrum of molecular signals, whereas the adaptive immune response is a specific immune response developed after pathogen recognition by the innate immune response.

### 1.7.1 Innate immune response

Innate resistance is an immediate protective response, consisting of a range of mechanisms to inactivate the invading pathogen (Stewart, 1997). It has many components, such as phagocyte cells (macrophage cells & neutrophil cells), natural killer cells (NK cells), the complement system and cytokines. Some of these components are developed upon MDV infection, and they exert a role in host protection from the virus.

#### a. Macrophage

Macrophage cells respond immediately upon infection with any pathogen and consequently secrete a group of cytokines that attack virus particles. After infection with MDV, the macrophage phagocytic activity and index increase (Powell et al., 1983). Moreover, their infiltration of the different organs in the infected birds, such as the bursa of Fabricius and lung parenchyma, increases (Abdul-Careem et al., 2008a; Abdul-Careem et al., 2009a). This increase in macrophage infiltration can

exert a negative effect on MDV replication, as it was shown earlier that macrophage cells were able to restrict MDV replication in duck embryo fibroblasts (DEF) (Lee, 1979). The negative effect on MDV replication could be a direct effect or also an indirect effect through the up-regulation inducible nitric oxide synthase (iNOS) secretion. Hence, the increasing of macrophage cells is always consistent with increasing of iNOS secretion (Djeraba et al., 2000; Xing and Schat, 2000b; Abdul-Careem et al., 2008a; Abdul-Careem et al., 2009a).

**b. NK cells**

NK cells are a specific population of lymphocytes which represent an important part of the non-specific immune response at the early stage of virus infection. Once the cells are attacked by the pathogen, NK cells respond directly by killing virus infected cells. Furthermore, it responds indirectly by secreting interferon-gamma, which stimulates the macrophage cells to kill the phagocytosed pathogens. In case of MDV infection, NK cells activity may be correlated with the bird's genetic resistance. One example, a genetically MDV-resistant chicken strain (N2a) has a higher NK cell response, than an MDV-susceptible chicken strain (P2a) upon MDV challenge (Sharma, 1981; Garcia-Camacho et al., 2003). Thus, MDV-genetic resistance can attribute to the high NK activity in the resistant breeds (Garcia-Camacho et al., 2003).

**c. Cytokines response**

Cytokines are a group of glycoproteins which are secreted by the cells as a result of their stimulation by a stimulus (Stewart, 1997). Macrophage cells are the main source of cytokine production. Some cytokines act as modulators for the innate immune response such as interferons, tumour necrosis factor (TNF) and interleukins, especially interleukin-1 (IL1) and IL12 (Stewart, 1997). Whereas, there are other cytokines that are considered related to both innate and adaptive immune responses, such as interferon-gamma. Innate immunity cytokines such as TNF and IL1 are mainly involved in attracting phagocytic cells to infection sites, while IL12 targets NK cells to mediate its interferon-gamma induction. Subsequently, interferon-

gamma activates macrophage cytokine secretion and also mediates antibody production. Additionally, upon virus infection, infected cells produce type I interferon (interferon-alpha and interferon-Beta), which inhibits virus replication. In this section, the cytokine response to MDV infection will be discussed in detail.

**Interferon**

Interferons are a group of cytokines that contribute to the innate non-specific antiviral immune response. They are produced immediately upon virus infection by infected cells. Subsequently, the secreted interferons trigger the surrounding cells to establish an antiviral state. Moreover, interferons can activate the cytotoxic activity of NK cells, which in turn kill virus infected cells. In mammals and birds, there are three types of interferon: type I (e.g. interferon-alpha and Beta), type II (interferon-gamma) and type III interferon (interferon λ). The interferon classification and mode of action will be discussed in more detail in Chapter 5. In this section, it will be focused more on the role of interferon in the regulation of MDV infection and the effect of MDV infection on the modulation of interferon secretion.

***Interferon-alpha (IFN-α)***

Herpesvirus infection is associated with the down-regulation of type 1 interferon, particularly IFN-α (Quere et al., 2005; Yu et al., 2005; Ambagala and Cohen, 2007; Wu et al., 2009b). Consistently, MDV infection also results in the down-regulation of IFN-α level in infected birds. Moreover, it is able to block the interferon response of the Newcastle disease vaccine, a potent IFN-α inducer (Quere et al., 2005). This effect on IFN-α is not correlated with the virulence of the MDV strain. Hence, the down-regulation of IFN-α mRNA was observed in genetically resistant chickens from 1 to 7 days post infection with either RB-1B MDV or HVT (Quere et al., 2005). This down-regulation or even absence of IFN-α transcripts was mainly detected in the spleens of MDV infected birds, particularly in the latent stage (Xing and Schat, 2000a; Heidari et al., 2008b). This effect on IFN-α level in MDV-infected birds may be due to certain molecules encoded by MDV which exert a negative regulatory action on the IFN-α pathway.

***Interferon-gamma (IFN-γ)***

Interferon-γ is considered to be a cytokine of both the innate and adaptive immune response. Many studies have suggested that MDV infection and/or vaccination are associated with an up-regulation of IFN-γ mRNA level in different organs, such as the spleen (Xing and Schat, 2000b; Abdul-Careem et al., 2007; Heidari et al., 2008b) and feather follicle epithelium (Abdul-Careem et al., 2008b; Abdul-Careem et al., 2008c; Abdul-Careem et al., 2009b). However, the up-regulation of IFN-γ level seems to correlate more with disease development than protection from the disease. For example, there was an increase in IFN-γ transcripts in spleens from MDV-vaccinated but diseased birds than the vaccinated and protected birds (Abdul-Careem et al., 2007).

The genetic type of the chickens is also considered a major factor contributing to the modulation of IFN-γ levels in different organs. After infection with oncogenic MDV or vaccination with HVT, the enhancement of IFN-γ transcripts was observed in the blood of genetically resistant chickens (Quere et al., 2005). In contrast to the blood, a significant increase in IFN-γ transcripts was observed in the feathers of genetically susceptible chickens, more than the genetically resistant line, after infection with a virulent MDV strain (JM-16) (Abdul-Careem et al., 2009b).

**Interleukin (IL)**

Interleukins are a group of proinflammtory cytokines which involved in both innate and adaptive immune responses. They are secreted from the immune cells such as macrophage and T-lymphocytes. Interleukin proteins work as immunomodulators through the regulation and differentiation of the immune cells.

MDV disease development, rather than protection from the disease, is associated with the up-regulation of a group of interleukins such as IL18, IL6, IL10, IL8 and IL1β (Abdul-Careem et al., 2007). This up-regulation was detected in different organs such as spleen, bursa of Fabricius, lung and FFE (Abdul-Careem et al., 2007; Abdul-Careem et al., 2008a; Abdul-Careem et al., 2008b; Heidari et al., 2008b;

Abdul-Careem et al., 2009a; Parvizi et al., 2009). Consistently, the genetic susceptibility of the bird also contributed to the increasing level of some interleukins, such as IL18. Hence, IL18 level was higher in genetically susceptible birds challenged with virulent HPRS-16 strain than genetically resistant birds challenged with the same strain (Kaiser et al., 2003).

**Inducible nitric oxide synthase (iNOS)**

iNOS is an eukaryotic enzyme that regulates some cellular-signalling molecules such as IRF1 and NF-kB. The expression of iNOS is not affected by MDV infection or vaccination (Abdul-Careem et al., 2007). Although, the previous study is not in agreement with other studies that showed a marked increase in the iNOS level in the spleens of MDV infected chicken with either serotypes 1, 2 or serotype 3 (Xing and Schat, 2000b; Heidari et al., 2008b). Also in more recent study, lungs of RB-1B infected chickens showed marked increase in the iNOS level (Abdul-Careem et al., 2009a).

### 1.7.2. Adaptive immune response

#### a. Cell-mediated immune response.

Upon infection with any pathogen, T-cells are activated by recognition of peptides presented by MHC molecule on surface of the antigen presenting cells (APCs). They are divided and differentiated into 5 types T helper, T cytotoxic, T regulatory, T memory and NK T cells. T helpers are $CD4^+$ cells, and they mainly help the other T and B-cells to divide, activate and exert their immune responses. T cytotoxic are $CD8^+$ cells, and their role are to destroy virus infected cells and tumour cells. T regulatory cells are a group of T-cells that function as a suppressor for the activated immune system. It can be $CD8^+$ or $CD4^+$ $CD25^+$ cells. It suppresses the immune system after attacking pathogens and preventing it from attacking the host tissues. T memory subpopulation is a group of antigen specific T-cells which can be $CD4^+$ or $CD8^+$. NK T-cells are a special kind of cells that communicate the adaptive immune with the innate immune responses. It can act in similar way to T helper or T

cytotoxic cells, and are mainly involved in eliminating tumour cells and herpesvirus infected cells.

MDV is a cell-associated virus and because of this cell-mediated immunity plays a potential role in controlling MDV infection. Both T helper cell and T cytotoxic cell immune responses are developed in response to MDV infection and/or vaccination.

Infection with virulent MDV strains results in a $CD4^+$ helper lymphocyte and $CD8^+$ cytotoxic lymphocyte (CTL) immune responses (Abdul-Careem et al., 2008a). The infiltration of both types of T-cells increased significantly at 10th dpi in the inter-follicular areas of the bursa of Fabricius in MDV infected chickens (Abdul-Careem et al., 2008a). This suggested that $CD8^+$ cells are responsible for clearing of the virus from the infected bursa, and $CD4^+$ cells provide suitable cytokines media for the virus clearance.

$CD4^+$ helper cells are usually differentiated into a group of effector cells, such as T helper-1 (Th-1) and T helper-2 (Th-2). T helper-1 functions mainly as source of interferon-γ, whereas T helper-2 functions mainly as a cytokine source, including IL4, IL13 and IL10. Since the process of the naïve $CD4^+$ cell differentiation and the subtype of $CD4^+$ cells required for the immune response is determined according to specific cytokines excretion pattern, chickens infection with a very virulent+ MDV strain induced cytokine secretion pattern specific for the Th-2 cell pathway. These cytokines are IL4, IL10, and IL13, which were up-regulated during the MDV-cytolytic infection stage (Heidari et al., 2008b).

The cytotoxic T cell response representing in $CD8^+$ CTLs is developed after MDV vaccination with either HVT, SB-1 or CVI988 vaccine, particularly $CD8\alpha^{high}TCR1^+$ phenotype (Omar and Schat, 1997; Morimura et al., 1998; Quere et al., 2005; Abdul-Careem et al., 2008c; Kano et al., 2009). Development of an MDV specific cytotoxic immune response is induced by the MDV glycoproteins: gB, gC, gE, gH, gL (Omar et al., 1998; Markowski-Grimsrud and Schat, 2002). For example, chickens

immunised with recombinant fowl pox virus expressing MDV gB induce $CD8^+$, $TCR\alpha\beta1^+$ and $CD4^-$ CTLs, which are specific for gB (Omar et al., 1998). All of the previous indicate that $CD8^+$ CTLs play a crucial role in the immune response to MDV, particularly after vaccination. On the other hand, the presence of $CD8^+$ cells is not enough to prevent the lymphomas induced by the oncogenic MDV strain (Morimura et al., 1998). Thus, CTLs could have an antiviral effect, but they appear to have no anti-tumour effect (Morimura et al., 1998). Interestingly, genetic resistance to MDV is correlated with the activity of CTLs. MDV genetically-susceptible birds challenged with the virulent strain of the virus had a lower level of MHC restricted CTLs than non-infected birds at 20 days post infection (Garcia-Camacho et al., 2003). In the same study, genetically-resistant birds challenged with MDV had a higher MHC restricted CTLs than control birds at 4, 8, 12, 16 and 20 dpi. Virulent strains of MDV have the ability to reduce the expression of MHC class I in CEF infected cells as a smart way to evade CTLs host immune response (Levy et al., 2003). On the other hand and in contrast to all other herpesvirus, MDV up-regulates MHC class II expression on the surface of infected cells (Niikura et al., 2007). This up-regulation occurs as a cellular response to MDV lytic infection.

**b. Humoral immune response**

The humoral immune response to MDV is either passively acquired through the maternal antibodies, or actively acquired through previous virus exposure. Previous exposure to MDV leads to the development of neutralising antibodies against the virus14 days post infection or vaccination (Zelnik et al., 2004; Wu et al., 2009a). Later on, the level of MDV-specific neutralising antibodies usually continues to increase (Zelnik et al., 2004; Wu et al., 2009a). Re-vaccinated birds usually have a higher level of neutralising antibodies than single vaccinated birds. This is because of the maternal antibodies neutralise some of the first day old vaccine antigenicity. Therefore, boosting of the immune system by a second vaccine dose could enhance the immune response (Wu et al., 2009a).

The role of humoral antibodies in controlling MDV infection is currently uncertain. Hence, it is stated that the cell-mediated immune response plays the main role in the bird protection from MDV infection, due to the virus cell-associated nature (Sharma et al., 1975). On the other hand, Zelnik and colleagues noticed that the high seroconversion of MDV-specific antibodies was correlated with protection of birds challenged with a very virulent MDV strain (Zelnik et al., 2004).

## 1.8 MDV vaccination

Marek's disease was the first tumour which can be prevented by vaccination (Schierman and McBride, 1979; Calnek and Schat, 1991). MDV vaccines are either attenuated MDV-1 vaccine (CVI988), non-virulent MDV-2 (SB-1) or HVT (FC126). These vaccines are either monovalent (composed of one virus strain), bivalent (composed of two virus strains) or trivalent vaccines (composed of three virus strains). Monovalent vaccines are HVT (FC126) or CVI988; bivalent vaccines are HVT (FC126) + CVI988 or HVT (FC126) + SB-1, while trivalent vaccines are HVT (FC126) + SB-1 + CVI988 vaccine or HVT (FC126) + SB-1 + Md11/75C.

### 1.8.1 Vaccination route and time

HVT is inoculated either subcutaneously (s/c) at hatching or *in ovo* (Sharma and Burmester, 1982; Sharma et al., 2002; Zhang and Sharma, 2003; Tan et al., 2007). A comparison study of the times and routes of HVT vaccination by Tan and colleagues found that embryo day (ED-11) and (ED-17) vaccination times gave a higher survival rate than ED-6 vaccination, with survival rates of 89.5%, 94.1% and 38.5%, respectively (Tan et al., 2007). Thus, vaccination at later stages of embryonic development is better than early embryonic vaccination. However, early *in ovo* vaccination, particularly before ED-14 could lead to the development of tolerance to the HVT vaccine (Zhang and Sharma, 2003). The protection levels which usually obtained from the subcutaneous 1 day old HVT vaccination and the *in ovo* vaccination are similar. Hence, comparable levels of protection were obtained from late *in ovo* and 1 day old HVT vaccination against challenges with MPF57 virulent

MDV strain (Tan et al., 2007). This was in contrast to an earlier study, where ED-18 HVT vaccinated chickens were more protected from MDV challenge than the 1 day old vaccinated chickens (Sharma and Burmester, 1982). These contrasting results may be due to the difference in the time of challenge in the both studies. In the former study, chickens were 8 days old when challenged, whereas they were 3 days old in the latter study. Thus, the *in ovo* vaccination of HVT gives better protection to an early challenge with MDV, while *in ovo* and at hatching vaccination gives similar protection in the case of a late challenge. In agreement with this conclusion, vaccination of HVT at hatching was able to protect chickens challenged with MDV virulent strain 5 days post vaccination. In contrast, the same vaccine was not able to protect the challenged chickens 2 days post vaccination (Islam et al., 2007). The authors suggested that 5 days is enough time for the viral vaccine replication and development of the host immune response.

Rispens (CVI988) was characterised as mild pathogenic classical serotype 1 Marek's disease virus strain (Bulow, 1977). It was tested as a vaccine for the first time in 1971 in the Netherlands (Rispens et al., 1972a; Rispens et al., 1972b). It has been found that this vaccine can give a high protective efficacy against MDV challenge and a high seroconversion. Thus, it provides a superior MDV protection rate, compared with the attenuated HPRS-16, HVT and R2/23 serotype-1 vaccine (Rispens et al., 1972a; Rispens et al., 1972b; Witter et al., 1995; Tan et al., 2007). The commercially available CVI988 vaccine was attenuated and turned to be completely avirulent by passaging in cell culture (Rispens et al., 1972a; Rispens et al., 1972b). However, inoculation of 10 times field dose, 10,000 plaque forming units (PFU), of the vaccine resulted in MDV lesions in the inoculated birds (Bulow, 1977; Pol et al., 1986). CVI988 vaccine is usually administrated directly subcutaneously (s/c) after hatching, even though previous studies proved that intramuscular (i.m) administration of this vaccine could give a better protection rate than the s/c route (Bulow, 1977). In addition, CVI988 was very effective when it was tested for the *in-ovo* vaccination (Zhang and Sharma, 2001). Similar to HVT, CVI988 *in ovo*

vaccination provides the birds with better protection against early MDV challenge more than the post hatching vaccination does (Zhang and Sharma, 2001).

It has been found that the very virulent strains of MDV could break the protection barrier induced by an MDV monovalent vaccine. In order to provide the birds with adequate protection against the very virulent strain of MDV, bivalent or poly vaccines are used. These vaccines consist of different serotypes of MDV, either trivalent (combination of serotypes 1, 2 and 3) or bivalent (combination of serotypes 1 and 3 or 2 and 3). The use of a combination of MDV serotypes is efficient and has no safety hazards for the inoculated birds (Witter and Lee, 1984). Interestingly, protective synergism was observed between HVT and SB-1 when they were inoculated together (Witter and Lee, 1984). In the same study, the inoculation of the trivalent vaccine or bivalent vaccine gave better immunity than the usage of each serotype alone. In addition, the combination vaccine of HVT and SB-1 was better for the growth rate and the general survivability of the birds when it is administrated *in-ovo* rather than post hatching (Sarma et al., 1995). Also, combination of HVT and CVI988 vaccines could provide the bird with adequate protection against the recently emerged very virulent strains of MDV (Geerligs et al., 1999). This was inconsistent with other data showed earlier by Witter and colleagues. Hence, there was no improvement in the protection or synergism when CVI988 vaccine combined with serotype 2 and or 3 vaccines (Witter et al., 1995). This inconsistency may be due to the difference in the passage level of the CVI988 strain and the virulence of the challenged viruses used in the two studies.

### 1.8.2 Disadvantages of MDV vaccines

Until now, the current vaccination strategy against MDV is successful for the prevention of the disease but not for control of an existing infection. There are three main disadvantages associated with MDV vaccination strategy (Figure 1.8) (Baigent et al., 2006b). Firstly, the current vaccines do not provide sterilising immunity, meaning the birds remain a potential source of infection even after vaccination (Baigent et al., 2006b; Tan et al., 2007). Secondly, the intensive vaccination leads to

the evolution of more virulent strains. Thirdly, with the exception of HVT, MDV vaccines are presented in a cell-associated form due to the higher efficacy of the system. The presence of the vaccines in this form has many drawbacks, such as the possible contamination of the cell line by latent vMDV or endogenous viruses such as Avian Leukosis virus. In addition, the cost of vaccine production is high, because it is prepared in primary chicken embryo cells from specific pathogen free (SPF) eggs. Handling of the vaccine is very difficult as it should be frozen slowly when in storage, transferred in liquid nitrogen and thawed rapidly to maintain the virus titre in the cell (Jarosinski et al., 2006).

### 1.8.3 New vaccination strategies

Due to the many emerging drawbacks of the current vaccination strategies, new strategies for the prevention and control of the disease need to be developed, for example new vaccines or vaccination programmes. Different vaccination programmes used over the world depending on the level of immunity required and thus mainly depends on the breeding practices used. Field trials proved that revaccination with either CVI988 or FC126 vaccine reduced the mortality rate from MDV compared to a single vaccination at 1 day old (Wu et al., 2009a). This could be due to an induction of vaccine immunity from the productive infection of the vaccines.

Nowadays, using recombinant DNA technology such as bacterial artificial chromosome (BAC) or cosmid is considered to be a good approach to overcome the disadvantages of the already existing vaccines. Hence, the construction of an MDV BAC or overlapping cosmid will help in developing recombinant vaccines lacking MDV genes inducing virulence and oncogenicity. Additionally, an MDV BAC could be used as a recombinant vaccine for MDV and a vector for many other diseases by engineering in genes for avian viruses such as Newcastle disease (ND) and avian influenza (AI).

In recent years, many MDV strains have been cloned as BAC or overlapping cosmid constructs, and tested for their vaccine efficacy. For example, overlapping cosmids containing recombinant Md5 strain lacking vIL8 (rMd5/ΔvIL8) have been generated and tested as a vaccine (Cui et al., 2005a). This mutant virus decreased MDV incidence in the challenged birds and gave a protection index higher than that provided by the CVI988 vaccine. Also, a recombinant virulent MDV lacking the Meq oncogene (rMd5ΔMeq) was tested (Lee et al., 2008). This Meq knock-out virus gave protection for maternal $Ab^+$ and maternal $Ab^-$ chickens higher than the protection provided by the Rispens strain (Lee et al., 2008). Virus reconstituted from this HVT BAC can confer 100% protection against challenge with virulent MDV strain (Baigent et al., 2006a). In addition, recombinant HVT encoding chicken IL2 was tested in comparison with the parental HVT strain (Tarpey et al., 2007). There was no significant difference in the protection index of birds challenged with RB-1B strain and vaccinated with either the parental HVT or HVT/IL2. However, the level of neutralising Ab was higher in HVT/IL2 vaccinated birds 7 weeks post vaccination. Many other BACs were reconstituted from different strains such as MDV-814 and CVI988 and tested as MDV vaccines. Most of the engineered strains produced level of protection similar to that of the parental virus (Petherbridge et al., 2003; Cui et al., 2009).

Few trials have been done to study the ability of MDV BAC to be used as a DNA vaccine (Tischer et al., 2002a; Petherbridge et al., 2003) The DNA vaccine derived from CVI988 or 584Ap80C attenuated MDV strain gave a lower protection rate and showed higher MDV incidence than the BAC-derived or parental virus, which was attributed to an inefficient delivery method (Tischer et al., 2002a; Petherbridge et al., 2003).

Insertion of molecules which inhibit viral replication in the MDV genome could be another approach to improve MDV vaccine efficacy. For example, a double stranded small interfering RNA (siRNA) could be used to inhibit some viral or cellular genes and thus virus replication (Lambeth et al., 2009b). Hence, short hairpin RNAs

(shRNAs) targeting MDV UL27 and UL29 were engineered on HVT BAC and tested as a recombinant MDV vaccine. These HVT-RNAi vaccines were able to give up to 50% survival rate against challenge with MDV virulent strain, comparable to the parental HVT vaccine, which gave a 60% survival rate (Lambeth et al., 2009b).

Quantitative real time-PCR (QRT-PCR) is considered a good tool for assessing responses to vaccination and can contribute to design of vaccines and vaccination strategies. It may therefore be a good predictor of protection (Baigent et al., 2006b; Islam et al., 2007; Tan et al., 2007).

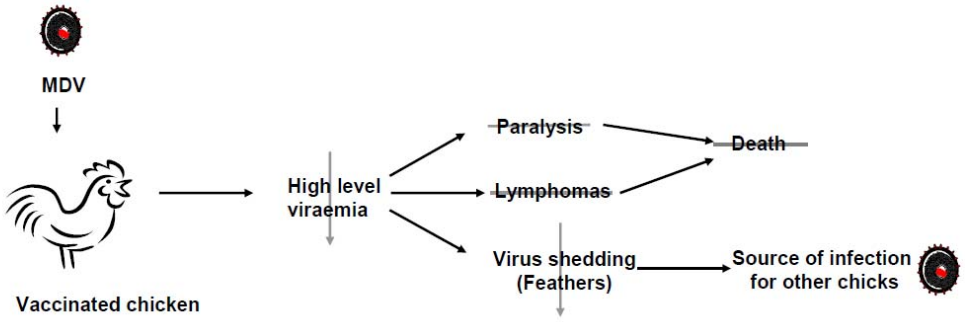


**Figure 1.8: The vaccination of MDV.** Figure illustrates how the MDV vaccine works as it inhibits the appearance of the clinical disease in case of infection but the host remains a potential source of infection for the other susceptible birds (Baigent et al., 2006b).

# 1.9 MDV genome

## 1.9.1 MDV genomic organisation

The genome of MDV is predicted to be about 174 kbp in size. The genome is composed of two regions of unique nucleotide sequences, each enclosed by inverted repeat sequences (Figure 1.9) (Cebrian et al., 1982). MDV was initially classified as a gamma-herpesvirus, as biologically it is similar to Epstein-Barr virus (EBV). Later, genetic analysis revealed its similarity to other alpha-herpesvirus such as herpes simplex virus-1 (HSV-1) (Cebrian et al., 1982). The UL region of the GA strain of MDV was analysed and found to encode 55 homologues to HSV ORFs and 12 ORFs unique for MDV (Lee et al., 2000a). This is in accordance with the first complete genomic sequence of the very virulent strain Md5 of Marek's disease virus serotype 1 (MDV-1), which also encodes 55 HSV homologues in the UL region (Tulman et al., 2000). However, Tulman and colleagues only predicted 6 unique MDV ORFs in the UL, in addition to 42 ORFs within the US and repeat regions (Tulman et al., 2000).

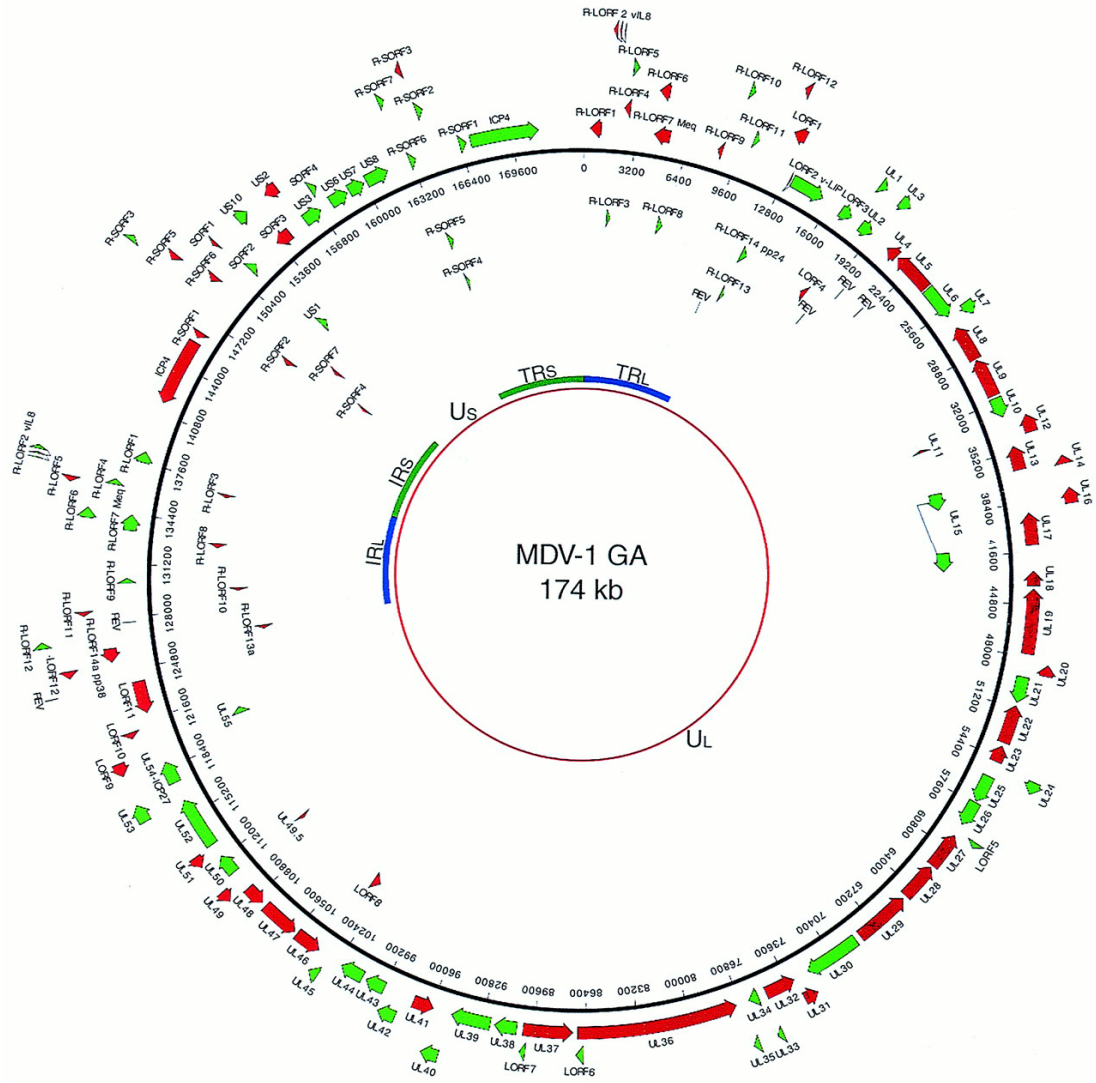


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**Figure 1.9: The genomic structure of MDV.** This Figure shows the genomic organisation of the GA strain of MDV. MDV genome is composed of two regions of unique nucleotide sequences UL and US regions, each enclosed by inverted repeat sequences RL and RS regions. This figure is adapted from (Lee et al., 2000a).

### 1.9.2 Gene products of MDV

Similar to the other herpesviruses, MDV genes are expressed in three kinetic classes: immediate-early, early and late. Except for a few, there are no available data for the kinetic expression of each MDV gene. In this section, MDV encoded proteins will be listed and discussed according to their organisation in the virus genome. As other herpesviruses, the MDV genome is composed of two sets of unique regions, unique long (UL) and unique short (US) regions. Both are flanked by two sets of repeat regions; repeat long and repeat short region, which are repeated twice in the genome. The regions are arranged as following: terminal repeat long (TRL), unique long (UL), internal repeat long (IRL), internal repeat short (IRS), unique short (US), and terminal repeat short (TRS) region (see Figure 1.9).

#### a. Unique long region (UL)

The unique long region of MDV-1 is composed of 113,508 bp and 113,563 bp in GA strain and Md5 strain, respectively (Lee et al., 2000a; Tulman et al., 2000) and encodes at least 67 proteins. From the sixty seven proteins, only 12 proteins are unique to MDV, and the rest of the ORFs have homologues in the other alpha-herpesviruses (see Appendix A), particularly HSV-1 (Lee et al., 2000a). Since the alpha-herpesvirus conserved MDV-ORFs have been named according to their homologues in the other herpesviruses as (ULs), the unique MDV ORFs which are located in the unique long region have been named as LORFs (Lee et al., 2000a). An additional spliced gene encoded in the unique long region of MDV-1 has been named as Lipase gene and will be discussed later in this section.

Although MDV's unique long region is fully sequenced, there are no available published data for the biological role of each MDV protein located in this region. For example, Lee and colleagues discovered that MDV's unique long region encodes homologues for the enzymes required for HSV-1 DNA metabolism, such as UL2, UL23, UL39, UL40 and UL50 (Lee et al., 2000a). However, the roles of these proteins have not been yet elucidated in the context of MDV replication. Therefore,

only a few genes from the UL region have known functions at the proteomic and viral level.

**Viral Lipase**

Viral Lipase is a spliced gene in the unique long region which is composed of 2 exons. The first encodes signal peptides, whilst the second encodes lipase activity. The protein is produced via splicing, with a length of 756 amino acids. It is a late gene, as it is sensitive to the inhibition of DNA replication (Kamil et al., 2005).

**UL13**

UL13 encodes a 60 kDa serine/threonine protein kinase and is located in the MDV-unique long region (Reddy et al., 1999). Studies with a virus reconstitution from a BAC with a frame shifted UL13 showed that it is unable to spread from one bird to another, suggesting that while UL13 is not required for tumour formation and feather follicles tropism (Blondeau et al., 2007; Spatz et al., 2007c), it may be needed for horizontal transmission in combination with other genes such as glycoprotein C (gC) and US2 (Jarosinski et al., 2007b; Spatz et al., 2007b).

**UL30**

UL30 is present in the unique long region and expresses a protein of a 135 kDa in size (Sui et al., 1995). Similar to its homologues in the other herpesviruses, it possesses three N-terminal domains with 3`-5` exonuclease activity and six C-terminal domains with catalytic activity (Sui et al., 1995). As such, MDV-UL30 has polymerase activity, and it is the polymerase gene as in other herpesviruses. Interestingly, UL30 is conserved in the whole *Mardivirus* genera, including MDV-2. MDV-2 UL30 encodes ORF of 1190 amino acids and has the same nine conserved functional domains required for the other herpsviruses polymerase (Izumiya et al., 1999).

**UL41**

UL41 is a tegument protein that acts as a virion host shut-off (vhs) protein in various herpesviruses including MDV (Gimeno and Silva, 2008). It possesses inherent mRNase activity which degrades the host mRNA. Deletion of UL41 from the MDV genome leads to a longer lytic phase *in vivo*, which may be attributed to the regulatory role of the vhs in the organisation of gene expression (Gimeno and Silva, 2008). It is very likely that the active form of UL41 has a negative effect on viral transcripts, and diminishing this form leads to an improvement in viral gene expression and replication. Hence, a single aa mutation in MDV-UL41 (R 337 C) diminishes host shut-off activity and increases MDV replication compared with wild-type virus (Mao et al., 2008).

**UL45, UL46, UL47, UL48, UL49, UL49.5 and UL50**

Seven ORFs sequenced in MDV's unique long region display a high degree of homology with the HSV-1 genes UL45, UL46, UL47, UL48, UL49, UL49.5 and UL50, some of which are functionally conserved (Yanagida et al., 1993). The growth of mutants lacking either UL46, UL47, UL48, UL49 gene or combination of them on chicken embryo skin cells (CESC) revealed that UL46, UL47 and UL48 are non-essential genes for virus growth and spread in cell culture (Dorange et al., 2002). However, plaques formed by these mutants were smaller than the plaques formed by the parental BAC. UL48 encodes the tegument protein (VP16), which has both a structural and a regulatory role in the other herpesviruses. Transcripts of the VP16 gene were detected in only one latently infected cell line (MSB-1) (Koptidesova et al., 1995; Ui et al., 1998). VP16 protein expression is not detected during infection of CESC, suggesting no clear evidence for UL48 expression during infection (Dorange et al., 2000). UL49 (VP22) is a tegument protein with a high degree of homology to its HSV-1, VZV, HVT and GaHV3 homologues which reaches to 24%, 29%, 56% and 59%, respectively (Yanagida et al., 1993; Dorange et al., 2000; Blondeau et al., 2008). It is localised in the nucleus and shows marked DNA binding activity (Dorange et al., 2000; O'Donnell et al., 2002). Deletion of UL49 produces a virus that is unable to grow in cell culture or spread from one cell to another (Dorange et

al., 2002; Blondeau et al., 2008). Thus, UL49 is an essential gene and is required for virus growth and spread.

**ICP27**

ICP27 was described by Ren and colleagues as a 55 kDa protein which has great similarities to HSV-1-ICP27 and VZV-ORF4, particularly in the C-terminus region (Ren et al., 1994). In contrast, MDV-ICP27 has a limited homology with its equivalent in the avian infectious laryngotracheitis virus (Johnson and Tyack, 1995).

**Glycoproteins**

The UL region of MDV encodes six glycoproteins, which have orthologues in the HSV-1 genome. These glycoproteins are gL, gM, gH, gB, gC and gK, and they are encoded by UL1, UL10, UL22, UL27, UL44 and UL53, respectively (Lee et al., 2000a). An additional ORF, UL49.5, also encodes a membrane protein, though to be gN. The glycoproteins have a high level of sequence similarity in MDV-1, MDV-2 and HVT, and there is no consistent mutation that is associated with virulence (Shamblin et al., 2004). MDV gK has glycoprotein features, including an N-terminal signal sequence, four N-linked glycosylation sites, and four potential transmembrane domains (Ren et al., 1994). Unlike other glycoproteins, MDV-gK has an immediate-early kinetic (Ren et al., 1994).

Glycoprotein B or UL27 was identified in serotype 1 by Ross and colleagues, their identification was based on the ORF sequence homology with a gB of the other alpha-herpesviruses, such as HSV-1, VZV and Pseudo rabiesvirus (PRV) (Ross et al., 1989). Later, gB of serotypes 2 and 3 were identified by Yoshida and colleagues, and their identification was based on the sequence homology with a gB of serotype 1 (Yoshida et al., 1994b). Interestingly, it was revealed that gB of MDV and MDV B antigen are the same, as anti-B Ag mAb and anti-gB serum cross-reacted with gB and MDV B Ag, respectively, and they recognised the same product on SDS-PAGE gel (Niikura et al., 1992). Glycoprotein B displayed a protective immunological role for chickens challenged with virulent MDV strains. Hence, different recombinant

constructs expressing gB were able to protect chickens from subsequent challenges with different virulent MDV strains (Nazerian et al., 1992; Ross et al., 1996; Liu et al., 1999). MDV-1 BAC lacking gB (20ΔgB) was able to grow in a single cell only in CEF and QM7 cell lines, therefore the mutant was not able to spread from one cell to another (Schumacher et al., 2000). MDV gM, UL10, encodes a protein of 47 kDa (Osterrieder, 1999). MDV gL or UL1 was identified by Yoshida and colleagues according to the ORF genomic organisation and the similarity between it and HSV-1 gL (Yoshida et al., 1994a). Whilst, MDV gL similarity with the orthologue is as low as 18%, it has a highly conserved region with VZV ORF60, equine herpesvirus 1 (EHV1) 62 and HSV-1 gL extending from amino acids 71 to 98 in MDV gL. Similar to other alpha-herpesviruses, MDV gH and gL form a hetero-oligomeric complex together (Yoshida et al., 1994a; Wu et al., 2001a). This complex has an important role in virus entry into host cells and cell-to-cell infection in herpesviruses. MDV gC or formally known as MDV Ag A was recognised as gC of MDV when IHARA and colleagues found 36.2% amino acids sequence similarity between it and gC of HSV-1 (Ihara et al., 1989). It was reported that gC expression has a negative effect on both virus growth and virus spread from cell to cell (Tischer et al., 2005). Hence, MDV lacking gC (MDVΔgC) produces wider plaque and has higher growth kinetics *in vitro* than the parental BAC. The authors attributed this result to the possibility of the effect of gC on the signalling cascade of interferon induction. However, the previous was just a suggestion and was not based on any evidence. *In vivo* experiments with MDV containing a frame shift mutation in gC revealed that it is required for the horizontal spread of the virus from one bird to another (Jarosinski et al., 2007b). However, the virus used in this experiment contains a frame shift mutation in UL13 gene and also lacks the US2 gene. The UL49.5 gene encodes a glycosylated protein product termed as glycoprotein N in ILT and PrV (Jons et al., 1996; Fuchs and Mettenleiter, 2005). Furthermore, it is considered to be a complex partner for gM in many herpesviruses, such as ILT, PrV, EHV 1 and bovine herpesvirus-1 (BoHV-1) (Jons et al., 1998; Wu et al., 1998; Rudolph et al., 2002; Fuchs and Mettenleiter, 2005). In MDV, virus growth in tissue culture and cell to cell spread requires both UL49.5 and gM (Tischer et al., 2002b). Hence, MDV BAC mutants lacking either

UL49.5 or gM were not able to spread from cell to cell in infected chicken and quail cells.

### b. Unique short region (US)

The unique short region is composed of 10,847 bp and 11,160 in Md5 and GA strains, respectively and is predicted to encode around 11 proteins in MDV-1. The unique short region (US) encodes 7 ORFs with homologues in the other alpha-herpesviruses (see Appendix A), namely US1, US2, US3, US6, US7, US8 and US10. Four additional MDV-specific ORFs have been identified in this region: SORF1, SORF2, SORF3 and SORF4 (Brunovskis and Velicer, 1995; Tulman et al., 2000).

**ICP22**

ICP22 (US1) is a regulatory protein that stimulates viral promoters in co-operation with other viral regulatory proteins, such as MDV ICP4. It is involved specifically in the transactivation of the ICP27 promoter and deletion of US1 resulted in a reduction in virus growth and yield (Parcells et al., 1994a; Kato et al., 2002).

**US2**

US2 was identified in 1991 and to date the protein has no distinct function in MDV biology and replication cycle. An MDV mutant containing a US2*lac* insertion has growth properties identical to the wild-type virus in cell culture (Cantello et al., 1991; Parcells et al., 1994a). Whilst reconstituted virus from a BAC of the RB-1B strain with US2 replaced by a mini F vector sequence is not transmitted horizontally (Jarosinski et al., 2007a). However, this BAC has frame shift mutations in other genes, such as UL13 and UL44 (gC) (Jarosinski et al., 2007a).

**US3**

US3 encodes the 44-48 kDa MDV serine threonine protein kinase (Sakaguchi et al., 1993; Jang et al., 1998; Schumacher et al., 2005). Similar to its homologues in other alpha-herpesviruses, it has anti-apoptotic properties mediated by its kinase activity (Schumacher et al., 2008). Moreover, it functions as the phospohorylation partner for

MDV phosphprotein (PP38), which acts as the US3 catalytic substrate. US3-negative BAC 20 MDV has a growth defect and smaller plaque size when infected in primary chickens cells (Schumacher et al., 2005; Schumacher et al., 2008). This defect in the virus replication cycle is due to the failure of the primary enveloped virus product to de-envelope at the outer nuclear membrane. Interestingly, the kinase site of US3 is responsible for the observed growth defect of the US3 MDV deletion mutant, as disruption of the US3 kinase lysine site resulted in growth characteristics similar to the full length US3 MDV deletion mutant (Schumacher et al., 2008). Additionally, US3 mediates the breakdown of the cellular actin cytoskeleton and stress fibres, a function which does not require the kinase activity of US3 (Schumacher et al., 2005; Schumacher et al., 2008).

**Glycoproteins**

Marek's disease virus US6, US7 and US8 encode gD, gE and gI, respectively. MDV gD prepared by *in vitro* transcription translation system and is 43 kDa molecular weight in size (Zelnik et al., 1999; Tan et al., 2001). gD protein expression is undetectable in MDV infected fibroblast cells, but it is detectable at the transcriptional level, possibly due to the cell associated nature of virus growth in cell culture (Parcells et al., 1994b; Zelnik et al., 1999; Tan et al., 2001). Unlike other MDV glycoproteins, gD is not an important gene for virus transmission or growth in cell culture (Parcells et al., 1994b; Anderson et al., 1998).

Unlike gD, both gI and gE are expressed in MDV infected cells as early as 24 hr post infection as either glycosylated or non-glycosylated forms (Schumacher et al., 2001). As in other herpesviruses, gE and gI form a heterodimer, and as such both are essential for transmission in cell culture (Dingwell et al., 1995; Mijnes et al., 1996).

As mentioned above, the MDV US region encodes four MDV-specific ORFs, which have no orthologues in the other herpesviruses: SORF1, SORF2, SORF3 and SORF4. The functions of these four proteins are not well studied, and so little information of their functions is available. It is believed that MDV SORF2 interacts

with the chicken growth hormone, and that this interaction may have a role in the virus genetic resistance (Liu et al., 2001).

### c. Repeat long regions

The MDV genome contains two similar long repeat regions called internal repeat long (IRL) and terminal repeat long (TRL) regions. This region of MDV is 12,584 bp to 13,065 bp long, in strains GA and Md5, respectively (Lee et al., 2000a; Tulman et al., 2000). The ORFs encoded in this area are named RLORFs, with the exception of some, which are named according to their molecular weight. A recently identified gene, viral telomerase, is named due to its homology to cellular telomerase. The exact biological functions of RL region encoded ORFs are currently unknown, but it is believed that the virulence factors of MDV are encoded within this region. Furthermore, the differences in genomic composition between the virulent strains and the vaccinal strains are mainly located in this region (Spatz and Silva, 2007). In this section, some of RL region's proteins, which play a vital role in virus pathogenesis and oncogenesis, will be discussed.

**RLORF2 (vIL8)**

vIL8 encodes a homologue to the CXC chemokine cellular IL8, and it was identified as a late spliced gene expressed during cytolytic infection (Parcells et al., 2001). It contains 134 amino acids, and it is composed of three exons. Exon I encodes the first 21 amino acids, which contains signal peptides, and exon II and III encode the sequence homologue to CXC chemokines IL8. Though MDV vIL8 is similar to the cellular IL8 in its sequence composition, it also acts as a secreted molecule and serves to attract chicken peripheral blood mononuclear cells (PBMC) (Parcells et al., 2001). Induction of lytic infection with n-butyrate in the MSB-1 cell line increases the level of vIL8 transcripts which suggests that it is involved in cytolytic infection (Parcells et al., 2001; Cui et al., 2004b). Studies using MDV mutants lacking a functional vIL8 (rMd5Δ vIL8 and RB-1BvIL8ΔsmGFP) showed that it is non-essential for MDV growth in cell culture (Parcells et al., 2001; Cortes and Cardona, 2004; Cui et al., 2004a). A lower efficiency of virus replication during the early

stages of infection resulted in a reduced rate of tumour formation in mutant-infected in comparison to wild-type-infected birds. Interestingly, rMd5Δ vIL8 used as a vaccine protects the birds from infection with very virulent + MDV strain (Cui et al., 2005b). Additionally, the absence of vIL8 expression in infected birds leads to an impairment and delaying in PP38 and Meq proteins expression in lymphoid organs and FFE (Cui et al., 2005b). This suggests a relationship between the expression of these three proteins, and this is will be discussed in Chapter 4.

**14kDa (PP14)**

14kDa is a spliced serotype 1 specific gene with immediate-early kinetics (Hong and Coussens, 1994; Hong et al., 1995). It is a cytoplasmic protein with a high degree of phosphorylation, hence it is named as PP14. 14 kDa was detected in an MDV-induced lymphoma cell line, so it is possible that it contributes to the maintenance of latency or transformation (Hong and Coussens, 1994).

**RLORF4**

RLORF4 is a 142 aa transmembrane protein, and it is considered one of the MDV virulence candidates based on several experiments. Firstly, deletion of RLORF4 from MDV BAC (pRB-1BΔRLORF4) attenuates the virus, causing an increase in plaque size and DNA replication rate (Jarosinski et al., 2005). Secondly, pRB-1BΔRLORF4 infection in chickens caused no MDV associated-death for up to 13 weeks post infection, and there was marked decrease in the appearance of MDV tumour lesions in these birds (Jarosinski et al., 2005). Finally, some naturally attenuated strains of MDV (R2/23, JM-16/P71 and 584Ap80C) contain a RLORF4 gene with deletions, generating a truncated protein (Jarosinski et al., 2003; Spatz and Silva, 2007). Recently, six splice-variants have been detected *in vivo* and *in vitro* encoding the N-terminal sequence of RLORF4 and exon II and/or III of vIL8, but it is not known if these transcripts encode functional proteins or not (Jarosinski and Schat, 2007a).

**RLORF5**

RLORF5a (IRL) and RLORF5b (TRL) are up-regulated in spleen tissue during latent infection (Heidari et al., 2008a). Taken with the observation that the deletion of RLORF5a has a little effect on virus replication suggests that this is a latent gene (Jarosinski et al., 2005). Similar to RLORF4, a splice-variant RLORF5a/vIL8b is detected in mRNA isolated from RB-1B chicken kidney cells and in MDV infected chickens (Jarosinski and Schat, 2007a). The functional difference between the wild-type and splice-variant is unknown.

**RLORF7 (Meq)**

Meq, (Marek's Eco Q) exists as two isoforms in the MDV genome (Jones et al., 1992; Lee et al., 2000b; Jarosinski and Schat, 2007b). The virulent strains possess a 339 amino acids (aa), whilst attenuated strains (CV1988/R6 and attenuated JM strains) possess a slightly longer Meq (L-Meq), which has a 180-bp sequence insertion (Lee et al., 2000b; Chang et al., 2005). The Meq protein contains several functional domains which play an important role in virus replication. It contains a basic leucine zipper (bZIP) domain near to its N-terminus and a proline-rich domain near to its C-terminus (Jones et al., 1992; Qian et al., 1995). Two additional basic regions, BR1 and BR2, contain nuclear and nucleolar localisation signals (NLS and NoLS). Meq mutants lacking both BR1 & BR2 remain localised in the cytoplasm (Liu et al., 1997; Lee et al., 2003). BR2 contains the nucleolar localisation signal (NoLS) of Meq protein, and its entire sequence is required for Meq-nucleolar localisation (Liu et al., 1997).

The C-terminal proline-rich domain in Meq (129 to 339 aa) has transactivation activity, particularly in the last C-terminal 33 aa (Qian et al., 1995). Additionally, Meq forms a homodimer or heterodimer with c-Jun which increases the transactivation of the Meq promoter (Qian et al., 1995). The Meq-bZIP motif is similar to the Jun/Fos family of transcription factors and has DNA binding affinity, enabling it to bind with many DNA sequences (Qian et al., 1995; Qian et al., 1996; Ajithdoss et al., 2009). Cyclic amplification of selected targets (CASTing) technique

found the Meq homodimer binds to MERE I and MERE II DNA motifs (Qian et al., 1996). MERE I represents a tetradecanoylphorbol acetate response element (TRE) and cyclic AMP response element (CRE) like sequences, whereas MERE II represents DNA sequence containing (ACACACA) sequences, which are present in the MDV origin of replication (Qian et al., 1996). These results indicate that the DNA binding properties of Meq enable control of virus infection and latency through binding with the MDV origin of replication.

Meq is considered to be the main MDV-oncoprotein and is highly expressed in an MDV tumour cell line (Ross et al., 1997; Liu et al., 1998; Gimeno et al., 2005; Brown et al., 2006). Firstly, Meq has anti-apoptotic activity, which is important for the protection of transformed cells from apoptosis (cell death) (Liu et al., 1998). Secondly, the over expression of Meq leads to the transformation of Rat-2, NIH3T3 and DF1 cell lines (Liu et al., 1998; Levy et al., 2005; Ajithdoss et al., 2009). Meq induced transformation with the same mechanism as v-Jun (avian retrovirus ASV17), chicken cDNA microarray on Meq-transformed cells identified an up-regulation in v-Jun transformation-associated genes such as JTAP-1, JAC, and HB-EGF (Levy et al., 2005). Interestingly, a Meq-c-Jun heterodimer abundantly exists in Meq-transformed fibroblasts, and removal of this by RNAi reduces the transformation potential of Meq by reducing JTAP-1, JAC, and HB-EGF levels (Levy et al., 2005). The formation of this heterodimer is therefore essential for tumour formation induced by MDV. Recombinant virulent MDV lacking the Meq oncogene (rMd5ΔMeq) was tested as an MDV vaccine (Lee et al., 2008). The Meq knock-out virus gave a higher level of protection to the vaccinated chickens than that provided by the Rispens strain (Lee et al., 2008).

Recently, it has been found that MDV encodes 8 miRNAs: five of them flank the Meq oncogene (Burnside et al., 2006). The Meq sequence encodes many transcript variants. Meq/vIL8 is composed of the DNA binding domain of Meq and the receptor binding site of vIL8 (Anobile et al., 2006). Both of Meq and Meq/vIL8 are localised in the nucleolus and nucleoplasm (Liu et al., 1997; Anobile et al., 2006).

Meq-sp is a spliced product, composed of 212 aa, and its C-terminal encoded by the second and the third exons of vIL8 (Peng and Shirazi, 1996). Finally, there is a novel transcript called ΔMeq which encodes the N-terminal 98 aa of Meq but lacks part of the basic leucine zipper and the transactivation domains (Okada et al., 2007). However, ΔMeq can interact physically with Meq and L-Meq, but this suppresses their transactivation activity (Okada et al., 2007). Many viral proteins have been found to physically interact with Meq, and these will be discussed later in Chapter 4.

**RLORF6 and 23KDa**

RLORF6 and 23KDa are two ORFs that overlap with Meq gene. The vaccinal strain CVI988 has a 177 bp insertion which is not present in the virulent strains Md5, Md11, GA and RB-1B (Spatz et al., 2007b). Microarray analysis showed that 23kDa was amongst the few genes that abundantly expressed in latent infection (Heidari et al., 2008a).

**RLORF12**

RLORF12 encodes a 115 aa protein in virulent MDV strains, where as it composed of 67aa in the IRL region of CVI988 strain (Spatz et al., 2007a). Moreover, RLORF12 binds with the growth-related transciptionally-related protein, TCTP protein, in the chicken genome (Niikura et al., 2004).

**PP24 and PP38**

PP24 and PP38 are two phosphoproteins encoded by MDV. PP38 is located between the UL and IRL regions of the MDV-1 genome, whilst PP24 is located between the UL and TRL regions of the MDV-1 genome (Chen et al., 1992; Zhu et al., 1994). PP38 encodes a 290 aa protein, present in the oncogenic MDV-1 but not in the non-oncogenic MDV-3-infected cells (Chen et al., 1992). It is transcribed leftward from the viral genome, whereas PP24 gene is transcribed rightward (Zhu et al., 1994). The expression of PP38 is associated with the appearance of two bands of approximately 41 and 38 KDa in the SDS PAGE, these are thought to represent the phosphorylated and non-phosphorylated forms, respectively (Li et al., 2006). PP38 is highly

expressed during lytic infection and also during the early stages of virus reactivation from latency (Parcells et al., 2003). Analysis of PP38 DNA sequences in 10 virulent MDV strains of differing pathotypes found one consistent glutamine at aa 107 in comparison to the CVI988 vaccine strain which has argenine instead. This argenine could be responsible for the big difference in antibody responses induced by native CVI988 (Zhizhong et al., 2004).

Induction of PP38 expression increased the metabolic activity of a quail cell line latently infected with MDV (QTP32). PP38 has no DNA binding domain or transactivating domain but it could act as a coactivator (Chen et al., 1992; Li et al., 2006). Both PP38 and PP24 present rightward to the origin of replication and they share the same promoter, which also controls the transcription of the 1.8-kb mRNA family. Both PP38 and PP24 are essential to activate this promoter (Ding et al., 2007), suggesting that these proteins physically bind together to form a complex, and this has been confirmed by co-immnuoprecipitation (Ding et al., 2008).

**Viral telomerase (vTR)**

MDV encodes two copies of viral telomerase (vTR), which exhibits 88% sequence identity to the chicken telomerase chTR. It consists of a protein subunit with reverse transcriptase activity and an RNA subunit (Trapp et al., 2006; Shkreli et al., 2007). It is expressed in both lytic and latent infection *in vitro* but responsible only for MDV induced T-cell lymphomagenesis *in vivo*, as a challenge with ΔvTR virus reduced tumour incidences by >60% (Trapp et al., 2006).

### d. Repeat short regions

Two repeat short regions are present in the MDV genome internal repeat short (IRS) and terminal repeat short (TRS) regions. It is composed of 12,264 bp in Md5 strain and encodes 12 genes, from them the immediate-early gene ICP4 (Tulman et al., 2000).

**ICP4**

ICP4 was detected by *in situ* hybridisation of RNA extracted from MDV-infected CEF plus FFE, liver, kidney and peripheral nerves from MDV infected birds (Endoh et al., 1996). Additionally, ICP4 transcripts were also detected in latently infected necrotising lymphoblasts from CNS lesions (Cho et al., 1999). ICP4 is considered sense for a group of antisense transcripts associated with the latent stage of virus infection known as Latency associated transcripts (LATs) (Cantello et al., 1994; Li et al., 1994). As well as encoding functional proteins, the LATs also encode 3 of the 8 miRNAs identified in MDV: miR6, miR7 and miR8 (Burnside et al., 2006). It has been suggested that MDV encoded miRNAs contribute to virus induced latency and tumour formation (Yao et al., 2009).

## 1.10 Aims of the thesis

As discussed earlier, MDV is a very important viral disease for two reasons. First, the virus causes an oncogenic avian disease that results in large economic losses to the poultry industry. Second, it provides a useful biomedical model for study of herpesvirus induced tumours such as EBV. The molecular functional analysis of the MDV genes will provide us with very useful data for understanding the fundamental basis behind MDV induced pathogenesis and oncogenesis. The aim of this study is to obtain a better understanding of the roles of individual MDV genes in the stages of virus replication and their role in the interplay between the MDV genes and the host innate immune response. The initial requirements are to clone the entire MDV library into different-suitable expression systems, allowing us to use them for different purposes. The first purpose is to transform the MDV genes into yeast cells and to test their interaction against each other. Using intraviral yeast-two-hybrid (Y2H) system, information about MDV replication cycles should be obtained. Additionally, it is hoped that the intraviral Y2H screen will provide information regarding MDV induced pathogenicity and transformation. The second purpose is to test the effect of the MDV genes on the chicken interferon signalling, using interferon-alpha as a model. By performing this screen, it is intended to discover MDV or host genes that

can modulate the chicken interferon-alpha response after MDV infection or vaccination. With such candidates, the next aim is to study the cellular mechanism behind their down-regulation of the interferon-alpha response. Subsequently, the study will investigate the effect of the absence of MDV-interferon antagonists on virus growth after interferon-alpha treatment. Taken together, these data from the intraviral and viral-host interaction assays will provide vaccine companies and drug designers with valuable targets in the MDV genome.

# Materials and Methods

## 2.1 Cell culture

### 2.1.1 Tissue culture Media and supplements

#### a. Basic Media

*DMEM*: Purchased from Lonza, supplied in 500 ml sterile bottles, supplemented with 4.5 g/L glucose and stored at 4°C.

*M199*: Purchased from Gibco, supplied in 500 ml sterile bottles and stored at 4°C.

#### b. Other sterile solutions and supplements

*Foetal Calf Serum (FCS)*: Purchased from Gibco, liquid supplied sterilised in 500 ml bottles. FCS was aliquoted into 50 ml sterile bottles and stored at -20°C.

*Glutamine*: Purchased from Lonza, L-glutamine solution was available in 100 ml sterile bottles supplied at 29.2 mg/ml in 0.85% NaCl, pH 4.7-6.0. The stock solution was stored at -20°C in 5 ml aliquots. 5 ml of L-glutamine was added to 500 ml of media.

*Sodium Pyruvate*: Purchased from Lonza, liquid supplied in 100 ml sterile bottles. The solution contains 100 mM of sodim pyruvate (Na Pyrvate). It was stored at 4°C, and 5 ml of Na Pyruvate was added to 500 ml media.

*Sodium Bicarbonate*: Purchased from Lonza, liquid supplied in 100 ml sterile bottles. The solution contains 7.5 gm sodium bicarbonate. It was stored at 4°C, and 5 ml was added to 500 ml media.

*Non-essential Amino Acid*: Purchased from Lonza, liquid supplied in 100 ml sterile bottles. The 100x solution was diluted with media to 1x solution and stored at 4°C.

*Penicillin/streptomycin*: Purchased from Lonza, Penicillin/streptomycin solution was formulated to contain 20000U/ml penicillin and 20000 μg/ml streptomycin. It was aliquoted into 5 ml aliquots and stored at -20°C. It was used at 5 ml per 500 ml media.

*Tryptose phosphate broth*: Purchased from Gibco, liquid supplied sterilised in 100 ml bottles. It was stored at 4°C. 50 ml of tryptose phosphate broth was added to 500 ml media.

*Phosphate-Buffered Saline (PBS)*: Purchased from Lonza, liquid supplied in 500 ml sterile bottles. PBS was formulated to contain 0.0067 M $PO_4$.and stored at 4°C.

*Trypsin EDTA*: Purchased from Lonza, Liquid supplied sterilised in 100 ml sterile bottles. Trypsin EDTA solution contains 200 mg/L EDTA and 170.000U/L trypsin.

### 2.1.2 Maintenance of cell lines

All cell lines were maintained in incubators at 37°C or 38.5°C supplied with 5% CO2. Cells were grown on a variety of tissue culture plasticware depending on the nature of the experiment.

Cultures were grown to 90-95% confluency, washed with PBS and trypsonised from the flasks with trypsin solution (Lonza, Belgium). Cells were incubated at 37°C or 38.5°C until all cells were dissociated from the flask. Cells were recovered by adding growth media containing 5 or 10% serum. Cells were centrifuged at 1000 rpm for 10 minutes in a benchtop centrifuge. The cells pellets were resuspended in growth medium and counted by use of a haemocytometer. Cells were seeded at a density required by the experimental design.

### 2.1.3 Freezing of cell lines

Cells were resuspended in 1 ml of pre-cooled freezing media. The freezing media consists of 70% growth media, 20% FCS and 10% DMSO (Fluka, Germany). Cells were aliquoted in sterile freezing ampoules (Nunc, UK) at a density $2x10^6$ cells/vial. Cells were transferred to a cryopreservation box (Mr Frosty, Nalgene) and slowly frozen overnight at -80°C. One day later, the cells were transferred to the liquid nitrogen storage tank.

### 2.1.4 Thawing of frozen cell lines

Frozen ampoules were thawed rapidly by warming in a 37°C water bath. Thawed cells were transferred into 15 ml tubes (Falcon, USA) and recovered by adding equal volumes of growth media slowly and then adjusted to 10 ml. Cell suspension were centrifuged for 1000 rpm for 10 minutes.

### 2.1.5 Cell lines

*QM7 (Quail muscle cell line)*:

QM7 cell line was used at a seeding density of $2x10^4$ and cultured with the following media:

| | |
|---|---|
| D-MEM | 500 ml |
| NEAA | 5 ml |
| Na Pyruvate | 5 ml |
| FCS | 50 ml |
| P/S | 5 ml |

*DF-1 (Chicken immortalised fibroblast cell line)*:

DF-1 cell line was used at a seeding density of $5x10^4$ and cultured with the following media:

| | |
|---|---|
| D-MEM | 500 ml |
| TPB | 50 ml |
| Glutamin | 5 ml |
| FCS | 50 ml |
| P/S | 5 ml |

*CEF (Chicken embryo fibroblast cells)*:

CEF cells were prepared from 10 days old specific-pathogen-free chicken embryos. The embryos were obtained from flock maintained at the Institute of Animal Health. CEF cells were used at a seeding density of 5 $x10^4$ and cultured with the following media:

| | |
|---|---|
| M199 | 500 ml |
| TPB | 50 ml |
| 7.5%NaHCO3 | 5 ml |
| FCS | 50 ml |
| P/S | 5 ml |

## 2.2 Transfection of plasmids into cells

### 2.2.1 Effectene transfection

Effectene reagent (Qiagen, UK) is a lipid based reagent that is suitable for the transfection of plasmid DNA into cultured eukaryotic cells. It is used with a special condensed DNA enhancer. The enhancer is used first to condense the DNA molecules, whilst the effectene reagent subsequently coats the condensed DNA molecules with cationic lipids.

The following protocol was used to transfect DF-1 cells plated in 48 well formats. Briefly, DF-1 cells were plated out into 48 well plates at a density $5x10^4$ cells per

well. Cells were incubated until reached to 80% confluency that it is considered ready for transfection. 150 ng of total DNA was diluted in EC buffer, following by addition of 1.2 µl enhancer. The diluted DNA was incubated for 5 minutes to allow formation of condensed DNA. Then, the effectene reagent was added at 3.5 µl per well. The transfection mixture was left at room temperature for 10-15 minutes to allow formation of the DNA transfection complex. During the last incubation, 200 µl of complete growth media was added to the cells of each well. After the lipid-DNA complex formation, 150 µl of complete growth media was added to the transfection complex. The diluted transfection complex was added in a dropwise manner and mixed by gentle swirling of each plate. Plates were incubated at 37°C with 5% $CO_2$. The transfection efficiency was checked after 24 hr using eGFP expression as a positive readout.

### 2.2.2 Polyfect transfection

Polyfect reagent (Qiagen, UK) is an activated-dendrimer transfection reagent. It assembles DNA into compact structures, optimising the entry of DNA into the cells. PolyFect–DNA complexes possess a net positive charge, which allows them to bind to negatively charged cellular receptors on the surface of eukaryotic cells.

The following protocol was used to transfect DF-1 cells plated in 24 or 6 well formats. Briefly, DF-1 cells were plated-out into 24 or 6 well plates at a seeding density of $1x10^5$ or $8x10^5$ cells per well, respectively. Cells were incubated until reached to 80% confluency that it is considered ready for transfection. Total DNA was diluted in DMEM at 500 ng or 2 µg for 24 and 6 well formats, respectively. Then, the polyfect reagent was added at 3 µl per well for 24 well formats and at 10 µl for 6 well formats. The transfection mixture was left at room temperature for 10-15 minutes to allow formation of the DNA transfection complex. During this incubation, suitable amount of growth media was added to the cells in each well, 400 µl for 24 well formats and 1.5 ml for 6 well formats. The diluted transfection complex was added in a dropwise manner and mixed by gentle swirling of each plate. Plates were

incubated at 37°C with 5% $CO_2$. The transfection efficiency was checked after 24 hr using eGFP expression as a positive readout.

### 2.2.3 Calcium phosphate transfection

QM7 cells were grown in 10 cm dishes to 60-70% confluency. 250 µl of 2x HBS pH 7.5 was added to 1.5 ml eppendorf tube. In another tube, 10 µg DNA was combined with 250 µl of 250 mM CaCl2. The tube with the 2x HBS was vortexed while the DNA/CaCl2 solution was added dropwise. The solution was incubated at RT for 30 minutes to allow the formation of the Calcium-DNA complex. Subsequently, the suspension was mixed with 6 ml fresh medium and was added to the cells after removal of the old medium. The next day, the transfection efficiency was assessed by eGFP expression.

## 2.3 Protein techniques

### 2.3.1 Buffers used in protein techniques

*NP-40 lysis buffer*: 20 mM Tris (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$ and 1% NP40 (IGEPAL). One tablet of cOmplete inhibitor tablets (Roche, UK) and 1 mM PMSF (Fluka, Germany) were added to 50 ml of NP40 buffer shortly before use.

*Sample extraction buffer ( 2x SEB)*: 90 mM Tris-Cl (pH 6.8). 20% Glycerol, 2% SDS, 0.02% Bromophenol blue and 10% Beta-mercaptoethanol. Stored at 4°C.

*Laemmli running buffer 10x*: 30.3 g Tris, 144.2 g Glycine, 10 g SDS. Water was added up to 1 L *and* pH was adjusted to 8.3.

*Transfer buffer 10x*: 29 g Tris, 144 g Glycine, 3.7 g SDS and water up to1 L. Transfer buffer 1x: 200 ml Transfer buffer 10x, 200 ml Methanol and water was added up to 1 L.

*TBST*: 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.2% Tween and water up to 1 L.

*Blocking buffer*: 5% skimmed milk powder (Fluka, Germany) dissolved in TBST solution

### 2.3.2 Materials and supplements used in protein techniques

*a. Antibodies*:

1- Anti-HA high affinity, rat monoclonal antibody, purchased from Roche Diagnostics. It was used at 1 μg per reaction for the immunoprecipitation, 1/1000 for immunoblotting and 1/2000 for immunofluorescent.

2- Anti-c-Myc, mouse monoclonal antibody, purchased from Santa Cruz Biotechnology. It was used at 1 μg per reaction for the immunoprecipitation, 1/1000 for immunoblotting and 1/2000 for immunofluorescent.

3- Anti-β-actin, rabbit monoclonal antibody, purchased from Cell Signalling. It was used at 1/2000 for immunoblotting.

4- Goat anti-mouse peroxidase coupled, purchased from Jackson, Hamburg, Germany. It was used at 1/3000 for immunoblotting.

5- Goat anti-rat peroxidase coupled, purchased from Jackson, Hamburg, Germany. It was used at 1/3000 for immunoblotting.

6- Goat anti-rabbit peroxidase coupled, purchased from Jackson, Hamburg, Germany. It was used at 1/3000 for immunoblotting.

7- Alexa fluor®488 goat anti-mouse IgG, purchased form Invitrogen. It was used at 1/4000 for immunofluorescent.

8- Alexa fluor®594 goat anti-rat IgG, purchased from Invitrogen. It was used at 1/4000 for immunofluorescent.

*b. Protease inhibitors*:

1- cOmplete, EDTA free, protease inhibitor cocktail tablets, purchased from Roche Diagnostics. One tablet was used for 50 ml of lysis buffer.

2- Phenylmethane sulfonyl fluoride, PMSF, purchased from Fluka. It was prepared as 0.1 M solution of PMSF in isopropyl alcohol and stored at -20°C. It was thawed at 55°C shortly before used.

*c. Beads*:

Protein G Sepharose 4 Fast Flow, purchased from GE Healthcare. 50 µl of 50% beads slurry were used per one reaction. Protein G-Sepharose was washed 3 times and resuspended with NP-40 lysis buffer to obtain 50% slurry.

*d. cycloheximide*:

Protein synthesis blocker, purchased from Fluka. It was diluted in DMSO and used at 50 µg/ml.

*e. MG132*:

Proteasomal-inhibitor drug, purchased from Calbiochem. It was diluted in DMSO (Fluka, Germany) and used at 100 µM.

*f. BCA protein assay reagent*:

Protein quantification kit, purchased from Thermo Scientific. It was used according to manufacturer's instructions.

*g. Protein Ladder*:

SeeBlue® Plus2 Pre-Stained Standard, purchased from invitrogen, supplied ready to use. 4 µl were loaded for small gels and 8 µl for large gels.

*h. Anti-fade reagent*:

Prolong Gold Anti-fade reagent with DAPI, purchased from Invitrogen, Single drop was place on the cells layer before sealing with cover slip.

### 2.3.3 Co-Immunoprecipitation

Co-Immunoprecipitation was performed using the plasmids pGBKT7 and pGADT7 with T7 promoter and recombinant vaccinia virus vTF-7 expressing the T7 RNA polymerase (NIH AIDS repository). QM7 cells were cultured on 10 cm dishes and infected with vTF-7 at an MOI of 10 in serum-free medium. One hour after infection, cells were transfected with 10 µg of each of the two destination plasmids by calcium phosphate transfection (Section 2.2.3). Expression was controlled using eGFP plasmid under the control of a T7 promoter. After 24 hr, cells were lysed by incubation in 1 ml of NP-40 lysis buffer for 30 minutes on ice. Lysates were centrifuged for 10 minutes at 1000 rpm and 4°C to remove unsolubilised material and pre-cleared with 50 µl of pre-equilibrated protein G-Sepharose by shaking for 1 hr. Lysates and beads were then centrifuged and the supernatant was collected and kept at 4ºC.

Supernatants were divided into two parts and by which proteins were precipitated from the supernatant by adding 1 µg of the anti-HA (Roche, UK) antibodies or the anti-c-Myc (Santa Cruz, UK) antibodies with 50 µl of protein G-sepharose beads, each overnight at 4°C. Beads were washed three times with ice-cold NP-40 buffer and were resuspended in 2x SDS protein sample buffer. Samples were boiled for 10 minutes and directly analysed by SDS-PAGE or stored at -20°C.

### 2.3.4 Preparation of protein extracts for western blot analysis

Cells were washed once with PBS before being lysed in lysis buffer containing protease inhibitors was applied to dishes in ice for 30 minutes. After which, cell lysates were harvested and centrifuged for 10 minutes at 1000 rpm and 4°C to remove unsolubilised material. Protein concentrations were determined using BCA

protein assay kit (Thermo scientific, UK). Samples were quantified using a polar star plate reader (Polar star optima, BMG Biotech) on the absorbance setting. Samples were resuspended in 2x SDS protein sample buffer and were boiled for 10 minutes and directly analysed by SDS-PAGE or stored at -20°C.

### 2.3.5 SDS-PAGE

Gel electrophoresis was performed with large gels using a twin vertical electrophoresis system (Galileo, Bioscience) with 12% gels. The Resolving gels 12% was prepared with 9.9 ml H2O, 12.0 ml 30% Acrylamide mix, 7.5 ml 1.5 M Tris (pH 8.8) and 0.3 ml 10% SDS. Prior to pouring the gel, 0.3 ml of 10% Ammonium persulfate (APS) and 0.012 ml of TEMED were added. The solution for generating the resolving gel was mixed and, after pouring, the gel was overlaid with isopropanol. After polymerisation, the isopropanol was removed and the stacking gels were prepared with 6.8 ml H2O, 1.7 ml 30% Acrylamide mix, 1.25 ml 1.0 M Tris (pH 6.8) and 0.1 ml 10% SDS. Prior to pouring the gel 0.1 ml 10% Ammonium persulfate and 0.01 ml TEMED were added. The stacking gel solution was poured on top of the separation gel and a comb was fixed. After polymerisation, the glass plates containing the gel were assembled in the gel electrophoresis apparatus. Samples were loaded on the gel together with a protein ladder. Separation was performed at 150 V constant current for 3 hr.

### 2.3.6 Western blot

Proteins were transferred to nitrocellulose membranes (Amersham Biosciences, UK), using the Trans-Blot semi dry Transfer Cell (Bio-Rad, UK). A piece of nitrocellulose membrane and six pieces of thin filter paper (Whatman, UK) of the same size as the gel were soaked with transfer buffer. Three pieces of the thin filter paper, the nitrocellulose membrane, the gel, and other three pieces of thin filter paper were packed and were placed on the platinum anode. The air bubbles were removed by rolling a pipette over the stack and subsequently the cathode was placed onto the stack followed by the safety cover. Blotting was performed with 15 V for 1 hr.

Unspecific binding sites were blocked by incubation in blocking buffer 2 hr at RT or overnight (o/n) at 4°C. Then incubation with the first antibody was performed in 5-10 ml TBST with 1/1000 antibody concentration. The membranes were washed 3 times 10 minutes each with TBST buffer. Incubation with the secondary antibodies was performed for one hour with 1/3000 secondary antibody diluted in 1% skimmed milk dissolved in TBST buffer. The membranes were washed again for 3 times 10 minutes each with TBST buffer. The blotted proteins were detected using the ECL Western blotting detection system (GE health care, UK), according to the manufacturer's instructions. The membranes were exposed to Fuji Medical X-Ray film (Fujifilm Europe GmbH) for different time periods and films were developed using OPTIMAX X-Ray Film Processor.

### 2.3.7 Immunofluorescence

DF-1 cells were grown in 8-well culture glass slide (BD Falcon, USA) and transfected with either HA-tagged plasmid, Myc-tagged plasmid or both plasmids. After 48 hr, cells were washed with PBS and fixed with 100% methanol incubation at -20°C for 20 minutes. Cells were blocked by 3% BSA diluted in PBS buffer for 1hr, then cells were stained for 1 hr with 1/2000 primary antibodies diluted in PBS. Cells were washed 3 times with PBS and then incubated for 1 hr with 1/4000 secondary fluorescent antibodies diluted in PBS. Cells were washed with PBS 3 times and mounted with Prolong Gold Anti-fade reagent with DAPI (Invitrogen, UK). Cells were examined using LEICA DM LB2 fluorescent microscopy.

## 2.4 Dual luciferase reporter assay

The dual luciferase system (Promega, UK) provides an efficient method to measure the response of a reporter plasmid. The system offsets problems encountered from low transfection efficiency and general transcription activity. It relies on the use of two luciferase plasmids one of which is the firefly reporter (*Photinus pyralis*) and the

second is the Renilla plasmid (*Renilla reniformis)*. Dual luciferase reporter assays were performed as the following.

Cells were plated out onto 48 well plates at a density of $5x10^4$ cells per well and incubated overnight at 37°C and 5% $CO_2$. The following day cells were washed with PBS. Transfection reactions were prepared as detailed in Section 2.2.1 containing 10-ng Renilla, 90 ng of tested DNA (MDV gene cloned into pCR3 expression vector) and 50 ng of luciferase reporter plasmid (DNA fragment from chicken Mx1 promoter cloned in luciferase reporter vector pHIIα). After 48 hr post transfection, cells were washed once with PBS and the complete growth media was exchanged with serum free media containing only 0.5% serum and optimised amount of units of chicken interferon-alpha (GenWay, USA). 15 hr later the media was aspirated and drained completely followed by addition of 50 µl of 1x Passive lysis buffer (PLB; Promega, UK). Then, plates were left for 1 hr on a shaker to allow for efficient lysis of the cells. The total cell lysate were transferred into 96 well black plate (BD Falcon, USA). Analysis was performed by addition of 30 µl of LARII reagent (Promega, UK) to each well. The reading was performed on a polar star plate reader (Polar star optima, BMG Biotech) on the luminescence setting. After the plate was read, 30 µl of Stop and Glo (Promega, UK) was added to each well to quench the firefly luciferase activity and read again on the same settings to measure Renilla luciferase activity. Relative luciferase activity was calculated by normalising the firefly luciferase value to that of the Renilla luciferase. To standardise the luciferase activity of samples within the same experiment set, the relative luciferase values were divided by the control sample value.

## 2.5 Polymerase chain reaction (PCR)

PCR is a technology by which specific DNA sequences are amplified. It is used to screen diagnostic samples for a particular DNA sequence or to amplify DNA products for cloning purposes. In this study, PCR was used to amplify MDV genes to be cloned into the Gateway® cloning system.

### 2.5.1 Primers

The primers used for open reading frame amplification were purchased from different companies (Metabion, MWG, Sigma-Aldrich and Operon).

**Table 2.5.1: The forward and the reverse primers used to amplify MDV genes and chicken interferon regulatory factor 7 (chIRF7) gene**

See Appendix B.

### 2.5.2 Enzymes and nucleotides mixture

*a. Expand Long Template PCR System*: Purchased from Roche diagnostics. Kit contains Expand Long Template Enzyme mix (150U) (containing thermo stable DNA polymerase with proofreading activity) and 10X Expand Long Template buffer (1, 2 and 3 with 17.5 mM MgCl2, 27.5 mM MgCl2 and 27.5 mM MgCl2 and detergents, respectively). The kit was stored at -20°C and all the buffers thawed and equilibrated at 37°C before use.

*b. Deoxynucleoside triphospahte set*: Purchased from Roche diagnostics, supplied as a clear, colorless solution of the sodium salts of dATP, dCTP, dGTP, and dTTP, each at a concentration of 10 mM in water for a total volume of 200 µl (pH 8.3).

### 2.5.3 Primer designing and nested PCR

The nucleotide sequences for all ORFs of CVI988 strain were obtained from *(http://www.ncbi.nlm.nih.gov/)* with accession number DQ530348. The full sequence of the RB-1B strain was kindly provided by Dr Venu Gobal Nair (Institute for Animal Health, Compton). The prediction of RB-1B viral ORFs was performed using the program Emboss (*http://pollux.mpk.med.uni-muenchen.de/cgi-in/W2H*). Each viral ORF was amplified by nested polymerase chain reaction (PCR) using viral BACs of the RB-1B and CVI988 strains as templates. For some RB-1B specific genes, cDNA generated by reverse transcription of RNA isolated from infected chicken embryo fibroblasts or cDNA from phage library were used as template. All

of the templates were kindly obtained from Prof Venu Gobal Nair (Institute for Animal Health, Compton). Primers were designed using an oligonucleotide properties calculator *(http: // www. basic.northwestern. edu/ biotools/ oligocalc. html) and* adjusting the annealing temperature to be approximately 55ºC. Two primer pairs were designed. The first primer-pair is the gene specific primers and contains the internal parts of the attB1 and attB2 recombination sites. The second pair of primers contains the external parts of the attB1 and attB2 recombination site in addition to a 12 nucleotide overlap with the internal forward and reverse primers (Table 2.5.3).

**Table 2.5.3: Primer construction for nested polymerase chain reaction (PCR)**

| |
|---|
| 1. attB1 internal for AAAAAGCAGGCTCCGCC**ATG**XXXXXXXXXXXXXXX |
| 2. attB1 internal rev AGAAAGCTGGGT**CTA**XXXXXXXXXXXXXXXXXXXX |
| 3. attB2 external for GGGGACAAGTTTGTACAAAAAAGCAGGCT |
| 4. attB2 external rev GGGGACCACTTTGTACAAGAAAGCTGGGT |

| | |
|---|---|
| yellow box: attB1/attB2 sequence | **red letters**: Kozak sequence |
| **bold letters**: ATG (for) and stop (rev) codon | XXXXX: homologous for and rev sequence (18–25 nucleotides, 3' end G or C, 55°C annealing temperature) |

The two PCR reactions were performed as described below, with 10 µl of the first reaction being used as a template for the second PCR reaction. Both reactions were performed using the same PCR-program (Figure 2.5).

**1st PCR:**

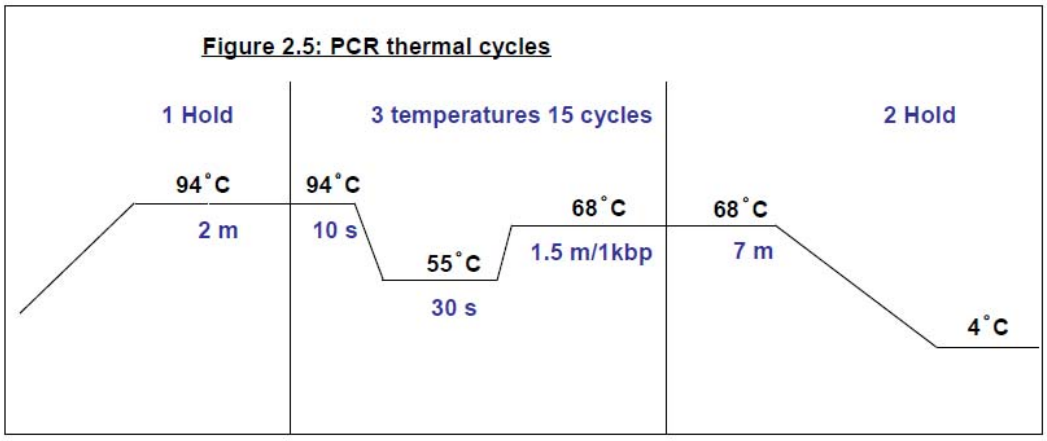
Components:

| | | |
|---|---|---|
| 10x buffer I | | 5 µl |
| Internal Forward Primer | 10 pmol/µl | 2 µl |
| Internal Reversed Primer | 10 pmol/µl | 2 µl |
| Template | 10 ng/µl | 2 µl |
| dNTP Mix | 10 mM | 1 µl |
| Polymerase | 5 U/µl | 0.3 µl |
| $H_2O$ | | add 50 µl |

**2nd PCR:**

Components:

| | | |
|---|---|---|
| 10x buffer I | | 5 µl |
| External Forward Primer | 10 pmol/µl | 1 µl |
| External Reversed Primer | 10 pmol/µl | 1 µl |
| Template (1st PCR Reaction) | | 10 µl |
| dNTP Mix | 10 mM | 1 µl |
| Polymerase | 5 U/µl | 0.3 µl |
| $H_2O$ | | add 50 µl |



### 2.5.4 Purification of PCR product

PCR products were purified either directly or by separation of the DNA fragment by 1% TAE agarose gel electrophoresis. The DNA band of the correct size was cut out from the gel. Both the direct purification and the gel purification were performed using (GE GFX PCR DNA Purification Kit) according to the manufacturer's instructions.

## 2.6 DNA techniques

### 2.6.1 Materials used in DNA techniques

**a. Molecular weight markers:**

1- Gene Ruler 100 bp DNA ladder: Purchased from MBI Fermentas.

2- Gene Ruler DNA 1 kbp ladder: Purchased from MBI Fermentas.

3- 1 kbp DNA ladder: Purchased from New England Biolabs.

**b. Kits:**

1- Plasmid DNA Purification NucleoBond Kit: Purchased from Machery –Nagel.

2- QIAprep Spin Miniprep Kit: Purchased from Qiagen.

3- PureYield TM Plasmid Midiprep System Kit: Purchased from Promega.

4- GFXTM PCR DNA and Gel Band Purification Kit: Purchased from GE Healthcare.

**c. Enzymes:**

1- Gateway® BP clonase enzyme mixture: Purchased from Invitrogen.

2- Gateway® LR clonase enzyme mixture: Purchased from Invitrogen.

3- Restriction enzymes: BamHI, EcoRI and BanII: Purchased from New England, Biolabs.

4- Ribonuclease A: Purchased from Sigma-Aldrich.

5- Ribonuclease H: Purchased from Sigma-Aldrich.

### 2.6.2. Buffers and solutions used in DNA techniques

*1x TAE*: Composed of 40 mM Tris base, 20 mM Acetic acid and 2 mM EDTA. Distilled water was added up to 1 litre.

*1x TBE:* Composed of 89 mM Tris base, 89 mM Boric acid and 2 mM EDTA. Distilled water was added up to 1 litre.

*Solution I (Resuspension solution*): Composed of 50 mM glucose, 25 mM Tris- Cl pH 8.0 and 10 mM EDTA pH 8.0. Distilled water was added up to 1 litre.

*Solution II (Lysis solution)*: Composed of 0.2 N NaOH, 1% SDS. Distilled water was added up to 1 litre.

*Solution III (1 L*): 5 M potassium acetate, 15 ml glacial acetic acid and 285 ml of distilled water.

### 2.6.3 Vectors

**a. DONR vector:**

pDONR 207 (Gateway®, Invitrogen).

**b. Destination vector:**

pGADT7 and pGBKT7 (Gateway®, Invitrogen).

**c. Expression vector:**

1- non-tagged pCR3 expression vector (Gateway®, Invitrogen).

2- N-terminal HA-tagged pCR3 expression vector (Gateway®, Invitrogen).

3- N-terminal Myc-tagged pCR3 expression vector (Gateway®, Invitrogen).

4- N-terminal protein-A-tagged pT-REx expression vector (Gateway®, Invitrogen).

5- N-terminal Renilla-tagged pcDNA expression vector (Gateway®, Invitrogen).

**d. Reporter vectors:**

1- pHSIIα: reporter vector encoding chicken ISRE motif in the upstream region of firefly luciferase.

2- pRLTK: reporter vector encodes Renilla luciferase.

### 2.6.4 Purification of plasmid DNA

***The pCVI988-Bac and pRB-1B-Bac*** were isolated by midiprep alkaline lyses according to manufacturer's instructions (NucleoBond, Machery –Nagel, Germany).

***pDONR 207, pGBKT7 and pGADT7*** were isolated by midiprep alkaline lysis according to manufacturer's instructions (PureYield TM Plasmid Midiprep System kit, Promega, UK).

***The cloned ORFs*** were isolated by plasmid DNA purification of QIAprep Miniprep Kit according to the manufacturer's instructions (Qiagen, UK) and by miniprep alkaline lysis method modified from (Sambrook and Russel, 2001).

A single bacterial colony was selected from the LB plates containing the appropriate antibiotics (Gentamycin, Ampicillin or Kanamycin) and was transferred to 1200 µl of LB broth with the same selectivity of the agar. The culture was incubated overnight at 37°C in 96 deep-well block with constant shaking. The broth was centrifuged at 3500 rpm for 10 minutes at 4°C. The supernatant was discarded and the bacterial pellet was resuspended in 300 µl of ice-cold solution I. For the alkaline lysis of the bacteria, 300 µl of solution II (lysis solution) were added and mixed thoroughly. The plate was incubated for 3-5 minutes then, 300 µl of ice-cold solution III (neutralisation solution) were added to stop the lyses. The mixtures were stored again on ice for 3-5 minutes and were centrifuged at 4000 rpm for 30 minutes at 4°C. Then, 800 µl of the supernatant was transferred in new plate and 580 µl of isopropanol was added to each well followed by a centrifugation at 4000 rpm for 45 minutes at 4°C. Subsequently, the supernatant was discarded and 500 µl of absolute

ethanol was added to each tube followed by a centrifugation at 3500 rpm for 5 minutes at 4°C. Finally, the DNA was dissolved in 50 µl of distilled water.

### 2.6.5 Determination of DNA concentration

The concentration and purity of the purified DNA was determined by measuring the UV absorbance at 260 and 280 nm. The DNA concentration was calculated with the $OD_{260nm}$ (1 $OD_{260nm}$ = 50 µg/ml dsDNA or 33 µg/ml ssDNA). The purity was estimated with the $OD_{260nm}/OD_{280nm}$ ratio, with a ratio of approximately 1.8, indicating a low degree of protein contamination.

### 2.6.6 Restriction endonuclease digestion

Restriction endonuclease reactions were performed according to the manufacturer's recommendations. In general, 1.5 µg DNA was digested for 2 hr at the appropriate temperature with 10-20U enzyme. Efficacy of the cleavage reaction was determined by 1% TAE agarose gel electrophoresis stained with ethidium bromide.

### 2.6.7 Cloning

#### a. BP reaction

Components:

| | |
|---|---|
| PCR- product | 2-7 µl |
| pDONR 207(150 ng/ µl) | 1 µl |
| $dH_2O$ | 1 µl |
| BP Clonase | 1 µl |

The reaction was incubated at 37°C overnight. 1 µl of the reaction was transformed into 50 µl of electrocompetent DH10B bacteria. Cells were recovered by adding 500 µl LB medium and incubating at 37°C for 1 hr whilst shaking. The whole volume of

transformation reaction was spread on LB plates containing 15 μg/ml gentamycin and incubated overnight.
DNA was isolated from a single colony by mini prep alkaline lyses. The plasmids were digested with the restriction endonuclease BanII to determine the correct size of the inserted fragment.

**b. LR reaction**

Components:

| | |
|---|---|
| Purified Entry Clone (150 ng/μl) | 1 μl |
| pGBKT7 | 1 μl |
| pGADT7 | 1 μl |
| dH2O | 1 μl |
| LR Clonase | 1 μl |

2 μl of the reaction mixture were transformed into 50 μl of electrocompetent DH10B bacteria. Cells were recovered by adding 500 μl LB medium and incubating at 37°C for 1 hr whilst shaking. The transformation reaction was divided in two parts. One was spread on LB plates containing 50 μg/ml kanamycin for pGBKT7 (bait vector), the other on ampicillin plates (50 μg/ml) for pGADT7 (prey vector). The plasmids were digested with the restriction endonucleases EcoRI and BamHI to determine the correct size of the inserted fragment.

### 2.6.8 Agarose gel electrophoresis

Analysis of DNA fragments and plasmids was performed by agarose gel electrophoresis in 1x TAE. While, the analysis of the Bac restriction digest pattern was performed by agarose gel electrophoresis in 1x TBE. In general, the agarose concentration was 1% in 1x TAE or 1x TBE. The agarose was solubilised by heating in a microwave oven. Ethidium bromide was added to a final concentration of 0.25 μg/ml (2.5 μl stock to 100 ml) just before pouring the gel. Probes were mixed with

0.17x volume loading buffer. Gels were run horizontally at 80-120 V. DNA was detected with UV light.

### 2.6.9 Sequencing of the genes

The sequencing of the genes was done commercially by GATC Biotech (Konstanz, Germany), using pDONR 207 vector forward and reverse primers.

## 2.7 Bacterial techniques

### 2.7.1 Bacterial media and solutions

*LB broth*: 10 g Bactotryptone, 5 g Yeast extracts, 10 g NaCl and water was added up to 1 L. The media was autoclaved and the kept at 4ºC.

*LB agar*: 10 g Bactotryptone, 5 g Yeast extracts, 10 g NaCl. Water was added up to 1 L and agar was added at 15 g per litre. The media was autoclaved and poured onto plastic plates (Sterilin limited, UK).

*2x YT media*: 16 g Bactotryptone, 10 g Yeast extracts, 5 g NaCl and water was added up to 1 L. The media was autoclaved and the kept at 4ºC.

*10% Glycerol*: Glycerol purchased from Sigma-Aldrich in liquid form. 100 ml of glycerol solution was diluted up to 1 L with distilled water to reach 10% concentration. The diluted solution was sterile filtrated.

### 2.7.2 Antibiotics

*Gentamycin*: Purchased from Sigma-Aldrich, liquid supplied and used at 15 µg/ml media.

*Ampicillin*: Purchased from Sigma-Aldrich, powder supplied. Stock solution was prepared containing 50 mg ampicillin per ml of sterile distilled water. 1 ml from the stock solution was added to 1 litre media.

*Chloramphenicol*: Purchased from Sigma-Aldrich, powder supplied. Stock solution was prepared containing 50 mg per ml of ethanol. The media was adjusted to contain 20 µg of chloramphenicol per ml media.

*Kanamycin*: Purchased from Sigma-Aldrich, powder supplied. Stock solution was prepared containing 50 mg kanamycin per ml of sterile distilled water. 1 ml from the stock solution was added to 1 litre media.

### 2.7.3 Bacteria

*Electrocompetent Escherichia coli DH10B*: Used for transformation of the amplified genes with pDONR 207, pGBKT7 and pGADT7.

*Electrocompetent Escherichia Coli DB 3.1*: Used for transformation of the vectors carrying the ccdB cassette.

### 2.7.4 Cultivation of bacteria

E. coli bacteria were grown in LB medium or on LB agar plates. Incubation was performed at 37°C with constant shaking in the case of liquid media.

### 2.7.5 Preparation of electrocompetent bacteria

A single clone of DH10B was picked and grown overnight in 50 ml of LB at 37°C. Subsequently, 10 ml of the overnight culture were inoculated into 1 L of 2x YT broth and incubated at 37˚C. At an OD600nm of 0.5 the culture was rapidly chilled on ice for 20 minutes.

The following process was performed at 4°C or on ice: The bacteria were transferred into centrifugation containers and centrifuged for 10 minutes at 6000 rpm. The supernatants were discarded and the bacterial pellets were resuspended in an equal volume of sterilised water. Then, the bacteria were centrifuged for 10 minutes at 6000 rpm. The supernatants were discarded and the pellets were resuspended in 250 ml of water followed by a centrifugation at 6000 rpm for 10 minutes. Then, the

supernatants were discarded and the pellets resuspended in 125 ml of 10% Glycerol. The suspensions from two centrifugation tube were first combined into one container and then were centrifuged at 6000 rpm for 10 minutes. In the final washing step, the supernatant was discarded and the cells were resuspended in 125 ml of 10% glycerol, followed by a centrifugation at 6000 rpm for 10 minutes. The cells were resuspended in 10% glycerol to give a final volume of 3-6 ml and then distributed into 1.5 ml microcentrifuge tubes. Finally, the cells were frozen in liquid nitrogen and stored at -80°C.

### 2.7.6 Electroporation

DNA was mixed with electrocompetent DH10B bacteria in 2 mm gap cuvettes. The cuvette containing the sample was placed on the shocking chamber of the gene pulser electroporation system and the mixture was electroporated at 2.5 V. Cells were recovered by adding 500 µl LB medium and incubating at 37°C for 1 hr whilst shaking.

## 2.8 Yeast techniques

### 2.8.1 Yeast media and solutions

*YEPD broth*: 10 g Yeast extracts, 20 g Peptone and 20 g Dextrose. Water was added up to1 L. The media was autoclaved and stored at 4°C.

*YEPD agar*: 10 g Yeast extracts, 20 g Peptone and 20 g Dextrose. Water was added up to1 L and agar was added up to 14 g/L. The media was autoclaved and poured onto plastic plates (Sterilin limited, UK) then stored at 4°C until required.

*SBEG solution*: 91.1 g Sorbitol, 5 ml Bicine 1 M (pH 8,35) and 15 ml Ethylenglycol (3%). Water was added up to 500 ml. The solution was sterile filtered and kept at 4°C.

*PEG/Bicine solution*: PEG 1000 (40%) and 200 mM Bicine (pH 8,35). The solution was sterile filtered and kept at 4°C.

*NB buffer*: 3 ml from 5 M NaCl stock solution and 1 ml from 1 M Bicine (pH 8, 35).Water was added up to 100 ml. The solution was sterile filtered and kept at 4°C.

*Dropout medium liquid medium*: 1.7 g Yeast nitrogen base without amino acids (Clontech, France), 5 g Ammonium sulphate, 20 g Dextrose and 1.4 g Dropout supplements. Water was added up to 1 L. Media was autoclaved and stored at 4°C.

*Dropout medium liquid medium +3AT*: The same ingredients as above plus 3 mM 3-aminotriazole.

*Dropout medium solid medium*: 1.7 g Yeast nitrogen base without amino acids (Clontech, France), 5 g Ammonium sulphate, 20 g Dextrose, 1.4 g Dropout supplements and 16 g agar. Water was added up to 1 L. Media was autoclaved and poured onto plastic plates (Sterilin, UK) then stored at 4°C.

*10x Leucine*: Purchased from Sigma-Aldrich, Stock solution was prepared contains 1000 mg Leucine per litre distilled water, (10x solutions). The working solution contained 100 mg per 1000 ml media.

*10x Tryptophan*: Purchased from Sigma-Aldrich, Stock solution was prepared containing 200 mg Tryptophan per litre distilled water, (10x solutions). The working solution contained 20 mg per 1000 ml media.

### 2.8.2 Yeast

Two yeast strains were used AH109 and Y187 provided by (Ragnhild Eskeland, MRC).

### 2.8.3 Competent yeast cells

To produce competent yeast cells, first a preculture had to be prepared:
10 ml YEPD-medium was inoculated by a colony of the yeast strains Y187 and AH109, respectively. The preculture was shaken overnight at 30°C.

The next day, the preculture was added to 250 ml of fresh YEPD-medium and grown at 30°C until it reached a density of 0,6 at $OD_{600.}$ The cells were harvested in ten 50 ml Falcon tubes at 2000 rpm (930 x g, 5 minutes, 4°C). The supernatant was removed and the cells were resuspended in 12.5 ml SBEG-solution (each) and subsequently pelleted once again. The resulting cell pellet was resuspended in 500 µl SBEG-solution. The 500 µl cell suspension was split into 100 µl aliquots for immediate use.

### 2.8.4 Transformation into yeast cells

For a transformation, the yeast was aliquoted in 96 deep wells block. Subsequently, 1 µg of the plasmid pGBKT7or pGADT7 was pipetted into the yeast cells of the appropriate yeast strain and mixed carefully by the pipette. Afterwards, 750 µl PEG/Bicine-solution was added and again mixed by the pipette. This reaction was incubated at 30°C for 1 hr, then at 45°C for 5 minutes. The next step was to pellet the cells for 2 minutes at 3500 rpm (approx 2700 x g, table centrifuge). The supernatant was removed carefully and the pellet was resuspended in 1 ml NB-buffer by pipette. Again, cells were pelleted, as before, but only 800 µl of the supernatant was removed. The pellet was resuspended in the remaining 200 µl supernatant and plated on an appropriate yeast plate.

### 2.8.5 Mating and selection by a robot device

The steps involving a robotic workstation (Biomek 2000; Beckman Coulter) were performed in the lab of Peter Uetz in the Institute for Toxicology and Genetic in Karlsruhe.

The prey array, consisting of 149 haploid yeast transformants, was divided into two 96 well plates; lacking leucine (-Leu) (the transformed yeast vector pGADT7 provides a leucine auxotrophy). The yeast-transformed bait array was treated the same way, apart from being placed on plates lacking tryptophan (-Trp). This procedure was done by hand in our lab.

By robot device, each element on the prey plate was duplicated. The duplication assures reproducibility of screening results. Bait colonies from -Trp plates were picked and grown overnight in 20 ml YEPD at 30°C. The bait-medium was transferred into empty microtiter plates. The pins of the 384-pin replicator were dipped into the BD fusion-expressing culture and placed directly onto a fresh single-well microtiter plate containing solid YEPD medium. This procedure was repeated for each of the 149 baits.

Between transfer steps, the tool must be sterilised by sequential immersion into a 20% bleach solution (20 sec), sterile water (1 sec), 95% ethanol (20 sec), and sterile water (1 sec). The level of these liquids should be 2 to 4 mm from the base of the pin and care must be taken that the ethanol does not evaporate.

In the next step, the prey array was picked with sterilised pins and transferred directly onto the mating α colonies expressing a single protein as a binding domain (BD) fusion, so that each of the BD yeast spots per plate received different active domain (AD) yeast cells. The plates were incubated for 1 or 2 days at 30°C to allow mating.

For selection, the colonies were transferred to single-well microtiter plates containing solid –Leu and -Trp dropout medium using the sterilised pinning tool. The colonies were grown for 2 days at 30°C until they were 1 mm in diameter. This was an essential control step because only diploid cells that contain Leu and Trp markers on pGADT7 and pGBKT7, respectively, will grow on this medium. This step also helps recovery of the colonies and increases the efficiency of the next selection step.

For the next step the colonies were transferred to a single-well microtiter plate containing solid –His, -Leu and -Trp (+3AT) dropout medium using the sterilised pinning tool and grow at 30°C for up to 10 days (or longer if there was little or no background growth) to select two-hybrid positive diploids.

The stringency of the screen can be varied by adding different amounts of 3AT, an inhibitor of the His3 gene product. In many cases (10% to 20%), the haploid strain expressing the BD fusion has transcriptional self-activation properties. These haploid strains can be titrated on plates lacking histidine and containing increasing amounts of 3AT (3 mM, 10 mM, 20 mM, 50 mM). The highest level of 3AT tolerated, should be added to the –His, -Leu and -Trp plates for selection of two-hybrid positive diploids. In many cases, the transcriptional activity is very strong (>200 mM 3AT), so that not all self-activators can be eliminated. The cellular genes Myc and Max, cloned into pGBKT7 and pGADT7, were included as a positive control. Empty pGADT7 and pGBKT7 vectors were included as a negative control. The interactions were scored positive by looking for growing colonies that were significantly above the background (by size) and that were present in 2 colonies.

## 2.9 RNA Techniques

### 2.9.1 Materials used in RNA techniques

**a. Kits:**

1- RNeasy Mini Kit: Purchased from Qiagen and was used according to the manufacturer's instruction.

2- QIA shredder column: Purchased from Qiagen.

3- Verso TM SYBR® Green 1-Step QRT-PCR Low ROX Kit: Purchased from Thermo Scientific and was used according to the manufacturer’s instruction.

**b. Enzymes:**

1- Super Script ™ III Reverse transcriptase: Purchased from Invitrogen and was used according to manufacturer's instruction.

### 2.9.2 RNA isolation and purification

RNA was isolated according to the manufacturer's instructions using (RNeasy Mini Kit, Qiagen). Briefly, cells were resuspended and disrupted in a high-salt denaturing lysis buffer containing guanidine isothiocyanate, and homogenised on a QIA shredder column (Qiagen, UK) by centrifuging at 13000 rpm for two minutes. Samples were added to the RNA-binding columns following the addition of 350 µl 70% ethanol, to create correct resin-binding conditions, and centrifuged at 10000 rpm for 15 seconds. Flow-though was discarded and bound to RNA was washed twice in buffer RW1 and twice in buffer RPE by centrifuging for 15 seconds at 10000 rpm. RNA was eluted in 30 µl of RNase-free water.

### 2.9.3 Reverse transcription

RNA was reverse transcribed to cDNA using Super Script ™ III Reverse transcriptase (Invitrogen, UK) and random dt21 oligos or gene specific oligos. The reverse transcription was performed according to the manufacturer's instructions. The resulting cDNA was incubated with 1 µl of E.coli RNase H at 37°C for 20 minutes for removal of the RNA complementary to the cDNA.

### 2.9.4 Quantitative real time-PCR (QRT-PCR)

Quantitative real time-PCR is a molecular technique, used to measure relative gene expression levels or DNA copy number in tested samples. The previous is reached via measuring the cycle number at which the increase in the fluorescence becomes detectable. This point is termed as the cycle threshold (Ct) value and the detection level, which is set by user, is termed threshold level.

**a. Primers:**

Primers were designed using Primer3 online tool (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The primers were purchased from (Metabion, Germany). The designed primers for interferon regulatory factor 7 (IRF7) and β-actin are listed in Table 2.9.4.

**Table 2.9.4: Primers used in QRT-PCR**

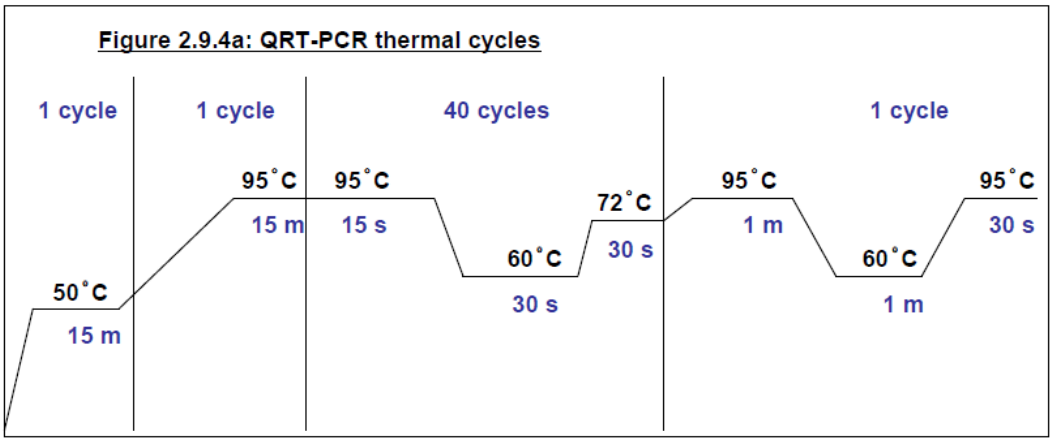
| Gene | Fwd | Rev |
|---|---|---|
| **IRF7** | CAG TGC TTC TCC AGC ACA AA | TGC ATG TGG TAT TGC TCG AT |
| **β-actin** | CCC CCG TGC TGT GTT CCC ATC TAT CG | GGG TGC TCC TCA GGG GCT ACT CTC AG |

**b. Optimisation of QRT-PCR:**

All amplifications were performed on a MX3000-P™ real-time PCR Strategene instrument. β-actin gene was used as an internal control for samples normalisation, then tested samples were normalised to calibrator samples. The PCR reaction was performed according to the description below.

Components:

| | | |
|---|---|---|
| Verso Enzyme Mix | | 0.25 µl |
| Forward Primer | 1 µM | 1.75 µl |
| Reversed Primer | 1 µM | 1.75 µl |
| Template | 15 ng/µl | 2.00 µl |
| 1-Step QPCR SYBR Low ROX Mix (2x) | | 12.50 µl |
| $dH_2O$ | | add 25.00 µl |




A standard curve assay was performed to determine the amplification efficiency of the used primers. The efficiency was calculated according to the difference of Ct values, obtained from double fold serial dilution of RNA template. Any efficiency ranged from 90-120% was considered ideal. As shown in Figure 2.9.4b, the amplification efficiency of IRF7 and β-actin were 79.5% and 107.7%, respectively.

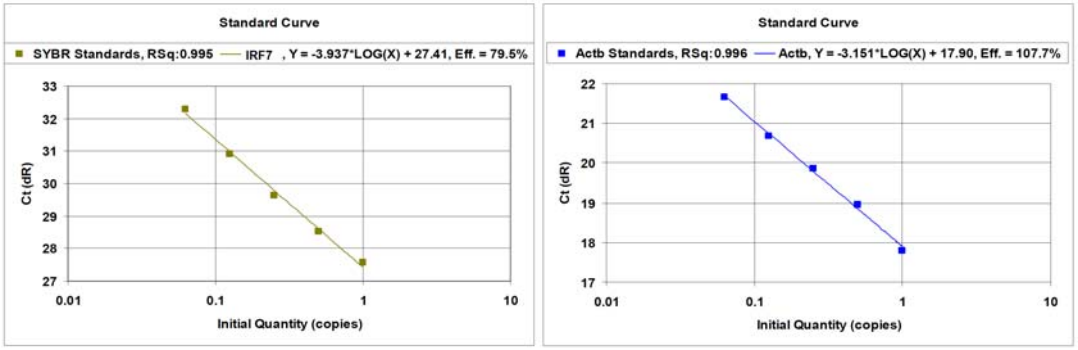



**Figure 2.9.4b: Standard curve of IRF7 and β-actin primers.** Standard curve graph compares the Ct values of five different concentrations from double fold serial diluted RNA template. Each dilution was run in duplicate, and the amplification was done using Verso TM SYBR® Green 1-Step QRT-PCR Low ROX Kit (Thermo Scientific, UK). The efficiency of the standard curves was calculated using MxPro software (Strategene, UK).

A dissociation curve assay was carried out for the primers at 60ºC annealing temperature for 1 minute to investigate if there was any non-specific amplification. As shown in Figure 2.9.4b, the dissociation curves of the both primers are representing a specific amplification.

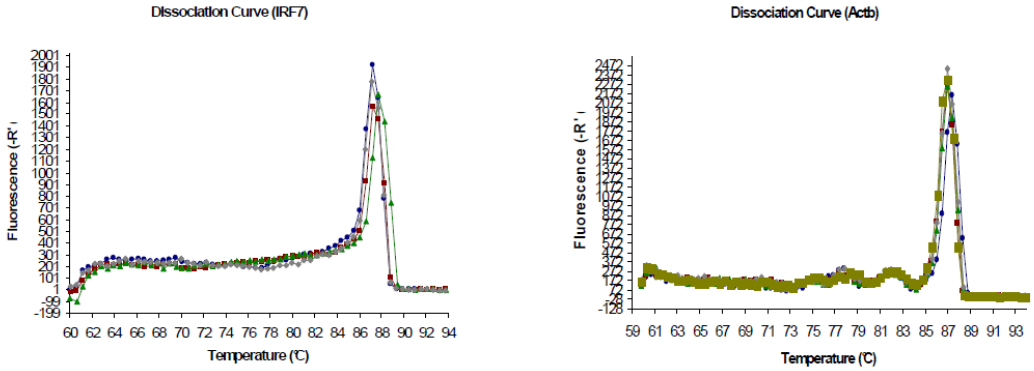



**Figure 2.9.4c: Dissociation curve of IRF7 and β-actin primers.** Dissociating curve graph show the specificity of the PCR amplification of five different concentrations from double fold serial diluted RNA template. Each dilution was run in duplicate, and the amplification was done using Verso TM SYBR® Green 1-Step QRT-PCR Low ROX Kit (Thermo Scientific, UK). The dissociation curves were analysed using MxPro software (Strategene, UK).

# 2.10 Virus techniques

## 2.10.1 Viruses

MDV strains pRB-1B5 (Petherbridge et al., 2004) and the Meq-deleted RB-1B5-D2 (Brown et al., 2006) viruses have been described previously. Viruses were propagated in CEF cells, and cells were cultured as described in Section 2.1.5.

## 2.10.2 Buffers used in virus staining

*a. Fixing solution*: Ice cold 1:1 acetone:methanol.

*b. Wash buffer*: PBS with 0.05% TWEEN 20 (PBST).

*c. Blocking buffer*: PBS with 5% FCS.

*d. Developing solution*: 0.513 ml of 0.1M sodium acetate (pH 4.8), 0.027 ml of 4 mg/ml 3-amino-9-ethylcarbazole solution diluted in dimethylformamide and 0.009 ml of $H_2O_2$. It was prepared shortly before use.

### 2.10.3 Materials and supplements used in virus techniques

**a. Antibodies:**

1- HB3 murine anti-gB antibody, used at 1/100 and diluted in blocking buffer.

2- Goat anti-mouse peroxidase coupled, purchased from Dako. It was used at 1/200 and diluted in blocking buffer.

**b. Cytokines:**

Chicken interferon-alpha-2 recombinant protein: Purchased from Genway Biotech. Recombinant protein prepared from E.coli and was supplied in 50 µl PBS. The solution containing $1x10^7$ U/mg was aliquoted into small 0.5 ml reaction tubes and stored at -20°C.

### 2.10.4 Staining CEF cells for MDV plaques

CEF cells infected with MDV were fixed with ice cold acetone: methanol for two minutes at room temperature. Fixative was removed, and cells were washed twice with PBS. Cells were blocked by incubation with the blocking buffer at room temperature for one hour, and then incubated with murine HB3 monoclonal Ab (primary antibody) at room temperature for one hour. Thereafter, cells were washed with the washing buffer three times and incubated with Goat anti-mouse peroxidase antibody (secondary antibody). After removal of the secondary antibody, cells were washed three times with the washing buffer and the plaques were detected by adding the developing solution. Plaques appeared as red spots which were distinguishable from the uninfected cells (Figure 2.10.4).

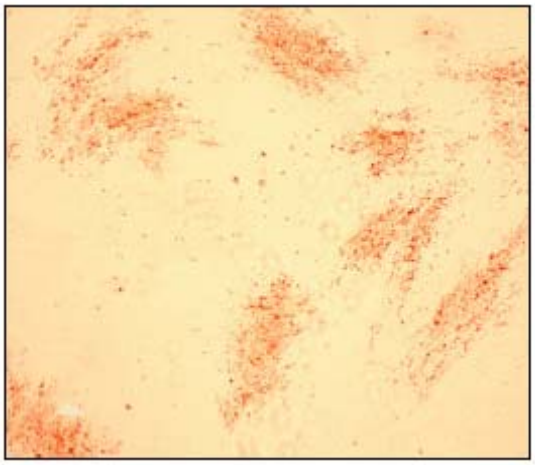


**Figure 2.10.4: Immunoperoxidase staining of MDV plaques.** CEF cells were infected with RB-1B MDV. Cells were fixed 5 dpi and stained with monoclonal Ab against glycoprotein B (HB3). The MDV-specific plaques were visualised and appeared as red spots after adding the developing solution which contains 3-amino-9-ethylcarbazole substrate.

# Cloning of the MDV ORFeome

## 3.1 Introduction

MDV encodes more than 100 genes that have both structural and regulatory roles. Cloning some of the MDV genes and testing them at the proteomic level led to discovery of new facts on the biology and life cycle of the virus. For example, cloning of MDV-UL9 gene and expressing it by *in vitro* transcription-translation system was the reason for discovering that it is the MDV origin binding protein (OBP) (Wu et al., 2001b). Another example is the Meq gene, MDV bZIP, in murine retroviral vector helped in discovering Meq transformation and anti-apoptotic properties (Liu et al., 1998). However, previous studies were performed to study individual or a small number of MDV genes. With the emergence of more virulent strains of MDV, it becomes a necessity to undertake functional analysis of MDV proteins on a genome-wide scale.

### 3.1.1 Gateway® cloning technology

Gateway® recombinatorial cloning provides the opportunity to clone large number of genes in a high-throughput manner. *Caenorhabditis elegans* ORFeome was the first to be cloned by using the Gateway® technology on a genome-wide scale (Walhout et al., 2000). Thereafter, many libraries were constructed for many organisms including many members of herpesvirus (Fossum et al., 2009).

Gateway® technology depends on att recombination sites, which are used by the lambda bacteriophage for excision and integration from the bacterial chromosome (Figure 3.1.1). Briefly, the insert is flanked by attB sites allowing the recombination into the entry vector which contains attP sites. After the first cloning step (BP recombination) the entry clone becomes flanked by attL sites, and the DNA can be easily subcloned into multiple destination vectors, which are flanked by attR sites (LR recombination). The formed expression clones, which are flanked by attB sites, can be used further for different purposes and expressed in different systems.

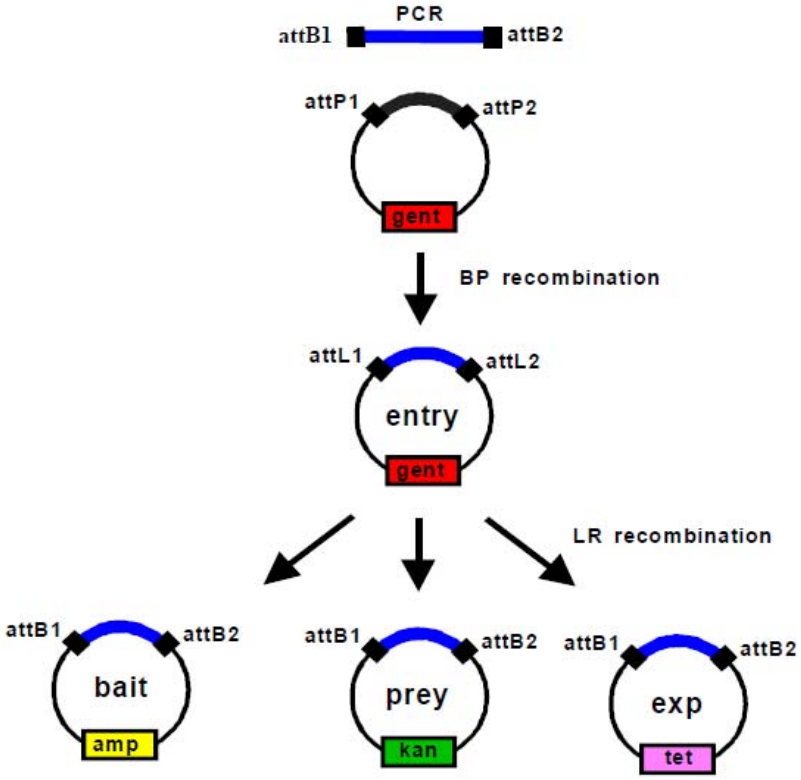



**Figure 3.1.1: Gateway® cloning.** Gateway® cloning is based on a recombination derived from the λ bacteriophage. First, the gene of interest (PCR product) is inserted into a DONR vector to create the entry clone via BP recombination reaction. Subsequently, the gene of interest can be subcloned into different destination-expression vectors by a second recombination reaction (LR recombination reaction).

### 3.1.2 Aim

Marek's disease virus has been completely sequenced and is fully annotated (Lee et al., 2000a; Tulman et al., 2000; Spatz et al., 2007b; Spatz et al., 2007c). The aim of the work presented in this chapter was to clone all predicted MDV genes into the entry vector pDONR 207 using Gateway® technology. Moreover, to subclone the entry clone collection into different destination and expression vectors to perform various functional assays. These functional assays will help in understanding the role played by each of the MDV genes and determining the essential genes for the MDV infection and pathogenicity.

## 3.2 Results

### 3.2.1 Amplification of MDV genes

To functionally analyse Marek's disease virus proteins, MDV genes were first amplified by polymerase chain reaction (PCR). In total, 122 genes were amplified as full-length genes (Figure 3.2.1b) or as fragments. In addition, 25 extra- or intracellular domains were amplified from the genes which were predicted to have transmembrane regions (Appendix C). The templates used for the amplification of MDV genes were two bacterial artificial chromosome (BAC) clones of the MDV CVI988 and RB-1B strains (Figure 3.2.1a), RB-1B RNA isolated from MDV-infected CEF and a RB-1B cDNA phage library. The primers were designed based on alignments of the RB-1B sequence with the published sequence of the CVI988, Md5 and Md11 strains with the accession number DQ530348, NC_002229 and AY510475, respectively. PCR conditions were first the same for all ORFs. Some ORFs, which could not be amplified under the standard conditions, were successfully amplified with touch-down PCR by lowering the annealing temperature 1ºC every cycle (from 60 to 50ºC). This was the case for RLORF9, PP38, SORF4, US8, the cytoplasmic domain of US6, the cytoplasmic domain of US8, the cytoplasmic domain of US7 and the cytoplasmic domain of UL34. The four spliced genes 14kDa, 14KDb, Lipase and vIL8 were amplified from cDNA generated by reverse

transcription of RB-1B RNA isolated from CEF infected with RB-1B strain. The reverse transcription was done using dt21 primers with the exception of the Lipase gene, in which the RNA was reverse transcribed using gene specific primers. The fifth spliced gene, UL15, could not be amplified from the RNA and the cDNA library as well. For UL15, two internal overlapping primers were designed. The first primer is the 3′ terminus for the first exon, including 11 bases from the 5′ terminus of the second exon and the second primers is the 5′ terminus of the second exon plus 11 bases from the 3′ terminus of the first exon. The two exons were amplified in separate reactions from the RB-1B BAC construct. Then, the two fragments were purified and used together in the second reaction as a template. US2 gene (the area of BAC insertion) and another version of viral Lipase were amplified from cDNA phage library of RB-1B strain. The very long ORFs like ICP4 (6000 bp), UL29 (3576) and UL36 (10047 bp) were amplified as domains. With all the displayed efforts, we were unable to amplify RLORF1, LORF1 or vTR. However, very short cytoplasmic domain was amplified from LORF1 gene.

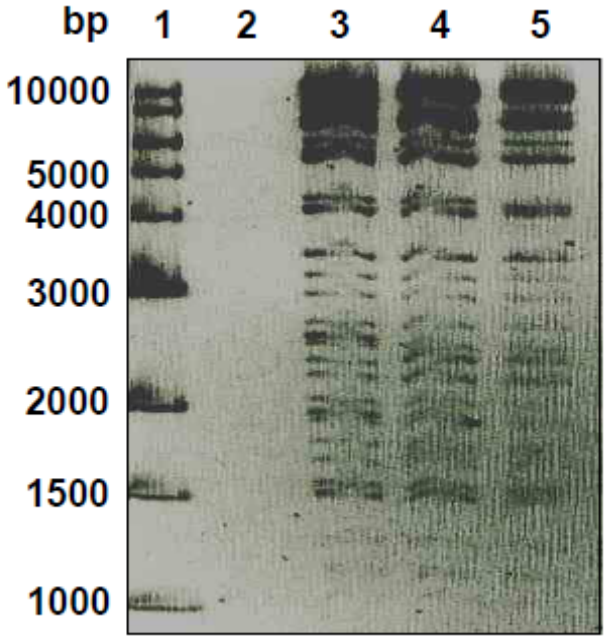



**Figure 3.2.1a: EcoRI digests of CVI988 BAC and RB-1B BAC DNA.** After DNA isolation using an alkaline lysis-based protocol and restriction digest with EcoRI, the digests were separated on a 1 % TBE agarose gel. After electrophoresis, the gel was stained with ethidium bromide. Lane 3 represents digested CVI BAC DNA, while lane 4 and 5 represent two different digested RB-1B BAC DNA. 1 kbp DNA ladder (NEB) was used as a reference (lane 1).

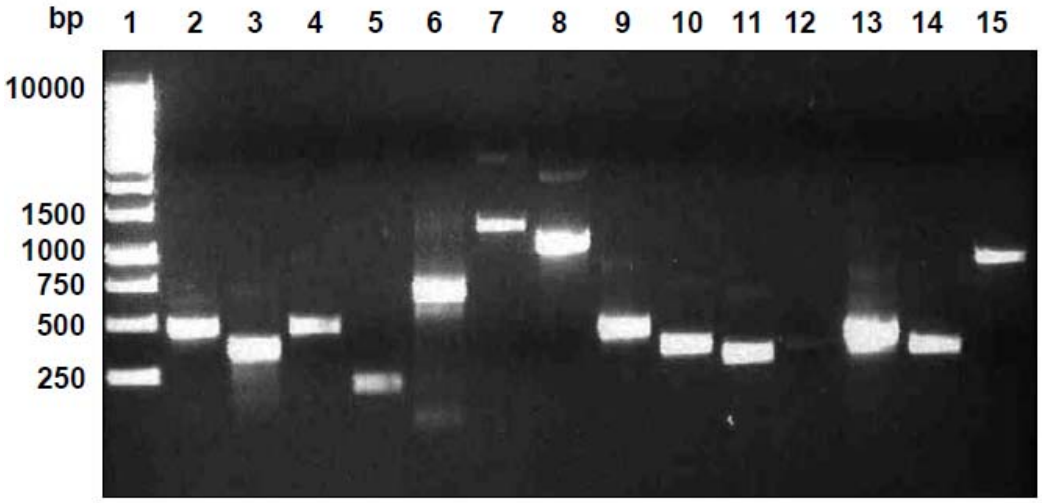



**Figure 3.2.1b**: **PCR products of MDV ORFs from the repeat long region.** Lane 1 1 kbp DNA ladder (Fermentas), lane 2 vIL8, lane 3 RLORF3, lane 4 RLORF4, lane 5 RLORF5, lane 6 RLORF6, lane 7 LMeq, lane 8 Meq, lane 9 RlORF8, lane 10 RLORF9, lane 11 RLORF10, lane 13 RLORF12, lane 14 RLORF13b and lane 15 RLORF14a (PP38).

### 3.2.2 Cloning of MDV genes into the pDONR 207 vector

Each PCR product was cloned into pDONR 207 by recombinatorial cloning. 119 genes have been cloned (Appendix C) as a full-length or as fragments, plus 25 domains for genes which were predicted to have transmembrane region(s). These clones represent the MDV entry clone library which can be subcloned into different destination and expression vectors to be used for different purposes. The cloning into the pDONR 207 vector was initially confirmed by restriction digest with BanII enzyme (Figures 3.2.2). The US7 gene could not be cloned as a full length ORF for unknown reason, but it was cloned as both extracellular and intracellular domains. Three genes were amplified by PCR but could not be cloned into pDONR 207 vector UL28, UL39 and LORF11.

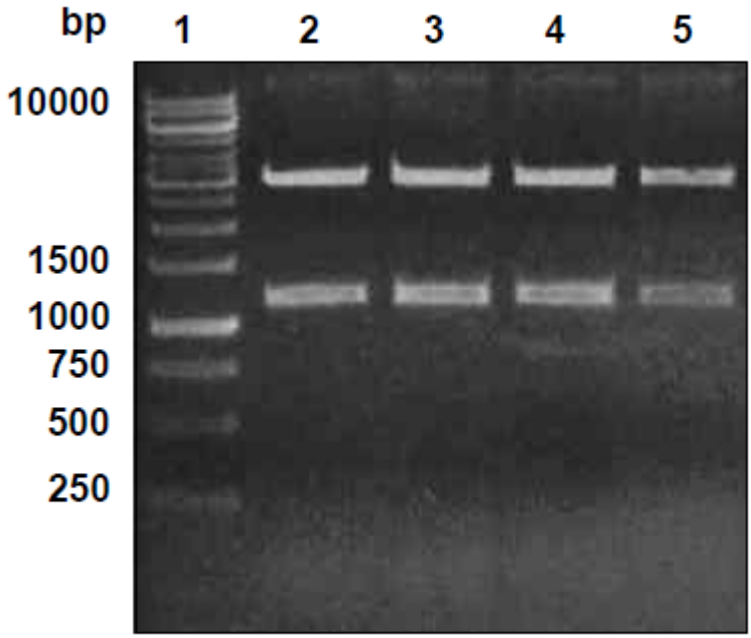



**Figure 3.2.2: BanII digests of UL49 clones in the entry vector pDONR 207.** Scanned image of ethidium bromide stained 1% TAE agarose gel which shows UL49 cloned into pDONR 207, and digested with BanII enzyme. Lane 1 contains 1 kbp DNA ladder (Fermentas), while lane 2-5 represent DNA isolated from four different clones.

### 3.2.3 Sequencing of the cloned entry vectors

All cloned ORFs in pDONR 207 were sequence-verified from both ends using forward and reverse primers (GATC Biotech, Konstanz, Germany). Clones with frame shift mutations were disposed, but not clones with silent (mutations not leading to an amino acid exchange) or missense mutations. A total of 118 full-length cDNA clones and 25 clones encoding protein domains of membrane-associated proteins were generated and sequence-verified (Figure 3.2.3). The sequencing results are listed in Table 3.2 in Appendix C.

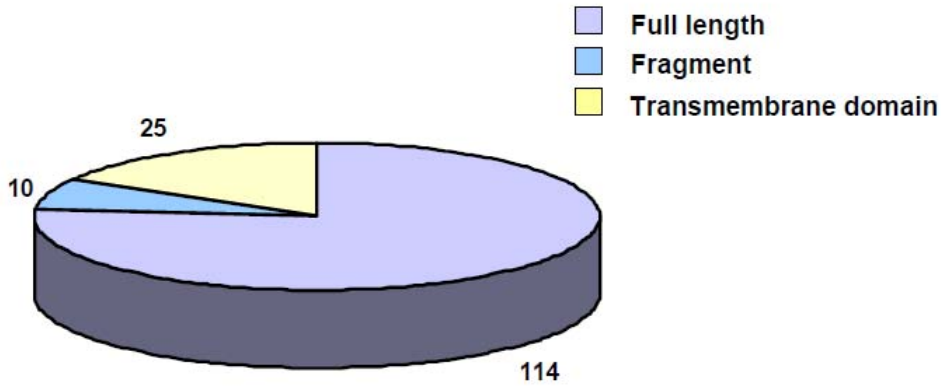



**Figure 3.2.3: Total 149 clones were verified by sequencing.** 149 constructs from MDV genome were cloned in pDONR 207 and were verified by sequencing using both the forward and the reverse primers of the pDONR 207. They represent 114 genes cloned as a full length, 4 genes cloned as fragments (10 fragments) and 25 transmembrane domains.

### 3.2.4 Cloning of the MDV entry clones into multiple destination vectors

#### a. Plasmids for Y2H and Co-IP

In order to use the Y2H system to investigate interactions between MDV proteins, the MDV entry clones were subcloned into the Y2H bait and prey vectors pGBKT7-DEST and pGADT7-DEST. Both vectors are destination vectors with different resistance genes, ampicillin in case of the prey vector and kanamycin in the bait vector. The prey and bait clones were confirmed by a double restriction digest using EcoRI and BamHI restriction enzymes (Figure 3.2.4a). All genes which had been cloned in the pDONR 207 vector, were successfully subcloned into both bait and prey vectors.

**b. Plasmids for LUMIER pull-down assay**

In order to use LUMIER pull-down assays for validation, a subset of MDV entry clones (27 genes) were subcloned into the LUMIER Renilla and protein A-tagged vectors pcDNA-Renilla and pT-REx-A. Both vectors are expression vectors with an ampicillin resistance. The Renilla-tagged clones were confirmed by a double restriction digest using XhoI and XbaI restriction enzymes, and the protein A-tagged clones were confirmed by a double restriction digest using XhoI and NheI restriction enzymes (Figure 3.2.4b).

**c. Plasmids for eukaryotic transient-expression**

In order to identify MDV protein(s) which inhibit interferon-alpha signalling, the MDV entry clones were subcloned into the non-tagged mammalian expression vector pCR3. pCR3 expression vector has two selective marker cassettes, ampicillin and kanamycin resistant cassettes. The expression clones were confirmed by a double restriction digest using XbaI and HindIII restriction enzymes (Figure 3.2.4c). All of the genes, which were subcloned into the pDONR 207 vector as a full length, were successfully cloned into pCR3 vector. Additionally, to investigate the expression of some MDV genes by immunoblotting, five MDV entry clones were successfully subcloned into HA N-terminal tagged pCR3 expression vector. These genes were Meq, LMeq, UL50, UL26 and UL12.

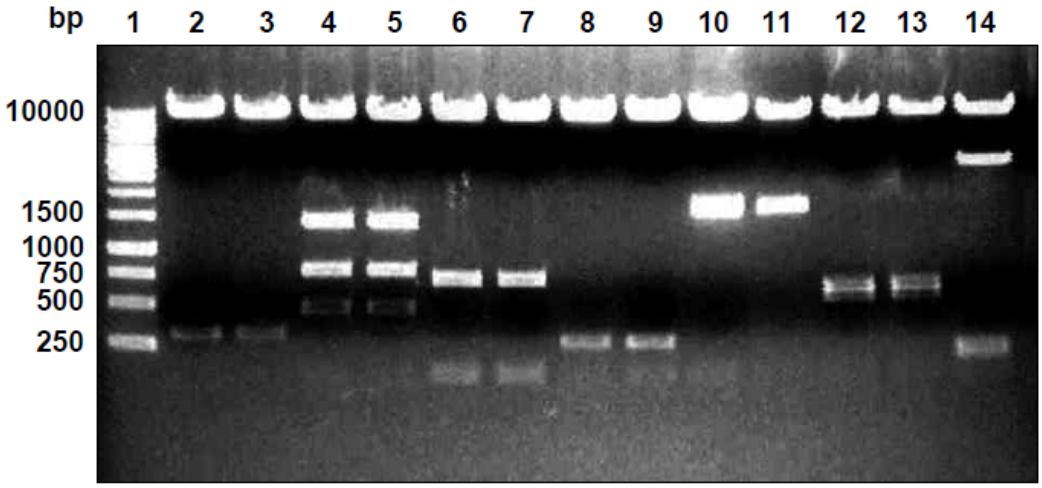



**Figure 3.2.4a: EcoRI and BamHI digests of different ORF clones in the prey vector.** Scanned image of an ethidium bromide stained 1% TAE agarose gel showing a subset of cloned genes in pGADT7-DEST digested with EcoRI and BamHI. Lane 1 1 Kbp DNA ladder (Fermentas), lane 2 and 3 the extra cellular domain of US7, lane 4 and 5 UL9, lane6 and 7 SORF4, lane 8 and 9 RLORF5, lane 10 and 11 UL44, lane 12 and 13 UL40 and lane14 UL37.

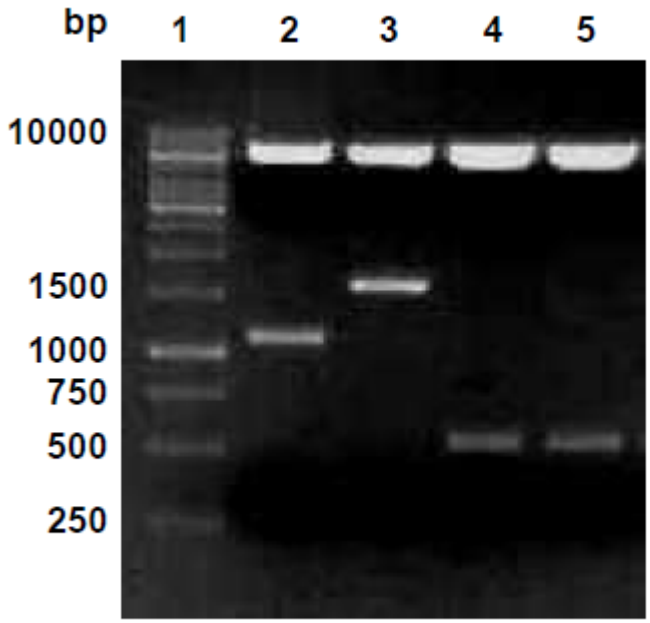



**Figure 3.2.4b: XhoI and XbaI digests of different ORF clones in the pcDNA-Renilla vector.** Scanned image of ethidium bromide stained 1% TAE agarose gel shows the cloned MDV genes in pcDNA-Renilla digested with XhoI and XbaI, Lane 1 1kbp DNA ladder (Fermantus), lane 2 Meq, lane 3 UL44 and lane 4 & 5 vIL8.

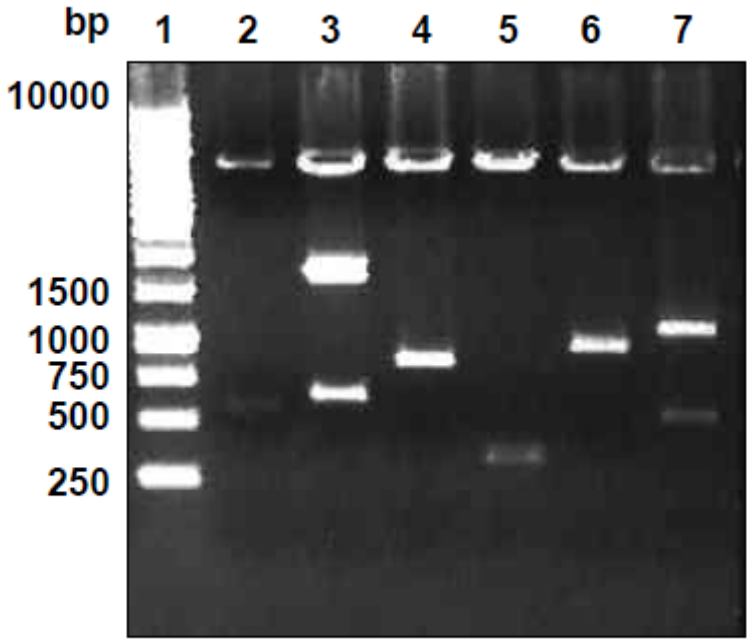



**Figure 3.2.4c: XbaI and HindIII digests of different ORF clones in the pCR3 vector.** This scanned image of an agarose gel stained with ethidium bromide shows the cloned MDV genes in pCR3 digested with XbaI and HindIII, Lane 1 1kbp DNA ladder (Fermantus), lane 2 RLORF8, lane 3 UL15, lane 4 UL3, lane 6 LORF9, lane 7 US8, lane 8 UL40 and lane 9 UL9.

## 3.3 Discussion

### 3.3.1 The importance of a functional analysis of MDV proteins

To achieve a better understanding of the biological role of individual viral proteins, the general objective of this thesis was to functionally characterise all MDV proteins by intraviral protein interactions and by their ability to suppress innate immune responses.

### 3.3.2 Choice of MDV genes, domains and templates

In order to study the function of each of the MDV genes, all ORFs in the viral genome had to be amplified and subcloned first. Two different MDV strains were used as templates for amplifying the viral ORFs. The first template was derived from the non-virulent, low-oncogenic and vaccine CVI988 strain, and the second from the very virulent and highly oncogenic RB-1B strain (both templates were kindly provided by Dr Venu Gobal Nair). Both strains belong to MDV serotype1 and share >90% sequence similarity. While previous studies have predicted MDV to encode more than 330 ORFs (Tulman et al., 2000; Spatz et al., 2007b), only 109 of these seem to be functional genes and of significance for genomic comparison between the pathotype (Spatz et al., 2007b). To avoid analysing short ORFs which may not expressed during MDV infection it has been decided to focus on the 109 functional genes. Comparative sequence analysis between the two strains revealed that most of the ORFs are identical in sequence composition. However, a small number of ORFs differed between the two strains. One example is RLORF7 (called Meq in the RB-1B strain and Long Meq in the CVI988 strain), which in the CVI988 contains an additional insertion of about 180 nucleotides. Moreover, some genes seem to be frame-shifted in the RB-1B strain due to deletion or insertion of one nucleotide, which subsequently changes the frame of the protein. This was observed for LORF1, UL13, UL44, US6, RLORF5a and SORF1. All the genes, which were identical between the two strains (identity> 98%), were cloned only from one of the strains. The genes that differed in sequence composition were cloned from the both the CVI988 and the RB-1B strains.

In the yeast-two-hybrid system both the bait and prey proteins have to be transported into the nucleus in order to transactivate the GAL4 promoter. However, proteins containing transmembrane domains may prevent the prey or bait proteins from reaching the nucleus, giving a higher degree of false negative results (Van Criekinge and Beyaert, 1999). To overcome this problem, all full length open reading frames were analysed for the presence of transmembrane domains using the TMHMM Server v. 2.0. Open reading frames predicted to contain a transmembrane domain were cloned both as full length protein and as fragments. Although the specificity and sensitivity of the prediction software are not 100%, it was still able to identify all known MDV glycoproteins with the exception of (UL1) or gL. Moreover, the server predicted some ORFs with unknown function to have transmembrane domains, i.e. RLORF3 and LORF6. This could be attributed to wrong prediction from the server. On the other hand, these ORFs function could be involved in the signalling process or act as glycoproteins.

### 3.3.3 Gene amplification and cloning

From the 125 functional genes from both strains, a total of 122 functional genes and 25 fragments from membrane-associated genes, were amplified from the viral templates. All gene products were amplified by nested PCR and analysed by gel electrophoresis on a 1% TAE agarose gel to verify the correct size of the gene product. Proof reading polymerase was used in the amplification process to overcome the problem of mutations associated with using the ordinary Taq polymerase. The annealing temperature was initially set to be 55˚C, since lower temperatures could increase the chance of unspecific binding of the primers to the template resulting in non-specific PCR product. On the other hand, much higher annealing temperatures could prevent the hybridisation between the primers and the template. For the genes which were not amplified with the initial setup, other annealing temperatures, templates and PCR conditions were tested until a specific gene product could be obtained. However, three genes were not amplified, despite experimenting with a variety of different conditions (RLORF1, LORF1, vTR). As the primers of these genes contain a high ratio of G+C nucleotides, it is possible that

the primers form secondary structure that resist the denaturation, and subsequently inhibit the annealing of the primers.

Most of the 122 functional MDV genes were cloned as full-length proteins or at least as fragments into pDONR 207 with the exception of UL28, UL39 and LORF11. Although the amplification of these three genes resulted in good PCR products, no positive colonies were received. The lengths of these ORFs (>2500 bp) suggests that the length of the gene to be cloned is one of the restrictive factors in recombinatorial cloning. On the other hand, several other genes which are longer have been successfully cloned into pDONR 207 such as UL19 (4182 bp). Also, the composition of the ORF (internal sequences with homology to att sites) could play a role when ORFs cannot be cloned.

### 3.3.4 Gene sequencing

All cloned ORFs were sequenced from the both ends using pDONR 207 forward and reverse primers. The sequencing result revealed that about 90% of the clones had the correct sequence and the correct reading frame. The remaining 10% of the clones had mutations in different regions. Some of the genes contained insertion or deletion mutation in the primer region resulting in frame shifts. The mutated genes were recloned and sequenced until the complete library was correct. Moreover, the sequencing revealed that the spliced Lipase gene (amplified from cDNA) still contained its intron. To solve this problem, the Lipase ORF was reamplified from a cDNA phage library and was subsequently cloned in the DONR vector. Unfortunately, the sequencing result was the same. This problem could be due to a DNA contamination of the template or due to the Lipase gene not being spliced in RB-1B strain. The later explanation is less likely due to the fact that the Lipase gene is highly conserved among the three serotypes of MDV (Tulman et al., 2000; Afonso et al., 2001; Izumiya et al., 2001). In total, 118 MDV genes from both CVI988 and RB-1B strains were cloned using the Gateway® system. This clone collection can now be used for a variety of purposes, including recombinant protein expression and functional assays.

# Genome-wide analysis for intraviral protein-protein interactions of MDV

## 4.1 Introduction

Herpesviruses have a complex structure, composed of nucleocapsid, tegument and outer envelope. Because of this complex structure, the process of herpesvirus replication is divided into several steps. Production of new viral particles starts with nucleic acid replication in the nucleus and nucleocapsid formation, followed by the egress of the capsid from the nucleus to the cytoplasm. There, tegument formation takes place with subsequent secondary envelopment, transport through secretary vesicles, virus budding and release. These processes depend mainly on a group of conserved interactions between different virion proteins. Genome-wide analysis for intraviral protein-protein interaction of herpesviruses had been investigated before for many mammalian herpesviruses such as herpes simplex virus-1 (HSV-1), Epstein-Barr virus (EBV), murine cytomegalovirus (mCMV) and Kaposi's sarcoma associated herpesvirus (KSHV) (Uetz et al., 2006a; Calderwood et al., 2007; Rozen et al., 2008; Fossum et al., 2009). However, there is very little information available on intraviral protein-protein interactions of avian herpesviruses. This chapter presents the results of a Y2H screen for intraviral protein interactions in MDV. The interactions detected will be discussed in terms of their role in MDV replication and biology. Particularly interactions conserved between MDV and other members of herpesviridae will be analysed and discussed in more detail.

### 4.1.1 Role of protein-protein interactions during herpesvirus replication

#### a. Role of protein-protein interactions in DNA replication

Herpesviruses encode two distinct polymerase subunits which are termed UL30 and UL42 in HSV-1. The two polymerase subunits bind together and form a complex which is required for viral DNA replication (Gottlieb and Challberg, 1994; Loregian et al., 2006). The biological relevance of this interaction has been studied in HSV-1

and equine herpesvirus (Gottlieb and Challberg, 1994; Loregian et al., 2006). In these viruses the interaction with UL42 increases the affinity of UL30 to nucleic acid and enhances its processivity.

### b. Role of protein-protein interactions in capsid egress from the nucleus

Once the production of new progeny viral genomes is completed by rolling circle replication, the formation of nucleocapsid starts. Subsequently, the assembled nucleocapsid is released from the nucleus to the cytoplasm (nuclear egress), where the tegumentation process occurs. UL31 encodes a nuclear and UL34 a membrane-associated phosphoprotein. The two proteins form a complex at the nuclear membrane of herpesvirus infected cells (Reynolds et al., 2001; Yamauchi et al., 2001; Fuchs et al., 2002c). This interaction has been identified in several members of herpesviridae, including Herpes simplex virus type I (HSV-1), Epstein-Barr virus (EBV), pseudorabies virus (PrV) and murine cytomegalovirus (CMV) (Ye and Roizman, 2000; Fuchs et al., 2002b; Reynolds et al., 2002; Lake and Hutt-Fletcher, 2004; Liang and Baines, 2005; Lotzerich et al., 2006). The interaction between UL31 and UL34 is thought to play a role in the egress of primary nucleocapsids from the nucleus to the cytoplasm. The amino acids 137 to 181 of HSV-1 UL34 are essential for its interaction with HSV-1 UL31 (Liang and Baines, 2005), whereas the amino acids 56 and 57 of M50, the UL34 homologue of mCMV, are considered responsible for its interaction with M53 (Bubeck et al., 2004). Orthologs of UL31 in the herpesviridae have an N-terminal variable region and four conserved regions (CR1-4). A protein complementation assay study revealed that the conserved region-1 (CR-1) of UL31 in various herpesviruses contains the specific binding site for UL34 proteins (Lotzerich et al., 2006; Schnee et al., 2006).

### c. Role of protein-protein interactions in tegumentation

Once the nucleocapsid has been transported to the cytoplasm, the tegument proteins start assembling around the nucleocapsids in a process called tegumentation. Until now the complete mechanism of herpesvirus tegumentation has not been determined,

but it mainly happens in the cytoplasm of virus-infected cells (Miranda-Saksena et al., 2002). However, several studies focusing on intraviral protein interaction identified a number of interactions occurring between different tegument proteins, in addition to interactions between tegument and capsid proteins or glycoproteins. For instance, the major and minor tegument protein of herpesviridae, UL36 and UL37 in HSV-1, physically bind together (Klupp et al., 2002; Vittone et al., 2005; Desai et al., 2008). This interaction is functionally relevant, as it contributes to virus assembly. For example, in PrV the UL36-UL37 interaction is considered important for early capsid tegumentation and secondary envelopment, although it is not essential for the latter (Klupp et al., 2002; Fuchs et al., 2004). Additionally, in HSV-1 localisation of UL37 at the cytoplasmic budding sites of Golgi complex depends mainly on its binding to UL36 (Desai et al., 2008).

UL11 is a membrane-associated tegument protein conserved in many different herpesviruses (Kopp et al., 2003). This protein is important for tegumentation and secondary envelopment. UL11 protein interacts with another tegument protein, UL16, during nucleocapsid tegumentation. Interestingly, UL11 of MDV, PrV and HSV-1 have been shown to interact with HSV-UL16, expressed from infected Vero cells, indicating that this interaction is conserved (Loomis et al., 2003; Vittone et al., 2005).

UL46, UL47, UL48 and UL49 are tegument proteins, which encode VP11/12, VP13/14, VP16 and VP22, respectively, in HSV-1 and other herpesviruses (McLean et al., 1990; Zhang and McKnight, 1993; Elliott et al., 1995). VP11/12 was recently was shown to be associated with both cellular membranes and viral capsids (Murphy et al., 2008). This association provides a very helpful model for the dynamic processes of nucleocapsid tegumentation and secondary envelopment (Murphy et al., 2008). It can co-localize with VP16 and VP22 in the cytoplasm, particularly at perinuclear regions (Kato et al., 2000; Murphy et al., 2008). This co-localisation suggests that both proteins together participate in tegumentation (Kato et al., 2000). VP16 (UL48) is a tegument protein, which has both a structural role and a

transregulatory role. The structural role is the involvement in the assembly of virion tegument; whereas the regulatory is the transcriptional activation of the other immediate early genes (see Section 1.3). Interestingly, both roles of VP16 are regulated by its interaction with other tegument proteins. The interaction of VP16 with VP13/14 (UL47) is required for transcriptional activation, as VP13/14 targets VP16 to the nucleus at an early stage of virus infection where VP16 starts the transregulation (Donnelly and Elliott, 2001). In addition, VP16 physically binds to VP22, which is required for virion tegumentation and the packaging of VP22 into the tegument (Elliott et al., 1995; Miranda-Saksena et al., 2002; Hafezi et al., 2005; Vittone et al., 2005; O'Regan et al., 2007; Taddeo et al., 2007).

Not only interactions between tegument proteins are required for tegumentation, but also interactions between tegument and glycoproteins. The first evidence for the presence of an interaction between glycoproteins and tegument proteins was published by Zhu and Courtney, who found by chemical crosslinking that gB, gD and gH of HSV-1 bind to the tegument complex VP16, VP11/12, VP13/14 and VP22 (Zhu and Courtney, 1994). Later, many other interactions have been discovered between tegument proteins and glycoproteins in other herpesviruses, such as bovine herpesvirus -1 (BoHV-1) and PrV (Fuchs et al., 2002a; Chi et al., 2005; Farnsworth et al., 2007; O'Regan et al., 2007; Kalthoff et al., 2008). Interestingly, PrV mutants lacking both glycoproteins E and M failed to incorporate UL49 into the tegument, highlighting the significance of glycoproteins for tegumentation (Fuchs et al., 2002a).

### d. Role of protein-protein interactions in secondary envelopment

The secondary envelopment is the process by which virus particles acquire their final lipoprotein layer by budding through a cellular membrane in the Golgi or trans-Golgi network (TGN) compartments (Miranda-Saksena et al., 2002). Several interactions between different glycoproteins and tegument proteins contribute significally to the secondary envelopment process, and many of them have been studied in more detail.

For example, the interactions of UL11 with different glycoproteins, such as gM, gE and gD, are considered to be important for the secondary envelopment of herpesviruses. These interactions mainly exist in alpha-herpesviruses, such as HSV-1 and PrV (Kopp et al., 2003; Kopp et al., 2004; Farnsworth et al., 2007; Leege et al., 2009). However, there is also some evidence that PP28, the UL11 homologue in human cytomegalovirus (beta-herpesvirus), is involved in secondary envelopment (Seo and Britt, 2007). By electron microscopy it has been shown that in cells infected with PrV lacking UL11, a large number of tegumented nucleocapsid can be found in the cytoplasm (Kopp et al., 2003). PrV and HSV-1 double deletion mutants lacking both UL11 and gM have lower virus titres than the wild-type virus and also show a marked decrease of the plaque size (Kopp et al., 2003; Leege et al., 2009). Furthermore, infection with these mutants results in cells with a large number of tegumented nucleocapsids accumulated in the cytoplasm indicating that the presence of both UL11 and gM is essential for the final virion envelopment (Kopp et al., 2003; Kopp et al., 2004; Leege et al., 2009).

### e. Role of protein-protein interactions in virus cell-to-cell spread

Interactions between herpesviral glycoproteins are not only crucial for the secondary envelopment process, but also for virus entry and cell-to-cell spread (syncytium formation). The four glycoproteins gD, gB, gH, gL form a complex together, which is essential for HSV-1 and HSV-2 entry and virus-envelope cell-membrane fusion (Muggeridge, 2000; Browne et al., 2001; Atanasiu et al., 2007; Gianni et al., 2007). Interestingly, the presence of these 4 glycoproteins in the same cell membrane is enough to produce cell fusion and syncythium formation in both viruses (Turner et al., 1998; Muggeridge, 2000; Browne et al., 2001; Atanasiu et al., 2007).

In several members of the alpha-herpesvirinae, glycoproteins E and I form a heterodimer complex. The presence of this complex is essential for cell-to-cell spread of the virus between host cells. For example, gE of Feline herpesvirus (FHV) can only act as a fusion protein in the presence of gI (Mijnes et al., 1996). In addition, a gE/gI negative mutant of HSV-1 can not spread efficiently from neuron to

neuron in rat retina, indicating the importance of this interaction for cell-to-cell spread (Dingwell et al., 1995).

### 4.1.2 Role of protein-protein interactions in the life cycle of MDV

There is not much information available on intraviral protein-protein interactions between MDV proteins. Only a few reports have identified a limited number of interactions between MDV viral proteins or with cellular proteins. One such example is the interaction between glycoproteins H and L of MDV which can form a hetero-oligomer complex. The presence of both glycoproteins is required for their expression and translocation from the endoplasmic reticulum (ER) lumen to the cell surface (Wu et al., 2001a). Therefore, the formation of this complex may be essential for MDV entry into cells and virus spread from cell to cell. In addition, UL11 of MDV is able to interact with HSV-1 UL16 (Loomis et al., 2003). Thus, it is possible that both UL11 and UL16 are structurally important in the process of tegumentation. PP38 and PP24, the two unique phosphoproteins of MDV, form a complex together (Ding et al., 2007; Ding et al., 2008). The presence of both proteins is important for the transactivation of the bidirectional promoter which is located between the PP24/PP38 gene and the 1.8 kb mRNA transcripts (Ding et al., 2007).

Meq, the major oncoprotein of MDV, is composed of an N-terminal basic leucine zipper domain and a C-terminal proline-rich transactivation and transrepression domain. The Meq gene encodes many different transcripts, such as Meq, Meq/vIL8 and ΔMeq (Peng and Shirazi, 1996; Anobile et al., 2006; Okada et al., 2007). Meq either forms a homodimer with itself or a heterodimer with the transcriptional factor c-Jun (Qian et al., 1995). The Meq/c-Jun heterodimer leads to an increased transactional activation of the Meq promoter in comparison to Meq homodimers (Qian et al., 1995). The transcriptional activation of the Meq promoter is mediated through the binding of these dimers to a AP-1-like motif present in the Meq promoter (Qian et al., 1995). Furthermore, transcriptional activation by the Meq/c-jun heterodimer is required for MDV replication and/or establishment and reactivation of the virus from the latency.

Meq can also interact with the 70 kilodalton heat shock protein (hsp70), however there is no well-defined role for this interaction yet (Zhao et al., 2009). It is possible that the Meq/hsp70 complex acts as a regulator of the cell cycle. Additionally, Meq interacts with the C-terminal binding protein (CtBp), and this interaction is functionally relevant (Brown et al., 2006). The interaction between Meq and CtBp is strongly correlated with Meq transrepression activity, and the inhibition of this interaction results in diminishing its inhibitory activity (Brown et al., 2006). Abolishing the interaction between Meq and CtBp in the RB-1B MDV virus results in a complete loss of oncogenicity and the ability to induce lymphoma in birds (Brown et al., 2006).

FRET studies have shown that Meq/vIL8 homodimers form in the nucleolus, nucleoplasm and cajal bodies of transfected CEF cells (Anobile et al., 2006). However, the definite role of this homodimer complex has not been elucidated yet. Meq and Meq/vIL8 can also form a heterodimeric complexes in vitro, which could have an effect on its regulatory function or its binding partners (Peng and Shirazi, 1996). Similar to Meq, Meq/vIL8 is able to interact with c-Jun (Peng and Shirazi, 1996). By gel shift assays it has been shown that the presence of Meq/vIL8 enhanced the DNA binding activity for the AP-1 recognition sequence of c-jun (Peng and Shirazi, 1996). However, the presence of Meq and Meq/vIL8 together does not have any effect on the AP-1 binding activity. These data suggest that Meq/vIL8 acts as a competitor for its dimerisation partners.

ΔMeq is another transcript derived from the Meq genomic sequence and composed of the N-terminal 98 amino acids of Meq and 30 aa derived from a different reading frame (Okada et al., 2007). ΔMeq inhibits the transactivation of the Meq and IL-2 promoters, which is induced by Meq and L-Meq. This negative regulatory effect of ΔMeq is through its direct physical binding with both Meq and L-Meq (Okada et al., 2007).

### 4.1.3 The Yeast-two-hybrid system (Y2H)

The yeast-two-hybrid system is an important method for studying protein-protein interactions, particularly for screening large number of interactions. It was first described by Fields and Songs as a novel system for studying protein-protein interactions by taking advantage of the properties of the GAL4 transactivator of the yeast Saccharomyces cerevisiae. This protein is a transcriptional activator, composed of two domains: an N-terminal domain which binds to specific DNA sequences and a C-terminal domain which is necessary to activate transcription. Fields and Songs generated a system of two hybrid proteins containing parts of GAL4: the GAL4 DNA-binding domain fused to a protein 'X' and a GAL4 activating region fused to a protein 'Y'. If X and Y interact, they will reconstitute a transcriptional activator and initiate transcription of an auxotrophy gene (Fields and Song, 1989) (Figure 4.1.3). Several Y2H screens in the recent years have identified very important interactions which have been crucial in understanding virus pathogenesis. For example, in IBDV (infectious bursal disease virus) the structural capsid protein (VP3) was observed to be the key organizer of Birnavirus structure, as it maintains critical interactions with all components of the viral particles: itself, VP2, VP1, and the two genomic dsRNAs (Tacken et al., 2002). Moreover, the crucial role of Nsp8 in the replication of the Sars coronavirus was shown using the Y2H system (von Brunn et al., 2007). Using the Y2H system, 123 intraviral protein interactions have been identified in KSHV, and 173 in VZV (Uetz et al., 2006b). The Y2H assay has previously been used to identify some interactions between MDV proteins and their cellular counterpart, such as the interaction between the chicken growth hormone and SORF2 (Liu et al., 2001), between US10 and stem cell antigen (LY6E) (Liu et al., 2003), between RLORF8 and CIQ binding protein, RLORF10 and MHC class II invariant chain, RLORF12 and growth related transciptionally related protein, RLORF13 and CIQ binding protein and LORF4 with MHC class II chain (Niikura et al., 2004).

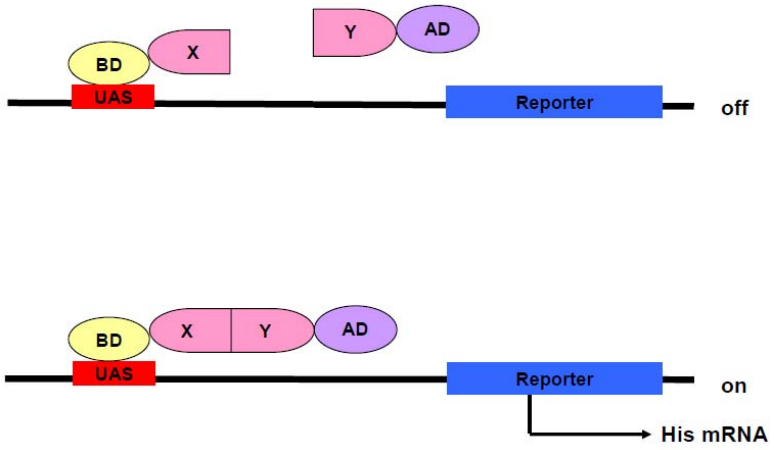



**Figure 4.1.3: The yeast-two-hybrid system.** The GAL4 DNA-binding domain (DB) fused to a protein of interest 'X' (named as bait protein) and a GAL4 activating domain (AD) fused to a protein of interest 'Y' (prey protein). If X and Y can interact together, they will reconstitute the GAL4 domain and initiate transcription of a gene under regulation of the up-stream activation sequence (UAS) (Fields and Song, 1989).

### 4.1.4 Aim

MDV causes an immunosuppressive lymphotropic disease in poultry, which severely affects health and welfare and causes great economic devastation in lifestock industry. It encodes more than 100 ORFs, a large percentage of which are only poorly characterised. The objective of this study was to provide a clearer and better understanding of the role of individual MDV proteins in virus replication and pathogenicity by studying their interactions with other MDV proteins. Based on the Y2H data, a MDV interactome map should be generated and positive Y2H interactions verified by a secondary biochemical assay.

## 4.2. Results

### 4.2.1 Identification of intraviral protein-protein interactions in MDV

In order to analyse physical interactions occurring between MDV proteins, MDV bait and prey clones (see Chapter 3) were transformed into yeast. Two different yeast strains were used to generate Y2H prey and bait arrays, AH109 (a mating type) for the preys and Y187 (α mating type) for the baits. The MDV clones collection was composed of 149 DNA constructs from MDV genes cloned as both pGADT7 (prey) and pGBKT7 (bait). This clone collection contains MDV genes which have been cloned as full-length proteins, fragments and extra- and intracellular domains for membrane-associated proteins. The transformed prey clones were grown on plates of single drop-out media lacking leucine and the transformed bait clones were grown on plates of single drop-out media lacking tryptophan. As both vectors have the appropriate cassettes of selectivity which make the transformed yeast grow on the selective plate lacking suitable amino acid. The transformed yeast clones were tested in collaboration with Dr. Peter Uetz and Thorsten Stellberger at the Institute of Toxicology and Genetics, Karlsruhe, Germany, in a robot-assisted Y2H assay in 96-well plate format. In a matrix analysis, all proteins were tested against each other. Since the Y2H assay can generate a number of false positive interactions, each pairwise interaction was tested in duplicates. Interactions in the Y2H screen were scored positive, if two of the two mating were positive. If only one replicate was positive, the interaction was scored non-reproducible and negative. The positive results from the Y2H array were visualised and analysed using the Cytoscape bioinformatics software.

From the list of positives, the interactions with 5 preys (UL33, UL35, UL37 of CVI988 strain, UL37 of RB-1B strain, SORF4 and the extracellular domain of B68) were removed due to their high prey count (above 30 interactions) which suggests that these preys are sticky and the interactions false positive. Of more than 20.000 tested interactions between the MDV proteins, 435 interactions were identified by

the Y2H screen (Figure 4.2.1). The full lists of positive interactions are listed in Appendix D.

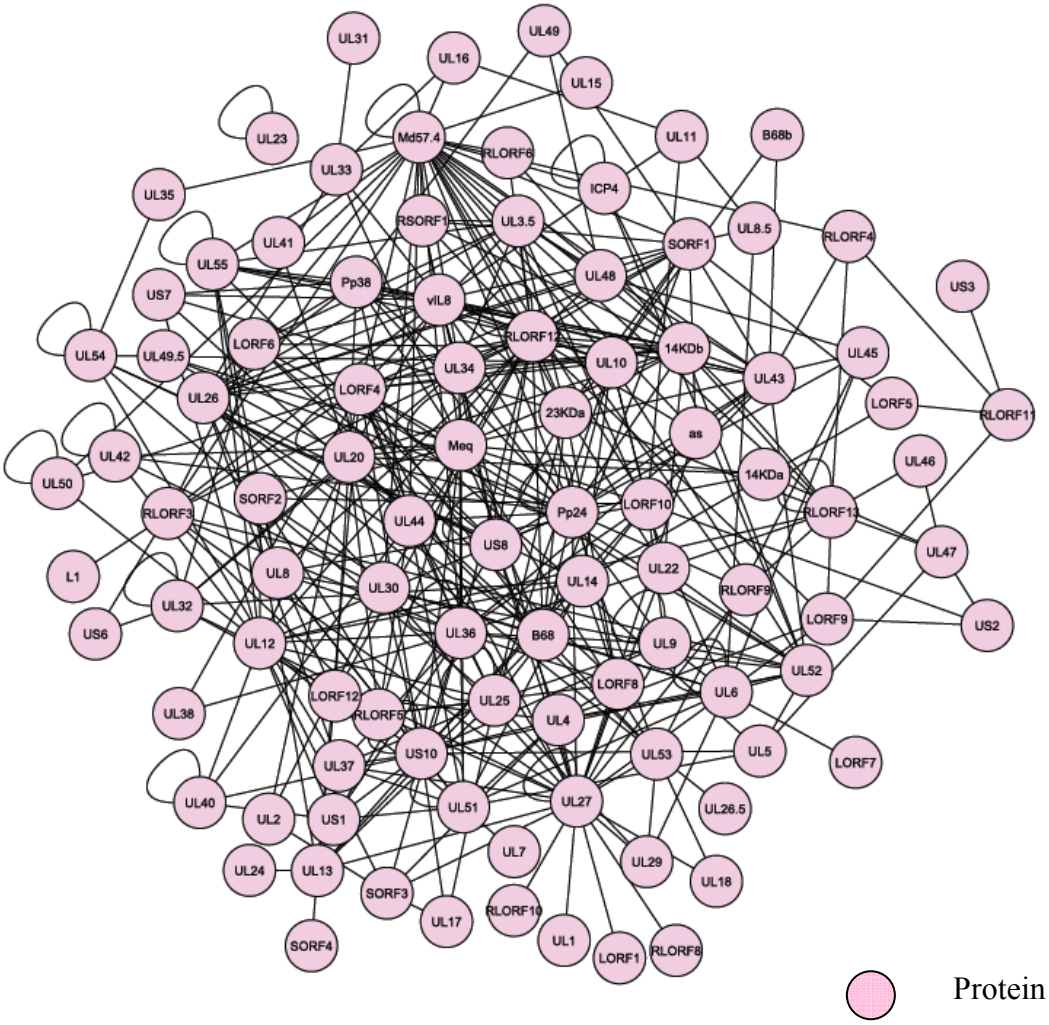



**Figure 4.2.1: MDV protein-protein interaction network.** MDV proteins, indicated as nodes, were tested against each other, using Y2H system. The MDV Y2H screen identified 435 interactions, indicated as edges, between MDV proteins. The results were analysed and visualised using Cytoscape, in order to construct an intraviral MDV interactome.

### 4.2.2 Network analysis of MDV protein-protein interaction interactome

The MDV network consists of 115 nodes (proteins) and 435 edges (interactions) and has a short characteristic path length of 2.68 (the characteristic path length is defined as the average number of edges between 2 random nodes in a network) and a small network diameter of 6 (Table 4.2.2).

| Network Parameters | MDV |
|---|---|
| Protein | 115 |
| Interactions | 435 |
| Interactions (without self-interactions) | 413 |
| Average degree | 7.57 |
| Average degree (without self-interactions) | 7.18 |
| Power coefficient | -0.94 |
| R2 | 0.80 |
| Characteristic path length | 2.68 |
| Diameter | 6 |
| Clustering coefficient | 0.25 |
| Enrichment over ER | 3.73 |
| Enrichment over ES | 1.34 |

**Table 4.2.2: Statistical analysis for the intraviral MDV interactome**
Average degree is the average number of interactions between two proteins in the network. The degree of a node in a network represents the number of edges connecting to that node. Nodes with the highest degrees are usually termed hubs. The diameter of a network is the maximum distance between any two nodes. Cellular protein-protein interaction network is a scale-free network with a degree distribution follows power-law decay:

$$P(k) \sim k^{-\gamma}$$

P (k) describes the number of proteins in the network which has a given degree (number of interactions), while γ indicates a coefficient which describes the decline of the graph. Since the MDV network is considered a small network, it can not be conclusively determined whether it follows the power-law distribution or not. The clustering coefficient was calculated according to the Watts and Strogatz model (Watts and Strogatz, 1998). For each protein, it is calculated as the number of interactions between neighbours of this protein divided by the number of possible interactions between these neighbours. The clustering coefficient of this network is calculated as the average clustering coefficient of proteins with at least two neighbours. The statistical network analysis was done by Caroline Friedel, Institute of Informatics, University of Munich, Germany.

### 4.2.3 Validation of MDV protein interactions by biochemical assays

#### a. Co-immunoprecipitation (Co-IP)

To further confirm the reliability of the MDV interactome, a subset of 37 interactions between MDV proteins were selected to be tested by co-immunoprecipitation (Co-IP). The Y2H bait and prey vectors were used to express the selected ORFs as Myc or HA N-terminal fusion protein under control of the T7 bacteriophage promoter. The bait and prey vectors of interacting proteins were transfected into quail muscle-7 (QM7) cells. Simultaneously, cells were infected with recombinant Vaccinia virus expressing T7 polymerase. Cell lysates were split into two parts and precipitated with either anti-c-Myc or anti-HA antibody. Each precipitate was separated on two polyacrylamide gels. On the left side of each gel the precipitating proteins and on the right side the co-precipitating proteins are indicated (Figure 4.2.3a). The direct precipitations were performed to control the expression levels and the correct size of the tested proteins. Out of the 37 viral protein-protein interactions identified with the Y2H-system, 27 interactions were also detected by Co-IP, which represent approximately 73% of Y2H positive (Figure 4.2.3b).

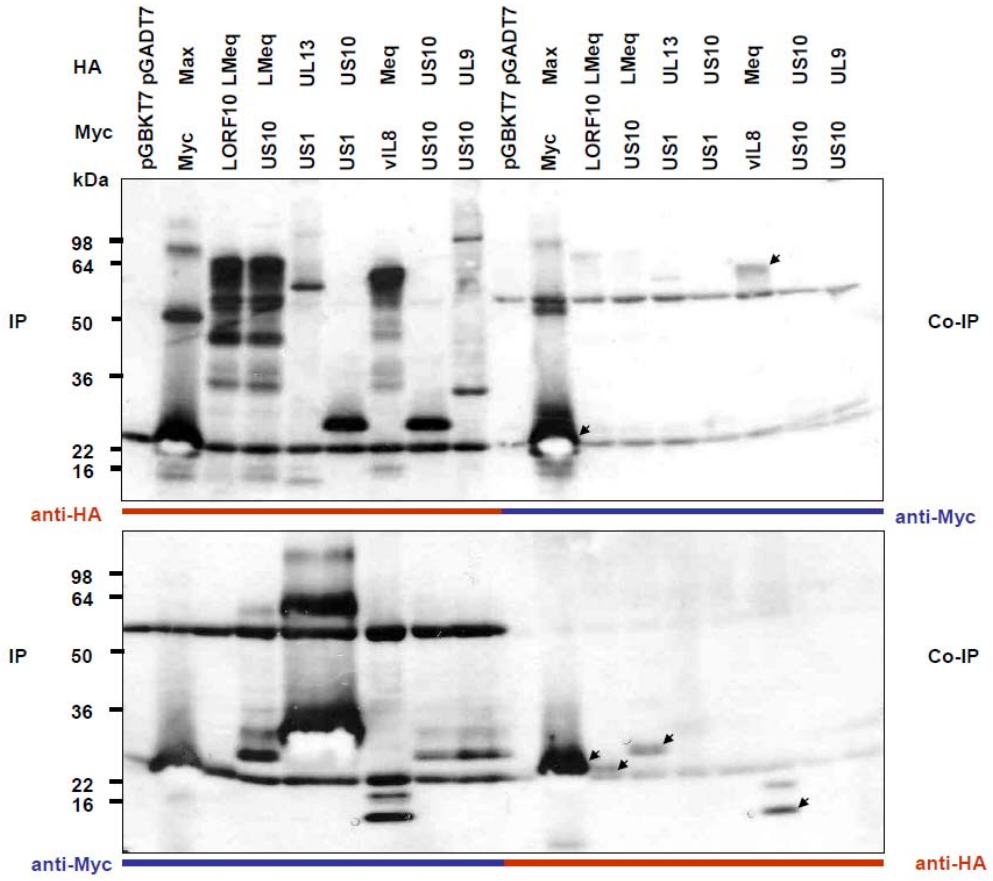



**Figure 4.2.3a: Validation of MDV protein interactions by Co-IP.** QM7 cells were infected with Vaccinia virus expressing T7 polymerase. One hour later, the cells were co-transfected with pGBKT7-LORF10 and pGADT7-LMeq, pGBKT7-US10 and pGADT7-LMeq, pGBKT7-US1 and pGADT7-UL13, pGBKT7-US1 and pGADT7-US10, pGBKT7-vIL8 and pGADT7-Meq, pGBKT7-US10 and pGADT7-US10 or pGBKT7-US10 and pGADT7-UL9. Twenty four hours post transfection, the whole- cell extracts were lysed and precipitated with either anti-c-Myc or anti-HA antibody. The precipitated samples separated on two polyacrylamide gels. On the left side of each gel the precipitating proteins and on the right side the co-precipitating proteins. pGBKT7 and pGADT7 Plasmids encoding cellular Myc and Max were used as a positive control. Empty pGBKT7 and pGADT7 vectors were used as a negative control. This experiment shows positive physical binding between LMeq with US10 or LORF10 and between Meq with vIL8.

**b. LUMIER pull-down assay**

To further confirm Y2H and Co-IP results, the same subset of interactions, which was tested by Co-IP, was sent to German Cancer Research Centre, Heidelberg, Germany to be assessed by LUMIER pull-down assay as another independent biochemical assay. This work has been done in collaboration with Dr. Manfred Koegl, German Cancer Research Centre, Heidelberg, Germany. The LUMIER pull-down assay is a biochemical assay for investigating protein-protein interactions which is based on an N-terminal Renilla luciferase fusion protein (pcDNA-Renilla), and an N-terminal protein A fusion protein (pT-REx-A). The protein A-tagged and the Renilla-tagged plasmids were transfected in 293T cells. Cells were lysed and the cellular lysates were split into two aliquots. The total luciferase activity was measured for the first aliquot, while the other aliquot were allowed to precipitate with IgG coated magnetic beads. The precipitated protein complexes were measured for luciferase activity, and the bound luciferase activity was calculated relative to the total luciferase activity: log (bound luciferase) / log (10% of total luciferase). Out of the thirty seven interacting partners detected by Y2H, twenty three were validated by LUMIER pull-down assay (approximately 60%) (Table 4.2.3). Of the positive Y2H interactions tested by Co-IP and LUMIER, 20 of 37 interactions were confirmed positive by both assays. Hence, the degree of overlapping was above 50% (Figure 4.2.3b).

**Table 4.2.3: Assessment of MDV interactome by biochemical assays**

| Interaction | | Co-IP | LUMIER | Overlapping |
|---|---|---|---|---|
| **Protein 1** | **Protein 2** | | | |
| UL9 | LORF10 | - | - | - |
| UL9 | US10 | + | - | - |
| UL9 | UL25 | - | + | - |
| UL25 | UL17 | + | + | + |
| UL54 | UL54 | + | + | + |
| LMeq | LORF10 | + | - | - |
| LMeq | US10 | + | + | + |
| US1 | UL13 | - | - | - |
| US1 | US10 | - | - | - |
| US10 | US10 | - | - | - |
| UL13 | US10 | - | + | - |
| UL54 | UL25 | + | + | + |
| UL17 | US10 | + | - | - |
| vIL8 | UL34 | + | + | + |
| vIL8 | UL20 | + | + | + |
| RlORF3 | Md57.4 | + | + | + |
| RlORF3 | vIL8 | - | + | - |
| RLORF3 | RLORF5b | + | - | - |
| PP24 | vIL8 | + | + | + |
| PP38 | UL44 | + | + | + |
| PP38 | UL34 | + | + | + |
| Meq | RLORF5b | + | - | - |
| Meq | PP24 | + | + | + |
| Meq | PP38 | + | + | + |
| Meq | RLORF3 | + | + | + |
| Meq | UL20 | - | - | - |
| Meq | UL53 | - | - | - |
| Meq | 14kDa | - | - | - |
| Meq | vIL8 | + | + | + |
| UL50 | UL12 | + | - | - |
| UL50 | UL50 | + | + | + |
| UL44 | UL25 | + | + | + |
| UL44 | Md57.4 | + | + | + |
| UL34 | Md57.4 | + | + | + |
| UL30 | UL42 | + | - | - |
| UL42 | Meq | + | + | + |
| UL11 | UL16 | + | + | + |
| | | **27** | **23** | **20** |

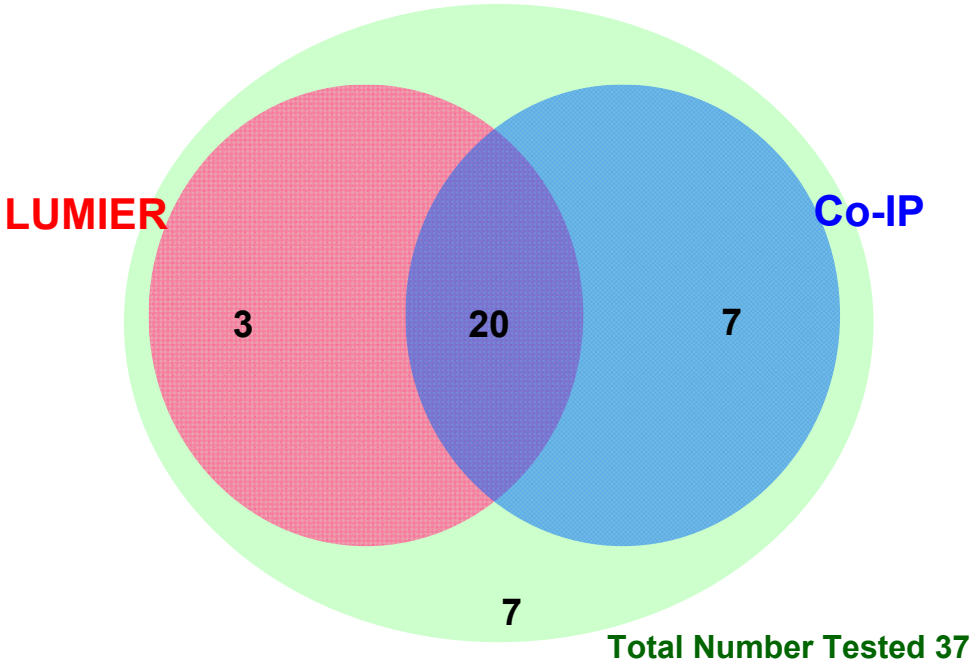



**Figure 4.2.3b: Confirmation of 81% of the Y2H interactions by either Co-IP or LUMIER.** Thirty seven interactions between protein pairs were analysed by two biochemical assays; LUMIER-pull down assay and co-immunoprecipitation. 27 interactions were positive by Co-IP (73%), and 23 interactions were positive by LUMIER (62%). From the 37 interactions, 20 interactions were positive by the both assays (54%), 30 interactions were positive by either of the two assays (81%), and 7 interactions were negative by the both assays (19%).

### 4.2.4 Extraction of biological data from the MDV interactome

#### a. Interactions among structural proteins.

Interactions among structural proteins may help elucidate the details of different steps during MDV replication and morphogenesis, such as DNA replication, capsid formation, DNA packaging, primary envelopment, tegumentation and secondary envelopment. Several of these interactions were observed in the Y2H analysis of MDV. For instance, UL33 (DNA packaging & cleavage protein and capsid-associated protein) was observed to interact with the minor capsid scaffold protein UL26. Three interactions were identified by the Y2H among the minor capsid scaffold protein (UL26) and different tegument proteins such as VP16 (UL48), minor tegument protein (UL37) and tegument protein (UL3.5). Two interactions were identified among minor capsid protein (UL6) and two tegument proteins UL3.5 and UL51. In addition, UL33 interacted with UL16, UL32 (DNA cleavage and packaging

protein) interacted with UL14, and finally the minor capsid protein UL38 was observed to interact with the major tegument protein UL36. Thirteen interactions were detected in the Y2H screen between the different tegument proteins. VP16 (UL48) was found to interact with UL3.5 and VP22 (UL49). Furthermore, the major and the minor tegument proteins (UL36 and UL37) were observed to interact together. In addition, the major tegument protein also interacted with itself. Moreover, both of the major and minor tegument proteins interacted with UL51 and UL25. UL7 interacted with UL51. Many more interactions between the tegument proteins were detected by Y2H as the following; UL11 and UL16, UL51 and UL14, UL17 and UL25, and UL46 and UL47.

Eleven interactions were detected in the Y2H screen between tegument proteins and glycoproteins or membrane-associated proteins. The major tegument protein (UL36) has been found to interact with glycoprotein H (gH) (UL22) and the two membrane-associated proteins UL34 and UL43. The minor tegument protein UL37 was found to interact with gB (UL27). Also, the tegument or the capsid-associated protein UL25 was found to interact with gC (UL44) and gM (UL10). The tegument protein UL3.5 was found to interact with the two membrane-associated proteins UL34 and UL43. The tegument proteins UL14, UL41 and UL51 interacted with gM (UL10), the membrane virus egress protein (UL20) and gC (UL44), respectively. Thirteen interactions were identified by Y2H assay between MDV glycoproteins with each other or with membrane-associated proteins. The glycoprotein B (UL27) interacted with itself, gC of the RB-1B strain, gK (UL53), gE (US8), gL (UL1) and gH (UL22). MDV gE (US8) was found to interact with the two membrane-associated proteins, UL34 and UL43. In addition to interacting with itself, the viral egress protein (UL20) was found to interact with gN (UL49.5) and gK (UL53). Glycoprotein C (UL44) of the CVI988 strain interacted with gI (US7) and gM (UL10) interacted with gH (UL22).

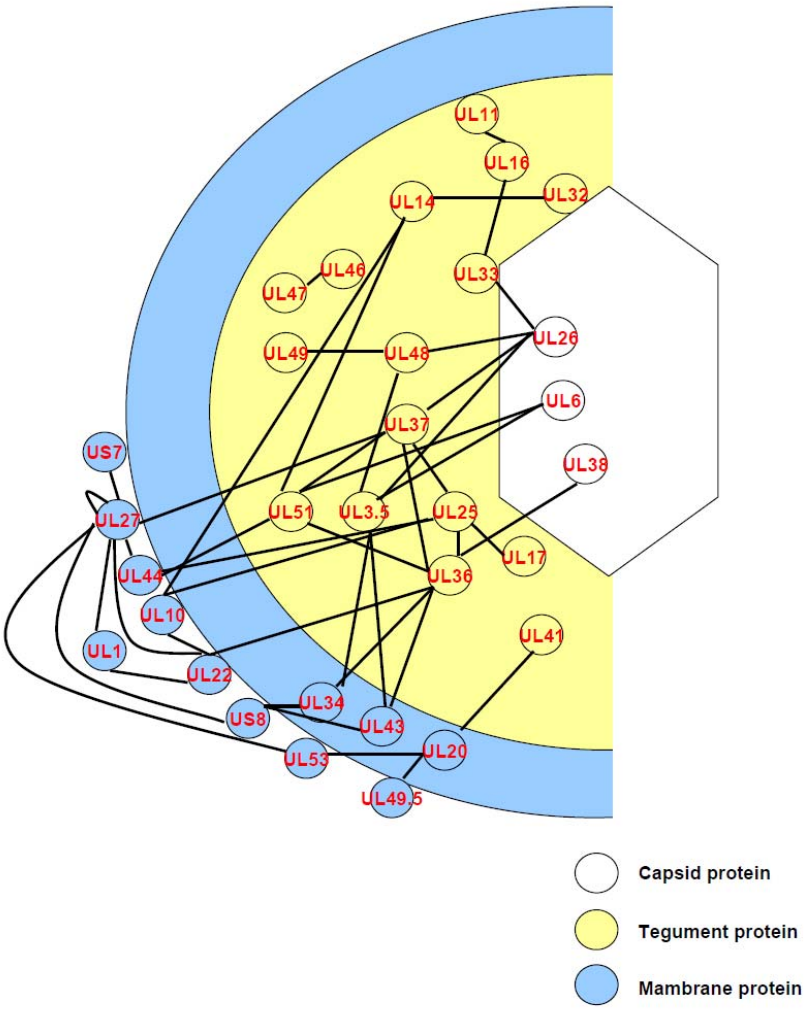



**Figure 4.2.4a: Interactions between MDV structural proteins**. Model illustrates the role of the MDV protein-protein interaction in the virion assembly. This figure illustrates the interaction between capsid or capsid-associated proteins with each other or with other tegument proteins (the tegument labelled by yellow colour). In addition, it indicates the interactions between tegument proteins with each other or with the tegument proteins and membrane-associated proteins (the envelope labelled by faint blue colour) and the interactions between glycoproteins or the membrane-associated proteins with each other.

### b. Protein interactions conserved between MDV and other members of herpesvirus subfamilies

Previous reports have detected a number of conserved interactions between herpesvirus core proteins, using the Y2H system and other assays (Calderwood et al., 2007; Fossum et al., 2009). The fact that MDV encodes 40 core proteins, which have homologues in the 3 subfamilies of herpesviridae, suggests that the MDV interactome could contain several conserved interactions. To assess this, a comparison was carried out between MDV intraviral protein interactions and the intraviral protein interactions of five viruses HSV-1, VZV, mCMV, EBV and KSHV. Those five viruses have been selected because they represent the three herpesviridae subfamilies (α, β and γ) and their intraviral protein interactions were detected using a similar Y2H assay setup as used for the MDV interaction network (Fossum et al., 2009). The intraviral MDV protein-protein interactions were compared to previously published interactions. In this analysis, 22 interactions identified between MDV core proteins were found to be conserved in HSV-1, VZV, mCMV, EBV and KSHV. The highest similarity was between MDV and VZV, with 14 intraviral protein-protein interactions conserved between the two viruses. The biologically more similar virus EBV had 8 conserved interactions with MDV, HSV-1 and mCMV had 5 conserved interactions with MDV, while KSHV only showed 2 conserved interactions (Table 4.2.4b). In comparison to previously published interactions, 16 MDV intraviral protein-protein interactions were overlapping with HSV-1, VZV, mCMV, EBV and KSHV (HSV-1 8, KSHV 8, EBV 6, mCMV 4, VZV 1) (Table 4.2.4b).

## Table 4.2.4b: Comparison of proteins interactions between core proteins

| | | Yeast-two-hybrid | | | | | Literature | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Protein1** | **Protein2** | **HSV-1** | **VZV** | **mCMV** | **EBV** | **KSHV** | **HSV-1** | **VZV** | **mCMV** | **EBV** | **KSHV** |
| UL42 | UL42 | - | + | - | - | - | - | - | + | + | + |
| UL42 | UL30 | - | - | - | - | - | + | - | + | - | + |
| UL42 | UL12 | - | - | - | - | - | - | - | - | + | - |
| UL38 | UL36 | - | - | - | - | - | - | - | - | - | + |
| UL37 | UL27 | - | - | - | + | - | - | - | - | - | - |
| UL37 | UL36 | - | + | - | + | - | + | - | - | - | + |
| UL37 | UL26 | - | + | - | - | - | - | - | - | - | - |
| UL36 | UL36 | - | - | - | + | - | - | - | - | + | + |
| UL36 | UL25 | - | + | + | - | - | - | - | - | - | - |
| UL36 | UL22 | - | - | - | - | - | - | - | - | - | + |
| UL33 | UL31 | - | + | + | + | + | - | - | - | - | - |
| UL33 | UL16 | + | + | - | - | - | - | - | - | - | - |
| UL33 | UL26 | - | + | - | - | - | - | - | - | - | - |
| UL32 | UL32 | - | + | - | + | - | - | - | - | - | - |
| UL32 | UL14 | - | + | - | - | - | - | - | - | - | - |
| UL27 | UL27 | - | - | - | - | - | + | - | - | - | - |
| UL54 | UL54 | + | - | + | - | + | + | - | - | + | + |
| UL53 | UL26 | + | - | - | - | - | - | - | - | - | - |
| UL52 | UL5 | - | - | - | - | - | + | - | + | + | - |
| UL52 | UL8 | - | - | - | - | - | + | - | + | - | - |
| UL51 | UL7 | - | + | - | + | - | - | - | - | - | - |
| UL51 | UL12 | - | - | - | + | - | - | - | - | - | - |
| UL51 | UL51 | - | + | - | - | - | - | - | - | - | - |
| UL51 | UL6 | - | - | - | - | - | - | - | - | + | - |
| UL49 | UL48 | - | - | - | - | - | + | - | - | - | - |
| UL23 | UL23 | - | + | - | - | - | - | - | - | - | + |
| UL25 | UL17 | - | - | + | - | - | - | - | - | - | - |
| UL25 | UL25 | - | + | - | - | - | - | - | - | - | - |
| UL20 | UL20 | - | + | - | - | - | - | - | - | - | - |
| UL16 | UL11 | + | - | + | + | - | + | - | - | - | - |
| UL14 | US10 | + | - | - | - | - | - | - | - | - | - |
| US8 | US8 | - | - | - | - | - | - | + | - | - | - |
| | | **5** | **14** | **5** | **8** | **2** | **8** | **1** | **4** | **6** | **8** |

### c. Meq protein interactions suggest a potential role in virus replication and pathogenesis

Meq is the MDV oncoprotein which is responsible for the transformation of infected cells and which is mainly expressed during latency. vIL8 is a chemokine homologue, and may be responsible for switching the MDV infection from B lymphocytes, in which the lytic replication is established, to T lymphocytes, where the latent infection and transformation occur. PP38 is mainly expressed during lytic infection, and it is suggested that it is responsible for virus reactivation from latency. In this Y2H study, it has been found that these genes, which are responsible for different stages of virus infection, interacted with each other and formed a dense network (Figure 4.2.4c). Meq interacted with itself, PP38, PP24 and vIL8 protein. In addition, vIL8 protein interacted with Meq and PP24. PP38 interacted with itself and its homologues PP24 and Meq. Interestingly, there was also an interaction detected between Meq and the small polymerase subunit UL42 of MDV.

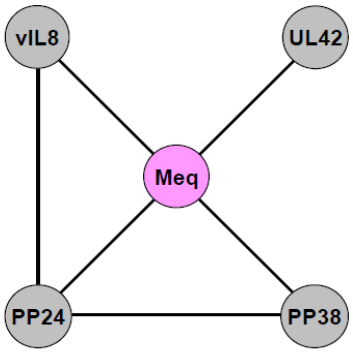



**Figure 4.2.4c: Meq protein interactions suggest a potential role in virus replication and pathogenesis.** Dense network formed between Meq, vIL8, PP38 and PP24 proteins.

## 4.3 Discussion

### 4.3.1 The significance of the identification of intraviral protein interactions in MDV

The main objective of this project has been to evaluate protein-protein interactions between all viral proteins of MDV. By cloning all the MDV ORFs and analysing them against each other in a Y2H assay, a better understanding of the biological role of the individual viral proteins should be obtained. Since most published studies in MDV focused on interactions between viral and cellular proteins (Liu et al., 2001; Liu et al., 2003; Niikura et al., 2004), not much has been known about intraviral interactions. However, several important intraviral interactions have been observed between orthologs in other herpesviral species. For example, the interaction between the two components of the nuclear egress complex, UL31 and UL34, had already been reported in Herpes simplex virus type 1 (HSV-1) (Reynolds et al., 2001), pseudorabies virus (PrV) (Fuchs et al., 2002), murine cytomegalovirus (mCMV) (Lotzerich et al., 2006), equine Herpesvirus 1 (EHV-1) (Neubauer et al., 2002) and Epstein-Barr virus (EBV) (Gonnella et al., 2005).

### 4.3.2 Genome-wide analysis of intraviral protein-protein interactions and identification of conserved interactions within Herpesviridae

Several studies examined intraviral protein-protein interactions as well as their biological and evolutionary roles in herpesviruses, for example in HSV-1, KSHV, EBV, mCMV and VZV (Rozen et al., 2008; Fossum et al., 2009). In this study, 435 interactions between 115 MDV proteins have been identified using the Y2H system. These 115 proteins were represented by 149 DNA constructs and amplified from two MDV strains, the virulent RB-1B and the CVI988 vaccine strain. In the comparison of the MDV interactome with the other herpesviral interactomes, a few conserved interactions were identified. The VZV interactome showed the highest similarity with the MDV interactome, which is consistent with the fact that both VZV and MDV are alpha-Herpesvirinae. Interestingly, the two viruses also share biological

characteristics, as both are strictly cell-associated. In the literature, only one of the MDV interactions had been reported for orthologs in VZV, which could be due to the low number of published interactions for both viruses (Olson et al., 1997). Interestingly, many of the conserved interactions between the core proteins have been identified before and showed biological roles in other herpesviruses, particularly HSV-1. For instance, the interaction between the two tegument proteins, UL11 and UL16, was detected in MDV, HSV-1, mCMV and EBV (Loomis et al., 2003; Vittone et al., 2005; Fossum et al., 2009). In HSV-1, this interaction was shown to be required for nucleocapsid tegumentation in the TGN (Loomis et al., 2003). HSV-1 UL16 was also able to interact with MDV UL11, PrV UL11 and HSV UL11 (Loomis et al., 2003). This is consistent with our data, suggesting that the UL11-UL16 interaction is conserved throughout the Herpesviridae, despite the low homology between the orthologs.

Another example is the interaction between the nuclear egress protein UL31 and UL33, which is conserved in the four viruses VZV, mCMV, EBV, KSHV (Fossum et al., 2009). Immunofluorescence studies done by Fossum and colleagues showed that M51, the UL33 homolog in mCMV, colocalizes with the nuclear egress complex M53 and M50, the UL31 and UL34 homologs in mCMV, in the nuclear membrane, suggesting that M51 may be a part of the nuclear egress complex (NEC). The Y2H interaction between UL33 and UL31 in MDV suggests that UL31 could have a similar role in MDV. Finally, the homodimer formation of the immediate early protein UL54 in MDV, has also been reported for HSV-1, mCMV, EBV and KSHV (Zhi et al., 1999; Malik and Clements, 2004; Fossum et al., 2009). This self-interacting property of UL54 could help its transregulatory function for early and late viral genes. In summary, several MDV core proteins showed interactions which have been shown before in other herpesviridae, suggesting that these interactions have a conserved role throughout the entire family.

### 4.3.3 Assessment of the reliability and the limitations of the Y2H system

The Y2H system can result in false positive and as well as in false negative results. False negative interactions are interactions which could not be identified by the Y2H system but are present in vivo and can be caused by a variety of reasons. First, the Y2H system only detects binary interactions which do not require additional binding partners as usually the case in protein complexes. Some interactions depend on post-translational modifications that may not occur in yeast such as the formation of disulphide bridges, glycosylation and phosphorylation. Moreover, the failure of the protein to reach the yeast nucleus due to the presence of localisation sequences (e.g. transmembrane regions) can cause false negative results (Van Criekinge and Beyaert, 1999). Finally, the addition of the DNA-binding domain in the bait fusion protein and the activation domain in the prey fusion protein can lead to incorrect folding which prevents protein interactions. False positive interactions do not occur in vivo and can be caused by the presence of protein domains with activation or DNA-binding properties in bait or prey proteins or by proteins which are particularly sticky (e.g. hydrophobic proteins). Whereas false negative Y2H interactions are difficult to avoid due to their systematic nature, false positives Y2H interactions can be much better controlled and reduced. Non-surprisingly, it is estimated that with the Y2H protocols used here the rate of false negatives is up to 80 or 90%, whereas the number of false positives only about 30%. Nonetheless, crucial Y2H interactions should always be confirmed by other techniques such as pull-down assays or co-immunoprecipitation (Co-IP).

In order to validate the MDV Y2H interaction results, two biochemical assays, Co-IP and LUMIER pull-down assays, were used. Thirty seven interactions between protein pairs were selected randomly and tested by both techniques. Among the thirty seven tested interactions, 30 interactions (81%) were confirmed by the both methods. Based on these results, it can be concluded that the MDV interactome data presented here have a very high reliability. The confirmation rate was higher than in the study on the mCMV, EBV, HSV, KSHV networks (Fossum et al., 2009), but comparable to a previous study on KSHV virion protein-protein interactions (Rozen et al., 2008).

### 4.3.4 A model for MDV virion assembly based on the MDV interactome

Herpesvirus replication and morphogenesis is a complicated process including nucleic acid replication, nucleocapsid formation, nuclear egress, tegumentation, secondary envelopment and virus release. Indeed, much information is lacking regarding the tegumentation and the secondary envelopment processes. Most of these steps of virus replication are based on interactions between different virion proteins, but until now no study has analysed the interactions between structural MDV proteins. The MDV interactome data generated in this study provide the opportunity to create models for each of the steps mentioned above.

The MDV interactome includes a large number of interactions between virion proteins. Unfortunately, several structural proteins including UL37 (small tegument protein), UL35 (capsid protein) and UL33 (capsid-associated protein) had a high prey count (number of baits interacting with a specific prey protein) and were thus excluded from the analysis. However, even after the removal of these interactions, there were still 46 interactions between MDV-structural virion proteins. These interactions could help elucidate the process of MDV virion assembly, starting from encapsidation, tegumentation and secondary envelopment. For instance, the MDV interactome revealed a physical interaction between UL33 and UL26, the minor capsid scaffold protein. UL33 is the DNA cleavage and packaging protein required for the encapsidation of HSV-1 virions (al-Kobaisi et al., 1991). In accordance with the MDV results, the VZV interactome also contained an interaction between ORF25 and ORF33, the orthologs for UL33 and UL26 (Fossum et al., 2009). Furthermore, there were 8 interactions between capsid proteins or capsid-associated tegument proteins with tegument proteins, representing the early stage of the nucleocapsid tegumentation. The tegument protein UL16, known to be associated with the mature capsid HSV-1 (Meckes and Wills, 2007), interacted with the DNA cleavage and packaging protein UL33. This interaction also has been detected in HSV-1 and VZV (Fossum et al., 2009). Another interesting finding from the MDV network involves the major tegument protein UL36. The observation that UL36 interacts with a capsid protein (UL38), several tegument proteins (UL37, UL25, UL5 and itself) and several

membrane-associated proteins (UL34, UL43 and gH) suggests that UL36 may have a central role in collecting these virion proteins during assembly. Again, this observation is consistent with findings reported for the KSHV large tegument protein ORF64 (Rozen et al., 2008; Fossum et al., 2009).

Moreover, the MDV Y2H analysis revealed thirteen interactions between different tegument proteins. Many of these interactions are conserved, and have been thoroughly studied among herpesviridae, such as the interactions between the small and the large tegument proteins UL37 and UL36. It has been reported that this interaction is required for the early capsid tegumentation in PrV and HSV-1 (Klupp et al., 2002; Fuchs et al., 2004; Vittone et al., 2005; Desai et al., 2008). Furthermore, UL36 recruits UL37 to the cytoplasmic budding sites of the Golgi complex, where the secondary envelopment happens (Desai et al., 2008). Another example is the UL48-UL49 interaction in MDV. This interaction was not detected in any previously published herpesvirus networks, but it was detected in other studies for HSV-1 protein-protein interactions (Elliott et al., 1995; Hafezi et al., 2005; Vittone et al., 2005; O'Regan et al., 2007; Taddeo et al., 2007). These studies discovered that the UL48-UL49 interaction is required for tegument assembly, and that VP16 is responsible for recruiting VP22 inside the virion tegument. Many other interactions between tegument proteins were also identified which are know to have a role in tegument assembly, such as UL11 with UL16, UL46 with UL47 and UL17 with UL25 (Loomis et al., 2003; Vittone et al., 2005; Thurlow et al., 2006; Fossum et al., 2009).

After the virion tegument has assembled around the nucleocapsid, the tegumented nucleocapsids are recruited to the TGN. The process of secondary envelopment occurs at TGN, mainly through the binding of tegument protein with glycoproteins. Since membrane proteins have an increased chance of false negative results due to their transmembrane regions (see Section 4.3.3), all glycoproteins were tested both as full-length proteins as well as domain fragments in our Y2H analysis to minimize this risk. Eleven interactions between tegument proteins and membrane or

glycoproteins could thus be detected in this study. Most of the identified interactions between MDV tegument proteins and glycoproteins had not been detected before in any other herpesvirus, and only the interaction between UL36 and gH had been published for KSHV (Rozen et al., 2008).

In summary, it is evident from the data mentioned above that the MDV interactome contains a series of interactions between the virion proteins which may represent different stages of virion assembly. Many of these interactions have been detected before in other members of the herpesviridae, particularly those involved in encapsidation and tegumentation.

### 4.3.5 Meq interactors suggest a potential role in virus pathogenesis

Meq is the main MDV protein responsible for oncogenesis and is expressed during latency. Because it contains a N-terminal basic leucine zipper domain, it has been suggested that it may form homodimers (Qian et al., 1995). In support of this, our Y2H screen identified an interaction between Meq bait and prey. PP38 and PP24 are two phosphoproteins which are mainly expressed during MDV lytic infection (Parcells et al., 2003). Ding and colleagues observed that PP38 and PP24 bind to each other and form a heterodimeric complex (Ding et al., 2008). Consistent with this observation, in the Y2H analysis PP24 and PP38 interacted with each other in both directions. PP24 also interacted with itself, suggesting that it dimerizes. Several papers are published on the relation between Meq and PP38. It has been suggested that PP38 can act as co-activator for Meq (Chen et al., 1992; Jarosinski et al., 2006). However, only limited scientific evidence exists supporting this hypothesis. The MDV interactome revealed an interaction between Meq and PP38, and this interaction was confirmed by Co-IP. Another interesting observation is that Meq and PP38 have many of the same interactors in the Y2H screen. Thus, the question arises if Meq and PP38 are co-workers or competitors? Another interesting finding was the interactions between vIL8, PP24 and Meq. While an interaction between vIL8 and PP38 was not detected in the Y2H system, an independent biochemical assay was used to re-examine the possibility that this interaction also occurs. In fact, by Co-IP it

was detected that both proteins interact with each other. This suggests that the vIL8-PP38 interaction was a false negative result in the Y2H screen, possibly for steric reasons. In accordance with these results, Cui and colleagues found that the absence of vIL8 expression in infected birds causes an impairment and delayed PP38 and Meq expression in infected organs (Cui et al., 2005b). This in vivo result indicates the importance of this interaction, and also suggests that there may be other important MDV protein-protein interactions not yet detected.

In summary, 435 interactions between MDV viral proteins were identified with the Y2H system in this study. Almost 80% of the Y2H interactions could be confirmed indicating that most of the observed interactions are indeed reproducible in biochemical assays. The MDV interactome contains many conserved interactions present in other members of the herpesviridae, confirming previous findings that core interactions are conserved to a large degree. While the MDV interactome contains a vast amount of biological useful data, further studies will need to address the biological importance and function of these interactions. In any case the MDV interactome will represent a valuable resource for future research into the pathogenesis of MDV.

# Identification of MDV proteins inhibiting chicken interferon-alpha signalling pathway

## 5.1 Introduction

Having the advantage of the MDV clone library, it was decided to study one side of the MDV-chicken interplay. This chapter focuses on the inhibitory effect of MDV proteins on the chicken interferon-alpha response. The reasons for this selection were as follows: firstly, many viruses, particularly herpesviridae members, encode proteins that are able to antagonise the induction of interferon-alpha. These proteins interfere with interferon-alpha signalling pathway at different levels, to date; no such proteins have been described for MDV. Secondly, the availability of the chicken ISRE reporter construct, which is known to respond by interferon-alpha treatment, makes a genome-wide study more feasible. In the next section, a brief introduction to the avian interferon system will be provided and also the effect of different viruses on the interferon-alpha signalling pathway.

### 5.1.1 Avian interferon background

Interferons are a family of cytokines, which are considered as a part of the non-specific, innate immune response. These cytokines are produced at early stages of viral infection, in order to induce an antiviral state in the host. In mammalian systems, interferons are classified into three types: type I, type II and the recently identified type III interferons. Type I interferons include IFN-α, IFN-β, IFN-δ, IFN-ω and IFN-τ, while type II interferons include IFN-γ, as a sole member. Type III interferons (IFN-λ), previously known as IL28/29, include three members IL28A, IL28B and IL29 (Sheppard et al., 2003).

Following the classification of interferons in mammals, avian interferons are also classified into three biologically distinct subtypes: type I, type II and type III

interferons (Sick et al., 1996; Lowenthal et al., 2001; Qi et al., 2010). Type I interferons encode two serologically distinct subtypes, formally known as ChIFN1 and ChIFN2. ChIFN1 is encoded by a family of 10 or more genes, whereas ChIFN2 is encoded by only one gene (Sick et al., 1998). Based on some sequence homology with mammalian interferon and the inducibility of their promoters in response to different stimuli, both of ChIFN1 and ChIFN2 were characterised as chicken interferon-alpha (chIFN-α) & chicken interferon-beta (chIFN-β), respectively (Sick et al., 1998). Briefly, the 5'-up stream region of ChIFN2 contains NF-KB and IRF1 binding sites and is strongly induced by virus infection (Sick et al., 1998; Lowenthal et al., 2001). On the other hand, the 5'-up stream region of ChIFN1 contains no NF-KB binding site, and this promoter has poor inducibility following virus stimulation (Sick et al., 1998). Both of chIFN-α and chIFN-β transcripts are induced by different stimuli such as virus infection and/or treatment with poly (I:C) (an artificial double stranded RNA). *In vitro*, chIFN-α and chIFN-β transcripts were detected in primary chicken macrophages stimulated with UV-inactivated Newcastle disease virus (NDV) (Sick et al., 1998). Furthermore, CEF cells strongly produce type I interferon in response to viral infections; such as NDV and yellow fever virus (YF 17DD) (Sick et al., 1996; Caride et al., 2008). Moreover, treatment of chicken leukocytes with poly (I:C) leads to up-regulation of chIFN-α and chIFN-β (Karpala et al., 2008).

Chicken interferon-alpha (chIFN-α) DNA is approximately 582 bp long, and encodes a protein of approximately 193 amino acids in length (Sick et al., 1996; Xia et al., 2004). The recombinant chIFN-α expresses a protein of 21 kDa in size (Xia et al., 2004). It induces antiviral effects in many chicken viral diseases, such as influenza, Marek's disease virus, Newcastle disease virus, infectious bronchitis virus and infectious bursal disease virus (Levy et al., 1999; Marcus et al., 1999; Browne et al., 2001; Pei et al., 2001; Xia et al., 2004). On the other hand, many avian viruses have evolved mechanisms to evade the effects of the endogenous interferon-alpha, secreted inside the bird's body (see Section 5.1.3). These mechanisms are mainly exerted via expression of viral proteins that have the ability to interrupt the

interferon-alpha signalling (Huang et al., 2003; Eldaghayes et al., 2006; Xing et al., 2008).

Chicken interferon-beta (chIFN-β) up-regulation plays a crucial role in the antiviral innate immune response. In chickens, infection with highly pathogenic avian influenza (HPAI) leads to the up-regulation of both TLR3 and chIFN-β mRNA (Karpala et al., 2008). This up-regulation was detected in the brains, lungs and spleens of infected birds 24 hr after infection (Karpala et al., 2008). In mammals, the up-regulation in IFN-β expression is associated with Toll-like receptor-3 (chTLR-3). Consistently, knock-down of chicken Toll-Like Receptor-3 (chTLR-3) from the DF-1 cell line leads to down-regulation of both TLR3 and chIFN-β at the transcriptional level (Karpala et al., 2008).

The chicken interferon-gamma (chIFN-γ) gene has 30-35% sequence homology with its mammalian counterpart (Schultz et al., 2004). The molecular weight of native and recombinant chIFN-γ protein varies from 17 to 27 kDa. This difference in molecular weight is attributed to differences in the expression systems used in the various studies; E. coli, chicken embryo cells or $CD4^{+}$ IFN-$\gamma^{high}$ hybridoma culture supernatant (Song et al., 1997).

Recombinant chIFN-γ was originally cloned from a chicken T-cell hybridoma and the chicken T cell line “855”, and the clones were used for studying its biological activity (Weining et al., 1996; Song et al., 1997). The result of these studies showed that chIFN-γ has antiviral properties, including the ability to induce activation of macrophages resulting in up-regulation of both MHC class I and class II expression and nitric oxide secretion (Weining et al., 1996; Song et al., 1997). Additionally, it up-regulates the guanylate binding protein (GBP) and IRF-1 in chicken T-cell lines and chick embryo cells (Weining et al., 1996).

The chicken interferon lambda (chIFN-λ) is poorly characterised in birds. In mammals, it has common biological properties with type I interferon such as the

signalling pathway and involvement in the antiviral response evoked by TLRs stimulation (Zhou et al., 2007; Ank et al., 2008).

### 5.1.2 Avian interferon pathway

Until now, there has been no direct evidence that the avian interferon-alpha pathway is similar to its mammalian counterpart. In mammalians, the interferon pathway initiates when IFN-α binds to its specific receptor, interferon-alpha receptor (IFNAR). Once binding is established on the surface of the target cells, the induction of the interferon stimulated genes (ISGs) are enhanced. Interferon stimulated genes factor 3 (ISGF3) translocates from the cytoplasm into the nucleus and binds to the IFN-stimulated regulatory element sequence (ISRE). This binding promotes the induction of a variety of IFN-α inducible genes including; antiviral proteins such as 2'5'oligoadenylate synthetase (2'5'OAS), protein kinase RNA (PKR), Mx1 proteins and transcriptional regulators such as IRF7. Interferon regulatory factor 7 is induced upon interferon treatment and is activated and phosphorylated as a part of the innate immune response to virus pathogen associated molecular patterns (PAMPs) such as double stranded RNA (dsRNA) or single stranded RNA (ssRNA). IRF7 forms a homodimer with itself and binds to the interferon promoter to induce the production of interferon-alpha. The later binds with IFNAR and initiates the pathway again in positive feedback mechanism between IRF7 and IFN-α (Figure 5.1) (Haller et al., 2007).

The interferon-inducible chicken Mx1 gene contains an interferon-inducible motif (ISRE) in its promoter. This motif is functionally active in chicken and monkey cells (Schumacher et al., 1994). The activation of the ISRE motif can be induced by various stimuli, such as exogenous interferon, poly (I:C) or NDV stimulation of chicken cells (Schumacher et al., 1994). Another defined DNA sequence motif that confers interferon inducibility, is known as the interferon consensus sequence (ICS). The chicken ICS motif is present in the sequence from 174 to 194 of the MHC class I gene BF-IV and can function as an interferon-inducible promoter (Zoller et al., 1992). In murine models, comparison between ICS motif and ISRE motif revealed

that ICS confers lesser interferon inducibility than the ISRE motif of the mammalian Mx1 gene promoter (Hug et al., 1988).

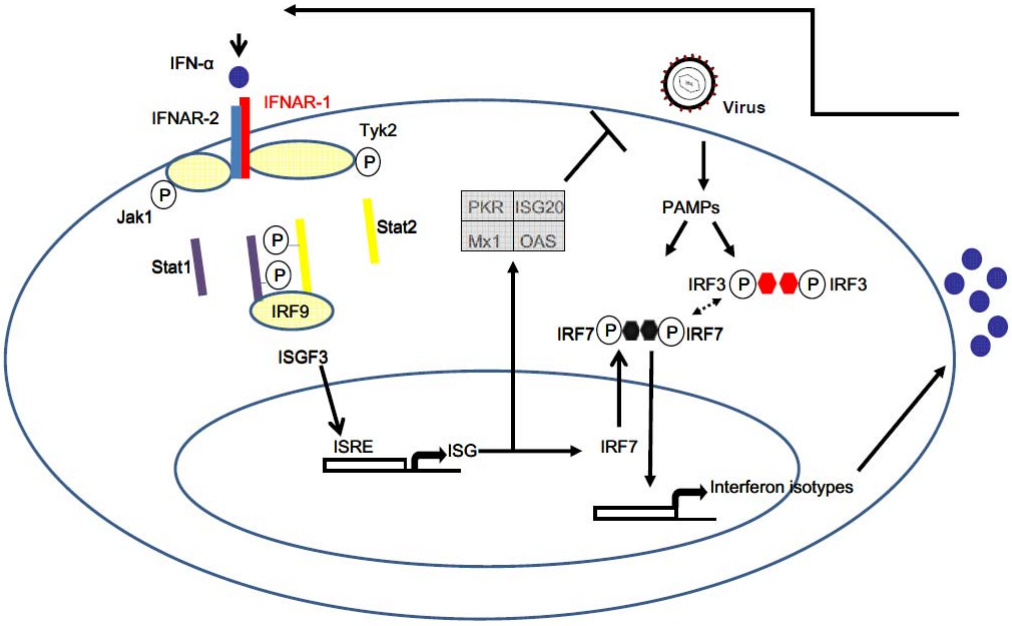



**Figure 5.1: IFN-α induced mammalian antiviral pathway.** Figure illustrates an IFN-α induced antiviral pathway, in which IFN-α binds to its specific receptor, interferon-alpha receptor (IFNAR). This binding results in activation of the JAK-STAT pathway, as interferon stimulated genes factor 3 (ISGF3) translocates from the cytoplasm into the nucleus. ISGF3 binds to IFN-stimulated regulatory element sequence (ISRE), and this binding promotes the induction of a variety of IFN-α inducible genes and transcriptional regulators such as IRF7. Interferon regulatory factor 7 binds to the interferon promoter to induce the production of interferon-alpha. The later binds IFNAR and initiates the pathway again in positive feedback mechanism between IRF7 and IFN-α (Haller et al., 2007).

### 5.1.3 Avian viruses and chIFN-α down-regulation

NS1, non structural 1 protein, from influenza virus, is a potent example of an interferon-alpha antagonist. Hence, infection of chicken macrophages with H9N2, a low pathogenic avian influenza strain, caused marginal down-regulation of the antiviral Mx1 protein. However, both the Mx1 and 2'5'oligoadenylate synthetase (2'5'OAS) genes were down-regulated in the lungs of infected chickens after

infection with the same strain (Xing et al., 2008). Differences in the cytokine levels produced by wild-type H9N2 virus or the NS1 deletion virus were analysed in the chicken macrophage cell line HTC (Xing et al., 2009). Interestingly, the level of interferon up-regulation was higher in the case of the deletion mutant than that of the wild-type virus (Xing et al., 2009). Furthermore, vaccination of chickens with H5N1 influenza strain possessing a truncated NS1 gene induced a protection against further challenges with a virulent wild-type H5N1 strain (Steel et al., 2009).

Newcastle disease virus (NDV) is an avian paramyxovirus and a negative stranded RNA virus. NDV RNA encodes 6 proteins; NP, P, M, F, HN and L, as well as two proteins resulting from alternative RNA editing of P protein. These two proteins were named V protein and W protein. The V protein directly contributes to virus virulence through its inhibitory effect on chIFN-α (Huang et al., 2003; Alamares et al., 2010). The carboxyl terminal part of the V protein was found to be responsible for this inhibition through the degradation of STAT1 (Huang et al., 2003). Interestingly, the V protein derived from mesogenic NDV (moderate virulence) exhibits greater antagonistic effects on interferon, compared with the V protein derived from lentogenic NDV (low virulence) (Alamares et al., 2010).

Avian reovirus is a double stranded RNA virus belonging to the reoviridae family. It causes many illnesses in chickens such as runting-stunting syndrome and viral arthritis syndrome. It is an immunosuppressive virus, and is also able to resist the antiviral effect of chicken interferon. The antiviral inhibitory effect of this virus is mainly due to one of the viral encoded proteins called σ A protein (Gonzalez-Lopez et al., 2003). This protein appears to have an essential role in the down-regulation of interferon-inducible genes through binding to double stranded RNA and negatively regulating the activity of PKR kinase. As a result of this, the presence of σ A protein in CEF cells confers protection of vaccinia virus from the antiviral effect following interferon treatment.

Infectious bursal disease (IBDV), another double stranded RNA virus, infects chickens and causes immunosuppression due to depletion of B lymphocytes. There are different hypotheses regarding the role of IBDV in the modulation of the interferon response, in terms of being down-regulator or up-regulator. However, a few studies have concluded that different strains of IBDV have different mechanisms to block the host chIFN-α response (Ragland et al., 2002; Eldaghayes et al., 2006; Li et al., 2007). Comparisons of the ability of the virulent and very virulent strains of IBDV, F52/70 and UK661 to modulate chIFN-α response was undertaken; the virulent strain was found to be a non-inducer of type I interferon, whereas the very virulent strain can be considered as a negative regulator of the type I interferon responses (Eldaghayes et al., 2006). On the contrary, type I interferon was up-regulated in spleen macrophages isolated from virulent IBDV infected chickens, particularly during the acute stage of infection (Kim et al., 1998). Chicken embryo cells infected with different MOI of Bursine-2, IBDV vaccine strain, has little or no induction of chIFN-α (Li et al., 2007). However, cytokine down-regulation in response to IBDV infection or vaccination requires further study. The IBDV protein or protiens responsible for modulation of the chicken cytokine response has yet to be identified.

### 5.1.4 Herpesviruses and interferon-alpha down-regulation

Herpesviruses usually have several strategies to protect themselves from the host immune response, especially the innate immune response. Many studies have reported herpesvirus encoded proteins that interfere at different levels of interferon signalling pathways, and few examples from them will be mentioned.

UL41 or virus host shutoff protein (vhs) is considered one example of a herpesvirus encoded protein, which affects the innate immune response. Orthologs of UL41 acts as a virus host shutoff protein in many herpesviruses, such as HSV-1, PrV, HSV-2 and herpesvirus papio 2 (HVP-2) (Fenwick and Owen, 1988; Kwong and Frenkel, 1989; Bigger and Martin, 2002; Murphy et al., 2003). UL41 is a tegument protein and functions as an RNase that accelerates RNA degradation of cellular mRNAs

(Elgadi et al., 1999). HSV-1 Δvhs was more sensitive to recombinant interferon-alpha (rIFN-α) and showed marked reduction in growth curves, compared with the wild-type virus (Pasieka et al., 2008). Similar results were also observed for HSV-1 ICP0, where an ICP0 deletion mutant was hypersensitive to IFN-α treatment (Mossman et al., 2000). As a result, the treatment with IFN-α caused marked reduction in the virus titre which equated to an overall 1000 fold reduction (Mossman et al., 2000).

VZV ORF63 plays a major role in the ability of VZV to suppress the host innate immune response (Ambagala and Cohen, 2007). One function of ORF63 is to inhibit the phosphorylation of the eukaryotic initiation factor-2α (eIF-2α), which is an essential process for inhibiting virus protein translation inside the cell.

EBV LF2 is a tegument protein that can evade the host immune response, through blocking IFN-α production (Wu et al., 2009b). LF2 protein binds directly to the C-terminal transactivation domain of IRF7, which is responsible for formation of IRF7 homodimers. Therefore, the binding of LF2 to IRF7 inhibits the formation of IRF7 homodimers and subsequently interferes with the transcriptional role of this dimer in initiating the innate immune response (Wu et al., 2009b). BZLF-1, an immediate early EBV protein, down-regulates IRF7 activity and as such the virus is able to tolerate the antiviral host response during lytic infection (Hahn et al., 2005). This down-regulation of IRF7 activity is as a result of the direct binding between the two proteins and the formation of IRF7/ BZLF-1 complex.

To better understand the MDV pathogenesis, it is necessary to carry out functional assays. These assays should aim to study how MDV proteins are involved in modulating the host immune response. While the mechanism of immune evasion is not yet understood, it is likely that MDV, similar to other herpesviruses, evades host immune responses by encoding viral protein(s), which interact with the host interferon pathway.

As mentioned previously (see Section 1.7.1c), there is some evidence for the down-regulation of interferon-alpha during the course of MDV infection. Thus, it was decided to study host pathogen interactions between MDV proteins and interferon-alpha signalling pathway.

### 5.1.5 Aim

The aim of the work presented in this chapter was to screen MDV proteins for inhibition of chIFN-α signalling. Potential antagonists of the chIFN-α signalling pathway will be studied in more detail, with an aim to describe the mechanisms behind chIFN-α inhibition. These candidates may later be considered as targets for vaccine development companies and the drug designer, in order to control the problem of MDV infections.

## 5.2 Results

### 5.2.1 The optimisation of the chIFN-α dose required to initiate IFN-α signalling

As mentioned in Section 5.1, the interferon-alpha signalling pathway is interferon-alpha-dependent pathway. In order to optimise the proper concentration of chIFN-α required for priming the interferon-alpha signalling pathway, a dose titration experiment was performed. Briefly, DF-1 cells were seeded in 48 well plates with $5x10^4$ cells per well 24 hr before transfection. When the cells reached 70-80% confluency, they were transfected with 90 ng of eGFP_pCR3 expression vector, 10-ng of Renilla luciferase and 50 ng of a reporter construct containing the chicken ISRE motif in the upstream region of firefly luciferase. The transfection reaction was prepared as detailed in Section 2.2.1. After 48 hr, the cells were stimulated with conditioned media containing different amounts of chIFN-α: 100U, 500U and 1000U. The experiment was carried out in triplicate for each dose concentration and 3 transfected wells were left as control without any interferon-alpha treatment. After 15 hr of chIFN-α treatment, the cells were harvested and lysed. The luciferase signal

was measured using the dual luciferase assay system (Promega) and the relative luciferase units (RLU) were calculated. All the luciferase values were normalised to the non-interferon treated samples, which represent basal interferon activity and is arbitrarily given a value of one. As shown in Figure 5.2.1, the amount of 1000U of chIFN-α induced the highest level of ISRE activation, which was around an 18 fold induction. Therefore, this was the amount which was selected to be used through out the screen.

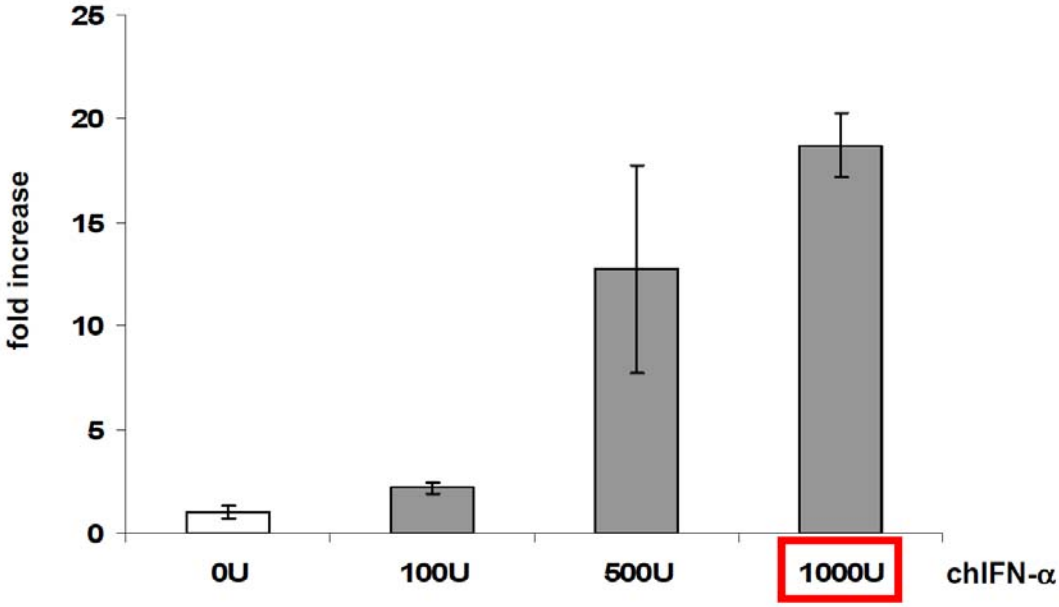



**Figure 5.2.1: Dose-dependent induction of the chicken Mx1 promoter.** Luciferase reporter assay was performed using the ISRE promoter reporter construct. DF-1 cells were seeded and transiently co-transfected with eGFP_pCR3 vector, Renilla luciferase vector and ISRE reporter vector. Transfected cells were grown for 48 hr prior to stimulation with different concentrations of chIFN-α (1000U, 500U, 100U and 0U), in triplicate. Cells were lysed after 15 hr following interferon treatment. Dual luciferase assay was performed. Values expressed as a fold difference relative to the eGFP non-interferon treated samples value which is given an arbitrary value of 1.

### 5.2.2 Effect of transient expression of individual MDV proteins on the inducibility of the ISRE motif of the chicken Mx1 promoter following chIFN-α treatment

To identify possible interferon-alpha antagonists encoded by the MDV genome, a reporter assay screen was carried out. The screen aimed to identify the effect of the expression of MDV proteins on the inducibility of the chicken ISRE motif after chIFN-α treatment. DF-1 cells grown in 48 well plates were transfected with chicken ISRE pHIIα firefly luciferase as a reporter construct and Renilla luciferase construct (pRLTK) as an internal transfection control, together with the individual MDV genes cloned into pCR3 expression vector (see Chapter 3). DF-1 cells were seeded in thirty six 48 well plates 24 hr before transfection. From each plate the internal 24 wells were used only to avoid an edge effect. The cells were transfected with the DNA transfection complex, composed of 90 ng DNA of the expression clone, 50 ng DNA of the ISRE reporter construct and 10 ng DNA of Renilla luciferase (pRLTK) plasmid per well. The transfection reaction was prepared as detailed in Section 2.2.1. Each of MDV genes was transfected in six wells. Forty eight hours post transfection, three wells were treated with conditioned media containing 1000U of recombinant chIFN-α. The other three wells were left without interferon treatment as a non-stimulated control.

Fifteen hours after the addition of chIFN-α, the cells were harvested and lysed. The firefly luciferase and Renilla signals were measured using the dual luciferase system. Transfection of the eGFP plasmid was used as a control for the normal basal level of interferon-alpha response.

All of the obtained luciferase values were normalised to the average of the eGFP interferon-treated samples value, which represents the normal activity and is arbitrarily given a value of one (see Appendix E). As shown in Figure 5.2.2, the results of the screen were plotted collectively, and the statistical analysis showed 11 MDV genes with significant down-regulation of the ISRE reporter. However, the cut-off level was placed below 0.5 fold of inhibition to minimise the number of the

hits. This screen discovered that from 106 tested genes; only 6 genes from the both MDV strains showed a decrease in ISRE interferon inducibility and that these genes gave values lower than the cut-off level. These genes were Meq, LMeq, UL50CVI, UL50RB-1B, UL12 and UL26. Both Meq and LMeq are considered homologues but with different reading frames in RB-1B virulent strain and CVI988 vaccinal strain, respectively. UL50, UL12 and UL26 encoded the dUTPas, deoxyribonuclease and capsid scaffold protein, respectively.

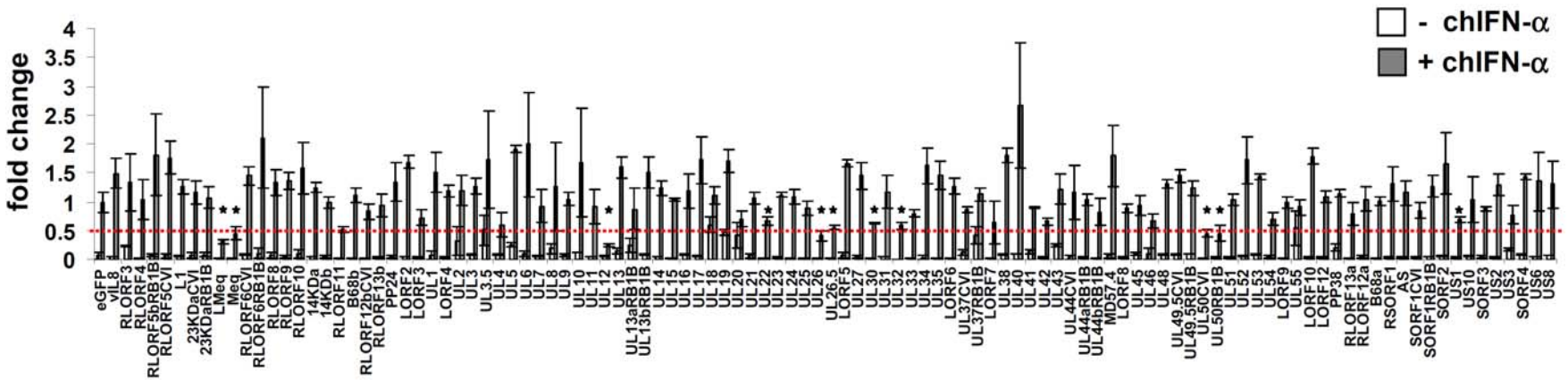



**Figure 5.2.2: Identification of MDV proteins blocking interferon-alpha induced activation of the chicken Mx1 promoter.** DF-1 cells were seeded and transiently co-transfected with the individual MDV genes or eGFP_pCR3 vector, plus ISRE reporter constructs and Renilla luciferase as an internal control. Transfected cells were grown for 48 hr prior to stimulation with 1000U of chIFN-α for each well. Cells were lysed and dual luciferase assay was performed. Values were normalised to Renilla luciferase and then expressed as a fold difference relative to the eGFP interferon-treated samples value, which is given an arbitrary value of 1. The white bars represent the non-interferon treated samples while the grey bars represent the interferon-treated samples. The red line represents the cut-off level 0.5. The statistical analysis was done using Welch's T test (*P<0.05).

### 5.2.3 Confirmation of the chIFN-α antagonists

To confirm the ISRE reporter assay screen results (see Section 5.2.2), the positive hits from the screen were retested in an independent experiment. DF-1 cells were seeded in 48 well plate with $5x10^4$ cells per well, 24 hr before the transfection. When the cells reach to 70-80% confluency, the five MDV genes, UL26, UL50, Meq, LMeq and UL12, were transfected in combination with firefly ISRE reporter and Renilla reporter. Forty eight hour later, the cells were treated with conditioning media containing 1000U of chIFN-α for each well. For each gene, three wells were left without interferon treatment as a non-stimulated control. The cells were lysed after 15 hr and the luciferase signal was measured using dual luciferase assay system.

As shown in Figure 5.2.3, the result of this independent reporter assay confirmed that all of the hits were reproducible and showed reduction in the response of the ISRE motif. The down-regulation was below or around the 0.5 cut-off level for all. Both Meq and LMeq seemed to be the most inhibitory interferon antagonists for the interferon-alpha pathway in this experimental setup.

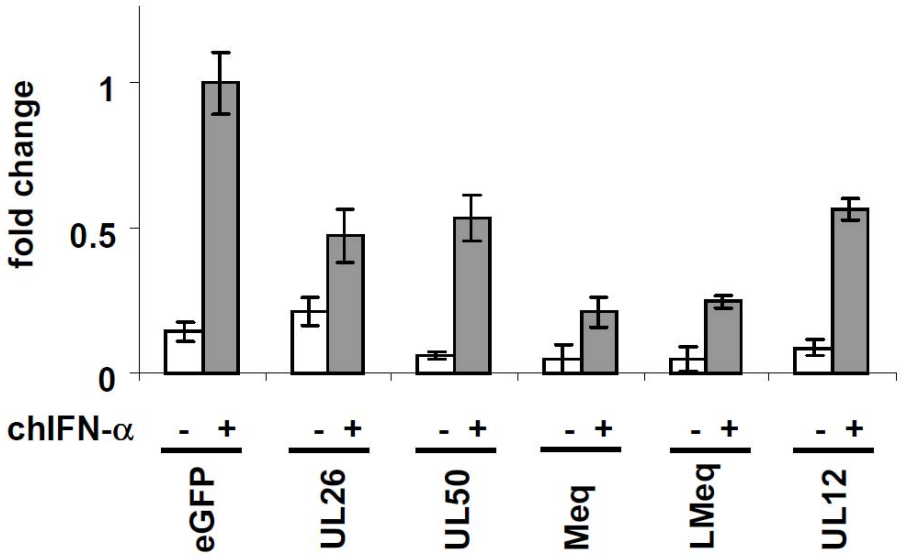



**Figure 5.2.3: Confirmation of the chIFN-α antagonists.** Luciferase reporter assay was performed using the ISRE promoter reporter construct. DF-1 cells were transiently co-transfected with UL50_pCR3, Meq_pCR3, LMeq_pCR3, UL26_pCR3, UL12_pCR3 or eGFP_pCR3 vector, plus ISRE reporter constructs and Renilla luciferase as an internal control. Transfected cells were grown for 48 hr prior to stimulation with 1000U of chIFN-α for each well. Cells were lysed and dual luciferase assay was performed. Values were normalised to Renilla luciferase and then expressed as a fold difference relative to the eGFP interferon-treated samples value, which is given an arbitrary value of 1.

### 5.2.4 Investigation of the expression of the MDV-interferon antagonists in DF-1 cell line

The findings outlined in Section 5.2.2 and Section 5.2.3 demonstrated that Meq, LMeq, UL26, UL50 and UL12 act as negative regulators for type I interferon signalling transduction. To study this finding in more detail, it was neccessary to confirm if these genes are truly expressed in the DF-1 cell line or not. To assess this, the entry clones of the seven genes were sub-cloned into an HA N-terminal-tagged pCR3 expression vector using Gateway® technology (as described in Section 3.2.4c). DF-1 cells were seeded in 24 well plates at a seeding density $1x10^5$ cells per well. After 24 hr, the cells were transfected with 500 ng of the expression vector of each gene. The transfection reaction was prepared as detailed in Section 2.2.2. Forty eight hours later the cells were lysed. Total cell lysates were harvested and subjected to SDS-PAGE, and immunoblotting using anti-HA primary antibody followed by HRP-

conjugated goat anti-rat secondary antibody. All the indicated genes were successfully expressed at the expected sizes (Figure 5.2.4). Meq and LMeq expressed protein products at or above 64 kDa in size, respectively. UL50 expressed a protein product of 50 kDa in size, and UL12 expressed a protein product of 55 kDa approximately. Finally, UL26 expressed a cleaved protein product of 36 kDa in size.

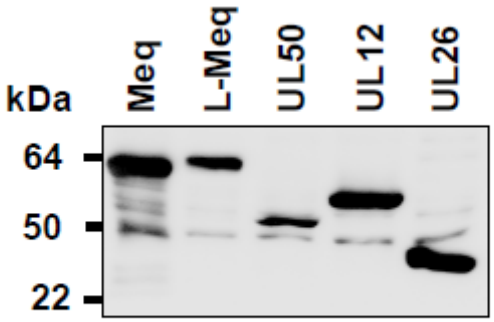



**Figure 5.2.4: Transient expression of MDV interferon-alpha antagonists in DF-1 cell line.** DF-1 cells were seeded and transiently transfected with MDV_Meq, MDV_LMeq, MDV_UL50, MDV_UL12 and MDV_UL26 cloned in HA tagged N-terminal pCR3 expression vector. Cells were grown for 48 hr, and then harvested and lysed. The cell lysates were subjected to SDS-PAGE and immunoblotting using anti-HA primary antibody followed by HRP-conjugated goat anti-rat secondary antibody.

### 5.2.5 Meq and LMeq reduce interferon inducibility of the ISRE motif in a dose-dependent manner

As shown in Sections 5.2.2 and 5.2.3, Meq and LMeq were able to repress the interferon responsiveness of the chicken ISRE motif. In order to assess whether Meq's and LMeq's ability to repress the interferon responsiveness of chicken ISRE motif was specific, another reporter assay was carried out. In this experiment, DF-1 cells were seeded in 48 well plates at a cell density of $5x10^4$ cells per well 24 hr before transfection. Thereafter, the cells were transfected with different concentrations of Meq and LMeq (90 ng, 45 ng, and 22.5 ng), in addition to ISRE firefly reporter construct (50 ng) and Renilla luciferase (10 ng) as an internal control. The transfection reaction was prepared as described in Section 2.2.1. eGFP pCR3 was used as a control plasmid and serves to normalise the total amount of DNA in

the transfection complex. Forty eight hours later, cells were treated with 1000U of chIFN-α and the luciferase signal was measured after 15 hours. All of the luciferase values were normalised to the eGFP transfected and interferon-treated samples, which represent the normal activity and is arbitrarily given a value of one.

As shown in Figures 5.2.5a, b, both Meq and LMeq expression robustly antagonise interferon-alpha inducibility of the ISRE motif in a dosage-dependent manner.

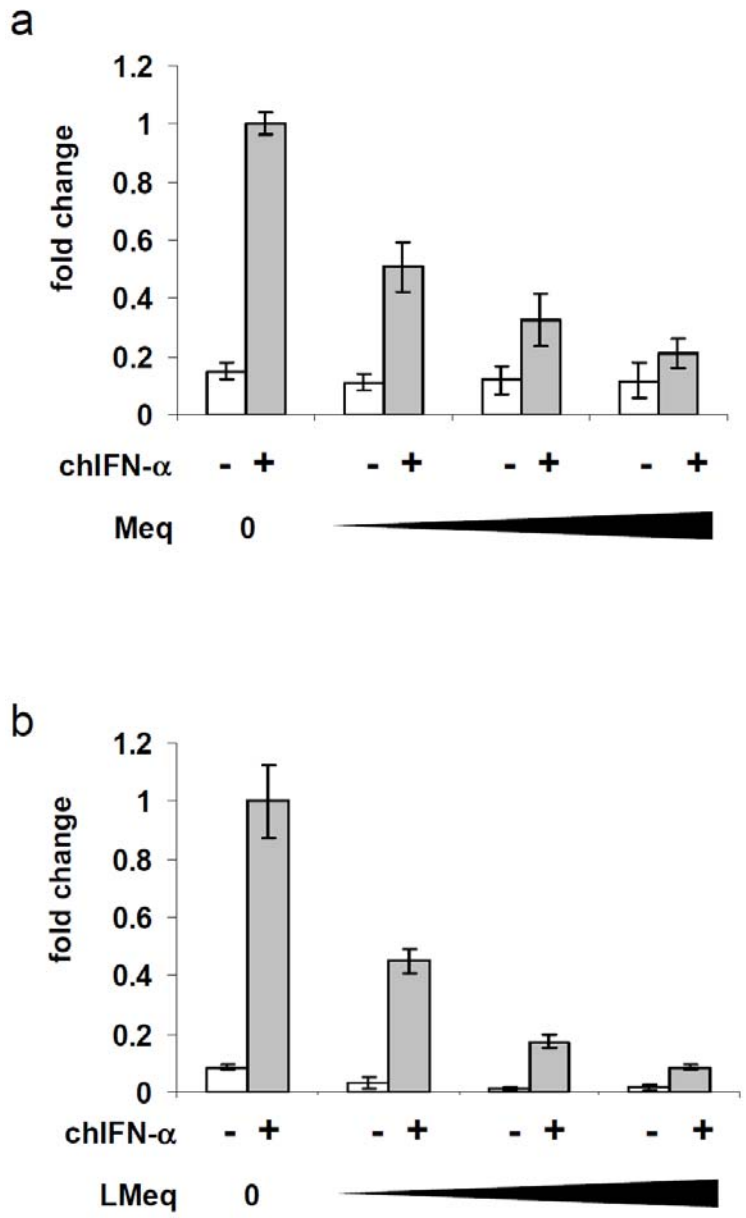



**Figure 5.2.5a, b: Meq (RB-1B) and LMeq (CVI988) inhibit the induction of ISRE by chIFN-α in dose dependent manner.** DF-1 cells were transfected with firefly ISRE reporter and Renilla pRLTK plasmids together with different concentrations of either Meq (a) or LMeq (b). At 48 hours post transfection, cells were treated with 1000 U of chIFN-α. Cells were harvested and lysed 15 hr later and luciferase activity was measured by a dual-luciferase reporter assay. All the data were normalised to Renilla luciferase activity. Results are the average of 3 samples. The white bars represent the non-interferon-treated samples while the grey bars represent the interferon-treated samples.

### 5.2.6 UL50 (dUTPase), UL26 (capsid scaffold) and UL12 (DNAse) proteins do not block activity of the ISRE response element in a dose-dependent manner.

As shown in Sections 5.2.2 and 5.2.3, MDV encoded dUTPase (UL50), DNAse (UL12) and capsid scaffold (UL26) proteins were able to repress the interferon responsiveness of chicken ISRE motif. In order to assess if these results were specific, a second and independent reporter assay was performed. In this experiment, DF-1 cells were seeded at a cell density of $5x10^4$ in two 48 well plates 24 hr before transfection. Thereafter, cells were transfected with different concentrations of UL50, UL12 or UL26 (90 ng, 45 ng and 22.5 ng), with constant concentration of the ISRE firefly reporter construct and Renilla luciferase as an internal control. The transfection reaction was prepared as described in Section 2.2.1. 48 hr post transfection, cells were treated with 1000U of chIFN-α per well and the luciferase signal was measured after 15 hr. The different concentrations of DNA were transfected in six wells of a 24 well plate and tested in triplicates with or without interferon-alpha treatment. All of the luciferase values were normalised to the eGFP transfected and interferon-treated samples, which represent the normal activity and is arbitrarily given a value of one. These three reporter assays failed to show interferon signalling inhibition in a dosage-dependent manner for the three proteins UL50, UL12 or UL26 (Figure 5.2.6a, b and c).

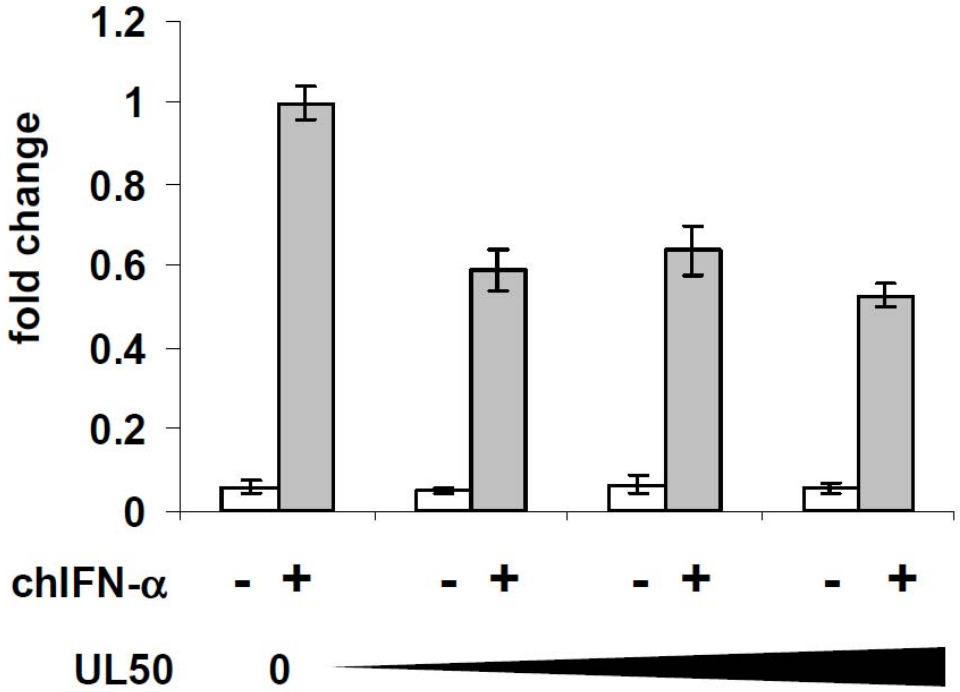



**Figure 5.2.6a: Transient expression of UL50 down-regulates ISRE responsiveness in non-dose dependent manner.** DF-1 cells were transfected with firefly ISRE reporter and Renilla pRLTK plasmids together with different concentrations of UL50 (90 ng, 45 ng and 22.5 ng). At 48hours post transfection, cells were treated with 1000U of chIFN-α. Cells were harvested and lysed 15 hr later and luciferase activity was measured by a dual-luciferase reporter assay. All the data were normalised to Renilla luciferase activity. Results are an average of 3 samples. The white bars represent the non-interferon treated samples while the grey bars represent the interferon-treated samples.

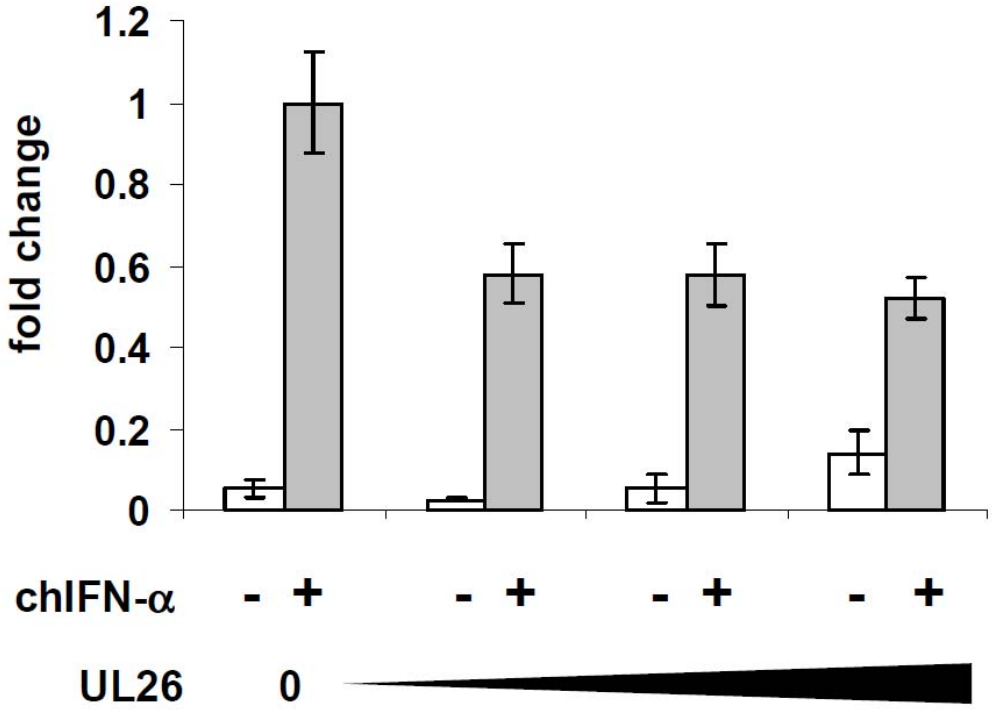



**Figure 5.2.6b: Transient expression of UL26 down-regulates ISRE responsiveness in non-dose dependent manner.** DF-1 cells were transfected with firefly ISRE reporter and Renilla pRLTK plasmids together with different concentrations of UL26 (90 ng, 45 ng and 22.5 ng). At 48hours post transfection, cells were treated with 1000U of chIFN-α. Cells were harvested and lysed 15 hr later and luciferase activity was measured by a dual-luciferase reporter assay. All the data were normalised to Renilla luciferase activity. Results are an average of 3 samples. The white bars represent the non-interferon treated samples while the grey bars represent the interferon-treated samples.

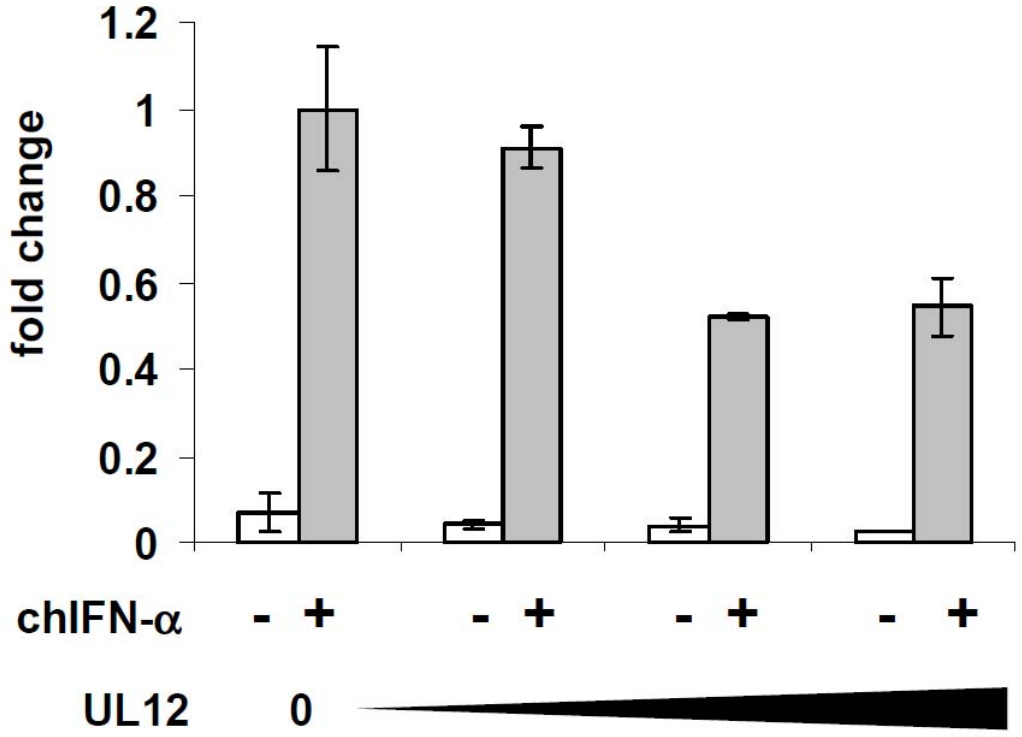



**Figure 5.2.6c: Transient expression of UL12 down-regulates ISRE responsiveness in non-dose dependent manner.** DF-1 cells were transfected with firefly ISRE reporter and Renilla pRLTK plasmids together with different concentrations of UL12 (90 ng, 45 ng and 22.5 ng). At 48hours post transfection, cells were treated with 1000U of chIFN-α. Cells were harvested and lysed 15 hr later and luciferase activity was measured by a dual-luciferase reporter assay. All the data were normalised to Renilla luciferase activity. Results are an average of 3 samples. The white bars represent the non-interferon treated samples while the grey bars represent the interferon-treated samples.

### 5.2.7 Meq N-terminal DNA binding and nuclear localisation domain responsible for down-regulation of chIFN-α response

From the above mentioned results, Meq and LMeq were the only MDV protein candidates which functioned as interferon-alpha antagonists in a dosage-dependent manner (see Section 5.2.5). The Meq protein is composed of 339 amino acids in the virulent strains. It contains a basic leucine zipper (bZIP) domain near its N-terminus and a proline-rich domain near its C-terminus (Jones et al., 1992; Qian et al., 1995). Moreover, Meq contains two additional basic regions (BR1 and BR2) near the N-

terminus, which also contain the nuclear localisation signals (Lee et al., 2003). In CV1988 and the attenuated JM strain, Meq is a slightly longer and termed LMeq, in which a 180-bp sequence is inserted in the proline rich region.

To identify the Meq domain responsible for the down-regulation of the chIFN-α response, a further reporter assay was carried out. In this reporter assay, three Meq mutants were used (kindly supplied as a gift from Dr Yoshihiro Izumiya, UC Davis cancer centre, USA). These mutants encode different parts of the Meq structure, and they were named N-151, C-151 and C-25. N-151 encodes the 151 N–terminus amino acids, C-151 encodes 189 C–Terminus amino acids, while C-25 encodes 315 C-terminus amino acids. DF-1 cells were transfectd with chicken ISRE pHIIα firefly luciferase plasmid as a reporter construct and the Renilla PRLTK plasmid as an internal transfection control together with the each of the mutants. DF-1 cells were seeded in 48 well plates and transfected with the DNA transfection complex after 24 hr. The DNA transfection complex was composed of 90 ng DNA of Meq mutant, 50 ng DNA of reporter construct and 10 ng DNA of pRLTK plasmid per well. The transfection reaction was prepared as detailed in Section 2.2.1. At 48 hours post transfection, the interferon-alpha pathway was primed with 1000U of chIFN-α per well. Fifteen hours later, the firefly luciferase and Renilla signals were measured using dual luciferase system. eGFP plasmid was used as a control open reading frame for the normal level of interferon-alpha response. The results were shown as the following; all the luciferase values were normalised to the average of eGFP interferon-treated samples value, which represents the normal activity and is arbitrarily given a value of one.

As shown in Figure 5.2.7, C-25 gave the same degree of down-regulation as the wild- type Meq. N-151 mutant gave some degree of down-regulation, but it was not equal to the wild-type, whereas C-151 did not retain any function as an interferon-alpha antagonist and gave a response equal to the control. From this data, it is clear that the last 189 C–Terminus amino acids and the first 25 N-terminal amino acids have no role in blocking interferon-alpha pathway. The sequence, which has a role in

down-regulation of interferon-alpha pathway, was identified as being located between amino acids 25 and amino acids 151.

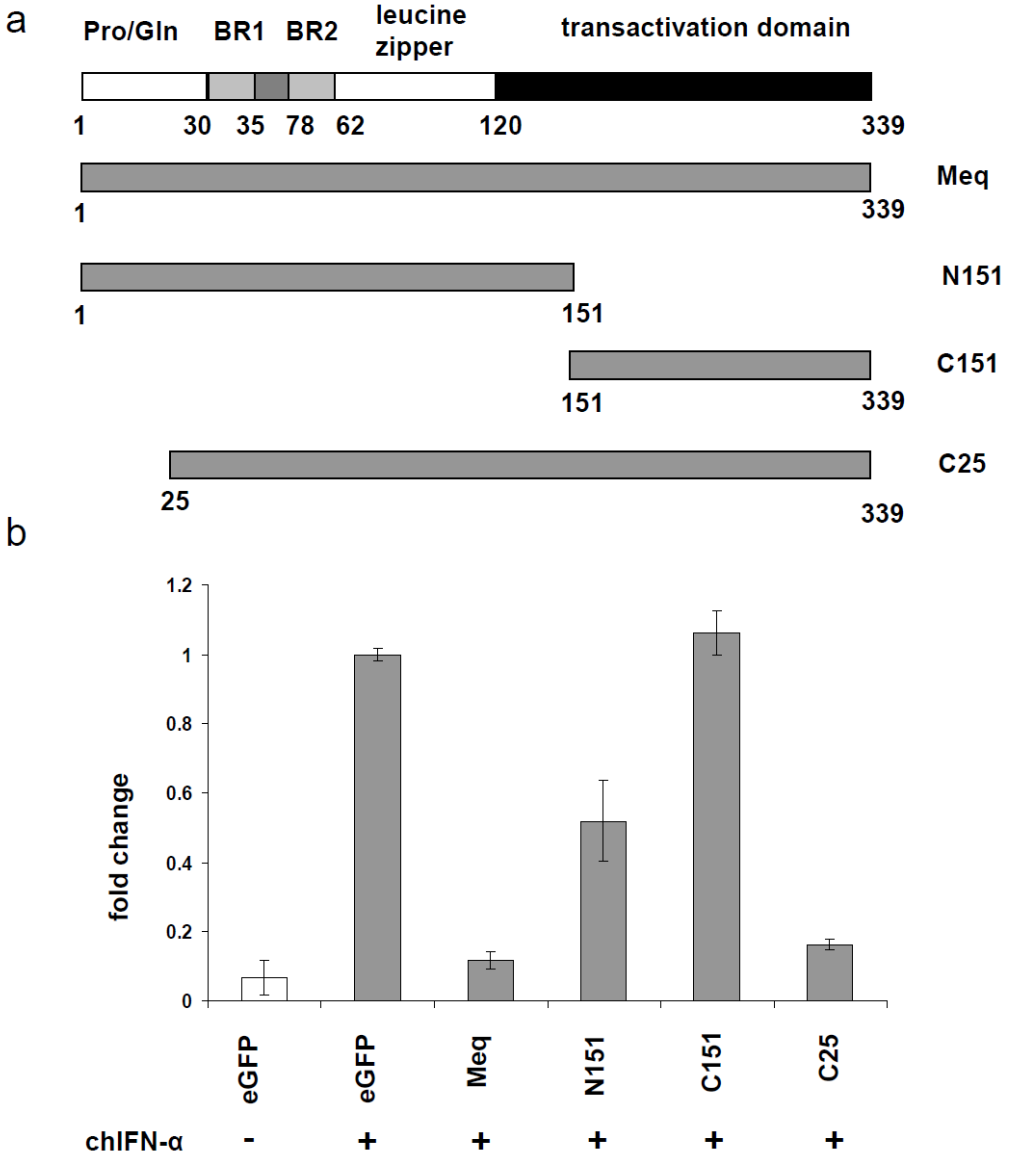



**Figure 5.2.7: Meq N-terminal domain is mandatory for down-regulation of chIFN-α.** DF-1 cells were transfected with firefly ISRE reporter and Renilla pRLTK plasmids together with different structural mutants of Meq DNA. Cells were grown for 48 hr prior to stimulation with 1000U of chIFN-α per well. After 15 hr of treatment, cells were lysed and dual luciferase assay was performed. All the data were normalised to Renilla luciferase activity. The white bar represents the non-interferon treated control while the grey bars represent the interferon-treated control and samples.

## 5.3 Discussion

Previous studies examining the function of MDV proteins have focused on knocking-out virus genes from the MDV genome, using BAC technology (Cui et al., 2005a; Jarosinski et al., 2007b; Lee et al., 2008; Cui et al., 2009). The constructed knock-out mutants were then tested *in vitro* and *in vivo* for their ability to induce lytic and latent infection. This study tested the ability of individual MDV genes to repress the interferon-alpha pathway in a single gene setting. By setting up a screen based on the dual luciferase reporter assay system, it was possible to identify MDV proteins that were able to down-regulate innate immune response, represented in chIFN-α signalling in DF-1 cells *in vitro*.

### 5.3.1 MDV and interferon-alpha response

MDV is an oncogenic disease which affects chickens. It is characterised by multiple stages of infection starting from lytic infection, latent infection through to tumour formation. To maintain a latent inside host cells, it has been suggested that MDV employs a mechanism that down-regulates innate and cell-mediated immune responses.

Interestingly, previous studies have revealed a reduction or absence in chIFN-α transcripts in spleen tissue from MDV infected birds (Xing and Schat, 2000a; Heidari et al., 2008b). Additionally, a blockage in the chIFN-α response to Newcastle disease vaccine has also been observed after infection with oncogenic MDV (Quere et al., 2005).

Most of herpesviridae members encode proteins that act as repressors of the innate immune response (Mossman et al., 2000; Pasieka et al., 2008; Wu et al., 2009b). Until now there have been no studies which show evidence of interactions between MDV encoded genes and the innate immune response. This study investigated the effects of MDV proteins on interferon-alpha signalling. A chicken fibroblast cell line was used as a model for the host interferon response, since it has been previously

tested for interferon-alpha inducibility by Schumacher and colleagues (Schumacher et al., 1994). The same reporter used in their study, identified five potential genes that can down-regulate the interferon responsiveness in our screens. These genes were Meq, LMeq, UL50, UL12 and UL26.

### 5.3.2 Meq and LMeq are inhibitors of the interferon-alpha pathway

Meq is the main MDV-oncoprotein (Liu et al., 1998; Gimeno et al., 2005; Brown et al., 2006), and is highly expressed in MDV-transformed T cells (Ross et al., 1997; Liu et al., 1998). It can independently transform Rat-2, NIH3T3 and DF-1 cell lines (Liu et al., 1998; Levy et al., 2005; Ajithdoss et al., 2009). Recombinant virulent MDV lacking Meq (rMdV5ΔMeq) provides birds with protection higher than the protection provided by the rispens vaccine strain (Lee et al., 2008).

In the MDV chIFN-α screen, Meq and its homologue in the vaccinal strain LMeq were the most potent down-regulators of the interferon response of the chicken ISRE promoter. This effect occurred in a dose-dependent manner, indicating the specificity and reproducibility of the result. The Meq transactivation domain exists in the C-terminal proline rich domain, located between 129 to 339 amino acids (Qian et al., 1995). This C-terminal domain contains two and a half repeats of proline rich sequences, located between 146 to 251 amino acids (Qian et al., 1995). The N-terminal region contains 2 basic regions (BR1 and BR2) and a leucine zipper domain. This domain of Meq is mainly responsible for the nuclear and nucleolar localisation of the protein (Liu et al., 1997; Lee et al., 2003), in addition to its DNA binding affinity (Qian et al., 1996; Ajithdoss et al., 2009). This study found that the sequence required for down-regulation of the chIFN-α pathway is located between amino acids 25 to 151. Furthermore, the C-terminal transregulatory domain, encoding the proline rich repeat, has no down-regulatory effect when it is transfected alone. Additionally, the first N-terminal 25 amino acids were not required for the down-regulation. Based on these findings it is more likely that the interferon antagonistic function requires either a Meq nuclear localisation signal, DNA binding activity or that this region contains a novel function responsible for the down-

regulation. Interestingly, another leucine zipper encoded protein in the herpesvirus family down-regulates interferon-alpha response (Hahn et al., 2005). BZLF-1, an EBV lytic protein, down-regulates the interferon-alpha pathway. This effect is exerted through interfering with IRF7 function. Although, BZLF-1 is a lytic protein, compared to Meq which is predominately latent, both are structurally related to one another.

### 5.3.3 UL50 encoded protein is a negative regulator of the interferon-alpha pathway in the virulent and the vaccinal strains

UL50 from the two strains, CVI988 and RB-1B, are almost identical, with the only differences located in the amino acids 2, 74 and 391. Hence, the aspartic acid, lysine and arginine in the CVI988 mutated to asparagine, arginine and glycine in RB-1B. MDV-UL50 encoded protein contains a dUTPase like domain, which is an important enzyme for DNA replication and repair. Although, dUTPase encoded proteins are present in many mammalian and avian herpesviruses (Fuchs et al., 2000; Zhao et al., 2008), they may play different roles in the different viruses. An ortholog of UL50 is an early gene in duck enteritis virus (DEV), and it is expressed in both the nucleus and cytoplasm (Zhao et al., 2008). In infectious laryngeotracheitis, it is a non-essential gene, and therefore not required for virus replication (Fuchs et al., 2000). Since there are no published data on the role of UL50 in MDV, this study provides the first evidence for the role of this gene in MDV. It was observed that the two genes cloned from the both strains were able to down-regulate the interferon-alpha responsiveness of the ISRE motif. However, this effect was not dose-dependent; indicating that even low expression levels of this protein is enough to induce the repression seen. Interestingly, the dUTPase encoded LF2 gene in EBV has been reported to have a similar effect on the interferon-alpha pathway (Wu et al., 2009b). LF2 is able to exert this effect through binding to IRF7 and interfering with the formation of IRF7-IRF7 homodimer complex (Wu et al., 2009b). Thus, it is very likely that both viruses use the dUTPase encoded proteins in order to evade the innate immune response during virus infection and latency. The mechanism behind this in MDV remains to be determined.

### 5.3.4 UL26 and UL12 encoded protein are negative regulators of the interferon-alpha pathway

UL26 is a capsid cleavage protein in many avian and mammalian herpesviruses (Weinheimer et al., 1993; Haanes et al., 1995; Dezelee et al., 1996; Kut and Rasschaert, 2004). It contains a protease domain near its N-terminal, which has proteolytic activity. The UL26 sequence also encodes another ORF at its C-terminal, named UL26.5, which has no protease domain. UL26.5 protein also exerts down-regulation, although slightly above the cut-off level. This indicates that the effect of UL26 in the screen has no relation to its proteolytic activity and that it is most likely due to activities within its C-terminal sequence. However, UL26 down-regulation of ISRE responsiveness was not dose-dependent, although these results could indicate that this effect needs only low level of the protein. There is no previous evidence of involvement of UL26 in the host immune response against herpesviridae, except in case of HSV-1, where mutant virus lacking UL26 was attenuated and produced efficient immune response in inoculated mice (Hippenmeyer et al., 1997).

The UL12 protein product has deoxyribounclease activity. It is an early gene in HSV-1 and PrV, although it has been defined as a late gene in BoHV-1 (Desloges and Simard, 2001). In the experiment reported here, UL12 induces down-regulation of the ISRE responsiveness. There is no recorded evidence for UL12 having a role in herpesvirus immune modulation. However, this could be the initial evidence for the role of this gene in modulation of the interferon response for MDV.

More than one MDV gene has been shown to interact with the interferon-alpha signalling pathway and is mainly immediate early or early kinetics. Expression of immune suppressors at an early stage may facilitate cell infection of the virus. Another interesting finding is that most of the identified proteins function as enzymes for DNA repair and metabolism. Although, it is not clear whether this is a coincidence or the fact that the integrity of the viral DNA is required for interferon blockage function. Finally, the only proteins exerting a dose-dependent chIFN-α down-regulation were Meq and its homologue in the vaccinal strain LMeq.

Therefore, it was decided to focus on Meq protein to investigate the mechanism behind this down-regulation.

# Elucidation of the role of Meq in the down-regulation of the interferon-alpha response

## 6.1 Introduction

Stimulation of the JAK-STAT interferon-alpha-dependent pathway results mainly in the production of two groups of antiviral factors. The first group act directly against viral infections. A classic example is Mx1 protein, which has an antiviral effect against Influenza virus, especially the strains of avian origin (Dittmann et al., 2008). The second group of the antiviral factors consists of transcriptional regulators such as interferon regulatory factors (IRFs), which regulate multiple aspects of the cell physiology including interferon production.

Many viruses encode inhibitors of JAK-STAT signalling pathway. These inhibitors can hinder the pathway either up- or down-stream level of ISRE promoter activation. An up-stream effect could be through interference with any stage of the pathway preceding ISRE activation such as the IFN-α receptor or one of the STAT proteins. One example is the paramyxovirus family V protein which targets the STAT proteins for proteasomal degradation by forming a STAT-ubiquitin ligase complex (Nishio et al., 2005; Ulane et al., 2005). Down-stream effects occur either via hindering the antiviral cytokines or the interferon regulatory factors. For example, the NS protein of the Rift Valley fever virus which is capable of inducing degradation of the PKR protein in a proteasome-dependent manner (Ikegami et al., 2009). Moreover, many viruses, particularly herpesviruses, negatively regulate IFN-α response through direct down-regulation of IRF7 at different levels.

### 6.1.1 Interferon regulatory factors (IRFs)

Up to now, only nine IRFs (IRF1, IRF2, IRF3, IRF4, IRF5, IRF6, IRF7, IRF8 and IRF9) have been identified in mammals. Their DNA structure is characterised by the presence of a conserved N-terminal DNA binding domain and a C-terminal IRF-

association domain. They are considered a group of transcription factors which mainly regulate immune signalling pathways, particularly interferon, chemokine, and proinflammatory cytokine signalling pathways. Their role in the signalling processes is exerted via binding with themselves and/or different adaptor molecules, which are important for the signalling pathways, such as TRIF, TRAF6 and MyD88 (Honda et al., 2004b; Takaoka et al., 2005; Honda and Taniguchi, 2006). Once binding occurs, the complex translocates from the cytoplasm to the nucleus and binds with IRF recognition sequences in the promoters of the interferon or interferon-inducible genes. For example, IRF9 binds to the STAT1 and the STAT2 proteins forming the interferon stimulated gene factor- 3 (ISGF3) complex. ISGF3 then binds to the ISRE sequence in the promoter of the interferon-inducible genes, thus inducing their transcription. However, not all of the IRFs are considered positive regulatory factors for immune signalling, as IRF7 and IRF3 are (Lin et al., 2000). IRF2, for example, is considered a negative regulator via suppression of the expression of many immunomodulatory molecules such as IL4 and TR2. This suppression is exerted via directly binding to their promoter sites (Elser et al., 2002; Honda et al., 2004a; Sul et al., 2008).

In addition, many IRFs play a direct or indirect role in other cellular regulatory processes, as reviewed in (Taniguchi et al., 2001; Savitsky et al., 2010). These regulatory functions include immune cell development and differentiation, cell cycle regulation, apoptosis and anti-oncogenesis (Taniguchi et al., 2001; Solis et al., 2006; Savitsky et al., 2010).

### 6.1.2 Interferon regulatory factor 7 (IRF7) and its interaction with viral proteins

Interferon regulatory factor 7 (IRF7) is considered as a key regulator for type 1 interferons signalling, particularly IFN-α (Au et al., 1998; Lin et al., 2000; Ning et al., 2005). It was initially described as a negative regulator for the Q promoter (QP) of EBV nuclear antigen-1 (EBNA-1) (Zhang and Pagano, 1997). Thereafter, the IRF7 transactivating activity of IFN-α, particularly after virus infection, was confirmed

(Au et al., 1998). It forms a heterodimeric complex with IRF3 or a homodimeric complex with itself, through its C-terminal domains, and binds with type 1 interferon promoters, particularly IFN-α promoter (Lin et al., 2000). Therefore, there is a positive feedback mechanism between IRF7 and type 1 interferon secretion, particularly IFN-α. Beside its role in interferon regulation, IRF7 exerts many other regulatory functions inside the cell. Recently, it has been suggested that IRF7 protein can contribute to the anti-tumour effector functions of macrophages (Solis et al., 2006). The latter can be via down-regulation of the pro-angiogenic genes such as vascular endothelial growth factor gene (VEGF) and up-regulation of apoptotic genes such as tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), resulting in an increase in death of tumour cells (Solis et al., 2006).

Due to the close relationship between type 1 interferons and IRF7, viruses can interfere with the innate immune response, particularly interferon response, via IRF7 down-regulation. Many RNA and DNA viruses express proteins that modulate IRF7 levels in order to escape the innate immunity. This modulation can happen at the RNA level via the negative regulation of IRF7 mRNA levels, as the LF2 protein of EBV (Wu et al., 2009b). Also, it could be at the protein level via negative regulation of IRF7 protein stability, as KSHV-RTA (Yu et al., 2005). Finally, it could be at the post translational level via affecting on IRF7 phosphorylation status, such as KSHV-ORF45 (Zhu et al., 2002).

### 6.1.3 Chicken interferon regulatory factor 7 (chIRF7)

Chicken interferon regulatory factor 7 (chIRF7) was identified by Grant and colleagues as the first example of non-mammalian interferon regulatory factors (Grant et al., 1995). It was initially named chicken interferon regulatory factor 3 (chIRF3), but higher DNA sequence homology with human IRF7 (33% to IRF3, but 42% to IRF7) led to being renamed chIRF7 (Grant et al., 2000). Like human IRF7, it has an N-terminal DNA binding domain (DBD) and a C-terminal IRF association domain (IAD). The N-terminal DNA binding domain binds specifically to the ISRE sequence present in the promoter of the chicken Mx1 gene, where it binds as a

homodimer and activates transcription (Grant et al., 1995; Grant et al., 2000). Therefore, the chIRF7 protein contributes in both interferon signalling and production processes. To date, there has been no study of the interplay between any avian viral protein and the chicken IRF7 (chIRF7). This study identifies a protein encoded by an avian virus that negatively regulates the chIRF7 level and therefore negatively regulates type 1 interferon production.

### 6.1.4 Aim

Viruses have evolved different strategies to interfere with components of the host immune system. The work described here identifies the mechanism behind Meq down-regulation for chIFN-α and in particular the interplay between Meq and chIRF7. This was achieved through investigation of Meq over-expression on both the RNA and protein levels of chIRF7. In addition, the localisation pattern of the two proteins, when they were co-expressed together in the same cell, was investigated.

## 6.2 Results

### 6.2.1 Transient expression of Meq or LMeq has no effect on the endogenous chIRF7 mRNA level

The luciferase reporter assays described in Chapter 5 outlined Meq and LMeq as antagonists of chIFN-α. In order to determine if this antagonism is due to a direct effect on chIRF7, the mRNA levels of chIRF7 in the presence or absence of Meq or LMeq were investigated. DF-1 cells grown o/n in 48 well plate were transfected with each Meq, LMeq or eGFP, six wells each. The transfection was done as described in Section 2.2.1. After 48 hr, three wells were treated with 1000U/well of chIFN-α or left untreated. After 15 hr, total RNA was extracted and quantitative real-time PCR was performed to measure the chIRF7 mRNA levels (see Section 2.9.4). A standard house keeping gene, β-actin, was used as an internal control for data normalisation. The quantitative real-time PCR showed a strong up-regulation of chIRF7 in the chIFN-α-treated samples compared with untreated samples (Figure 6.2.1). The

presence of Meq or LMeq did not influence the levels of chIRF7 mRNA in the untreated or the chIFN-α treated samples, compared to the eGFP transfected cells.

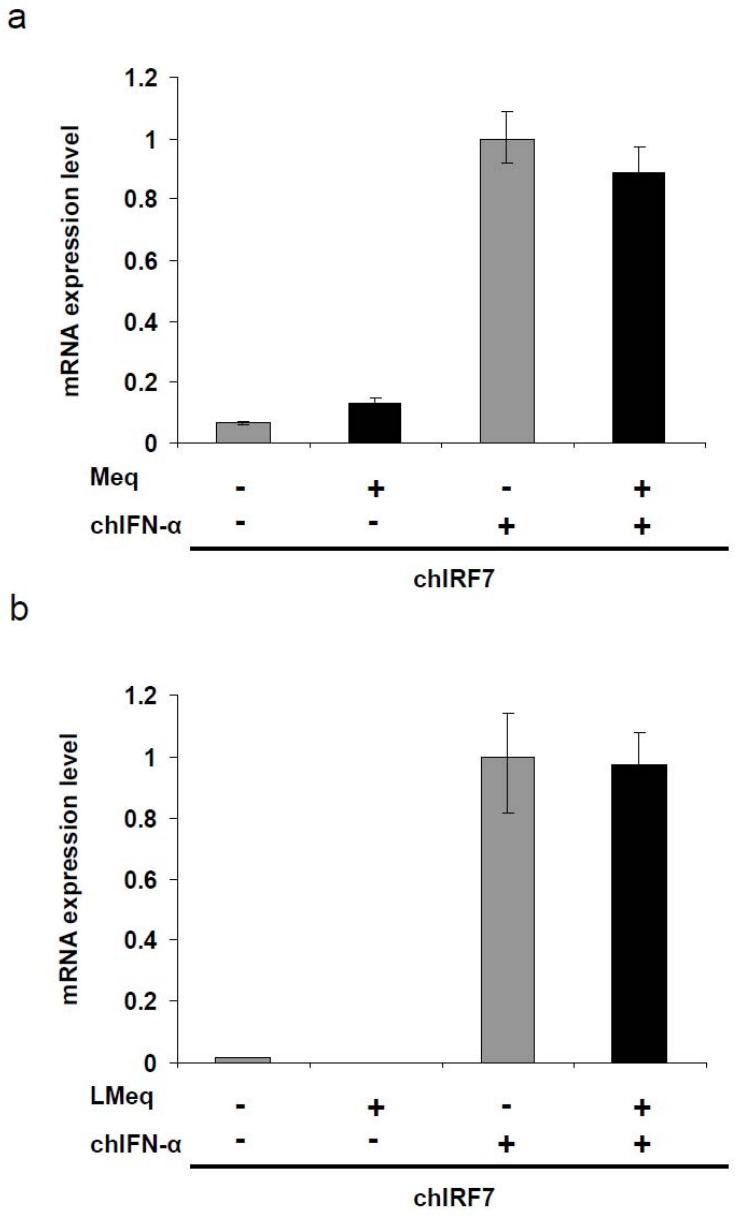



**Figure 6.2.1: Meq and LMeq have no effect on chIRF7 mRNA level.** DF-1 cells were transfected with either Meq (a) or LMeq (b). Transfected Cells were grown for 48 hr prior to stimulation with 1000U of chIFN-α per well. After 15 hr from chIFN-α treatment, total RNA was extracted and one-step quantitative real-time PCR was performed. All data were normalised to β-actin as an internal control, and values were expressed relative to the eGFP interferon-treated samples values. Grey bars represent the eGFP transfected samples while black bars represent the Meq (a) or LMeq (b) transfected samples.

### 6.2.2 Cloning and expression of chicken interferon regulatory factor 7 (chIRF7)

These data (Section 6.2.1) indicate that the transient expression of Meq or LMeq has no effect on chIRF7 mRNA level. To investigate the effect of Meq expression on chIRF7 protein level and due to the lack of commercially available specific antibody for the chIRF7 protein, it was necessary to clone a construct encoding the DNA sequences of the chIRF7 in a tagged vector. Three chicken cDNA clones (pgr1n.pk007.g20, C0000892J09_T7 and chEST39n20), encoding overlapping fragments of the chIRF7 DNA sequences, were obtained from Ark-Genomics laboratory (Roslin institute) to construct a full length cDNA. The three cDNA clones cover bases 1-671, 465-1145 and 907-1476, respectively. Semi-nested PCR was performed using the three cDNA clones as overlapping templates. Forward and reverse primers of the chIRF7 (see Appendix B) were designed based on published sequences of gallus gallus_IRF7 (accession number NM_205372.1). PCR conditions were as described in Section 2.5.3.

The amplified fragment was cloned in pDONR 207 using Gateway® cloning technology, and the cloning was confirmed by BanII restriction digest and sequencing (Figure 6.2.2a,b). Sequencing was performed commercially using the forward and the reverse primers of pDONR 207 vector (GATC Biotech). The sequence of the cloned chIRF7 was correct for all three examined clones, with the exception of a single mutation present in all clones. This mutation was in the 254 aa, in which isoleucine has shifted to valine. As it was present in all three clones sequence, this consistent mutation is likely existed in the template used in the amplification process. Only one of the sequenced clones was subcloned into GATEWAY® compatible destination vectors, such HA-tagged pCR3 vector, Myc-tagged pCR3 expression vector, pGBKT7 (bait vector) and pGADT7 (prey vector). Cloning was confirmed by restriction digestion using EcoRV (pCR3) or EcoRI & BamHI (pGBKT7and pGADT7).

To test the expression of the cloned chIRF7, DF-1 cells were seeded in a 24 well plate at $1x10^5$ cells per well. After 24 hr the cells were transfected with 500 ng of HA-tagged N-terminal chIRF7 (HA-chIRF7) expression plasmid. Forty eight hours later, total cell lysates were harvested and subjected to SDS-PAGE and immunoblotting using an anti-HA antibody followed by HRP-conjugated goat anti-rat secondary antibody. As shown in Figure 6.2.2c, the chIRF7 is strongly expressed with a molecular weight of ~ 64 kDa.

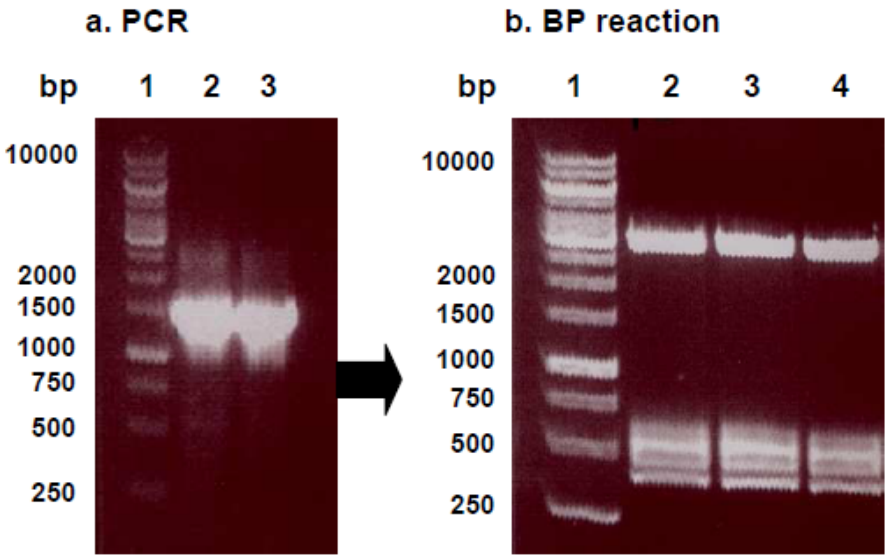



**Figure 6.2.2a,b: Cloning of chIRF7 into the Gateway® system.** Scanned images of ethidium bromide stained 1% TAE agarose gel which show: (a) PCR product of chIRF7; lane 1 contains 1 kbp DNA ladder (Fermentas), lane 2 and 3 contain chIRF7 DNA, (b) BanII digests of chIRF7 clones in the entry vector pDONR 207; lane 1 contains 1 kbp DNA ladder (Fermentas), while lane 2-4 represent DNA isolated from three different clones.

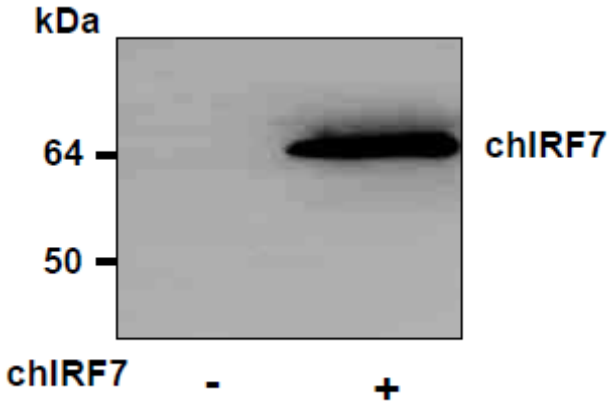



**Figure 6.2.2c: Expression of chIRF7 in the DF-1 chicken cell line.** DF-1 cells grown in 24 well plate were transfected with 500 ng DNA of HA-tagged N-terminal chIRF7 expression plasmid. After 48 hr, cells were harvested and lysed. The cell lysates were subjected to SDS-PAGE and immunoblotting using anti-HA primary antibody followed by HRP-conjugated goat anti-rat secondary antibody.

### 6.2.3 Co-IP has not detect direct interaction between Meq and chIRF7

The presence of direct protein-protein interaction between Meq and chIRF7 was investigated using a co-immunoprecipitation experiment. Meq_pGADT7 (prey) was transfected with empty pGBKT7 plasmid (negative control for the non-specific pull-down) or chIRF7_pGBKT7 (bait) into QM7 cells. Also, chIRF7_pGBKT7 (bait vector) was transfected with empty pGADT7 plasmid (negative control for the non-specific pull-down) into QM7 cells. The transfection was done using calcium phosphate transfection method (see Section 2.2.3). Simultaneously, cells were infected with recombinant vaccinia virus expressing T7 polymerase. Cell lysates were split into two parts and precipitated with either anti-c-Myc or anti-HA antibody (See section 2.3.3). Each precipitate was separated on two polyacrylamide gels. On the left side of the gel the precipitating proteins, whereas on the right side the co-precipitating proteins. The precipitation was performed to control the expression levels and the correct size of the tested protein. Co-IP has not detected direct interaction between Meq and chIRF7. Hence, either Meq or chIRF7 failed to co-

precipitate with the empty plasmids (negative controls) and with each other (Figure 6.2.3).

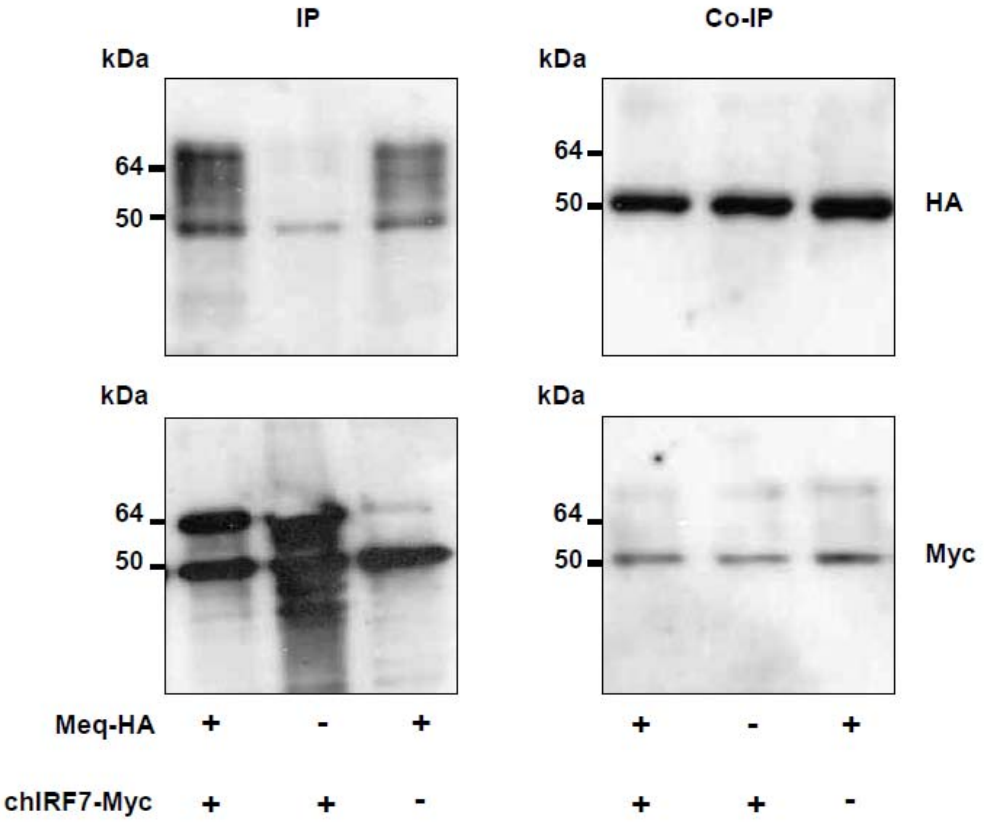



**Figure 6.2.3: No direct interaction between Meq and chIRF7 detected by Co-IP.** QM7 cells were co-transfected with Meq_pGADT7 & chIRF7_pGBKT7, chIRF7_pGBKT7 & empty pGADT7 and Meq_pGADT7 & empty pGBKT7. The cells were simultaneously infected with recombinant vaccinia virus expressing T7 polymerase. Twenty four hours post transfection, cells were lysed and whole cell extracts were precipitated with either anti-c-Myc or anti-HA antibody. Neither Meq nor chIRF7 were found to co-precipitate.

### 6.2.4 Meq down-regulates chIRF7 at the protein level

In the Co-IP experiment, it was observed that while Meq and chIRF7 did not directly interact, the amount of the precipitated chIRF7 was lower in the presence of Meq (Figure 6.2.3). This observation led to an investigation of the effect of Meq expression on chIRF7 protein level. DF-1 cells were seeded into 6 well plate at a density $8x10^5$ cells per well. After 24 hr, five wells were transfected with constant amount of HA-tagged N-terminal chIRF7 expression plasmid (HA-chIRF7) and increasing amounts of Myc-Meq. The transfection was done as described in Section 2.2.2. The total amount of the transfected DNA was 2 μg per well (1 μg HA-tagged N-terminal chIRF7 expression plasmid and 0 ng, 250 ng, 500 ng, 750 ng or 1 μg of Myc-tagged Meq expression plasmid). eGFP control plasmid was used to fill up the total amount of DNA up to 2 μg. After 72 hr, the total cell lysates were harvested, and the total protein concentrations were measured. The total amount of the loaded protein was 10 μg of each sample, and it was loaded twice into two SDS gels. Thereafter, the samples were subjected to SDS-PAGE and immunoblotting. The immunoblotting was performed using either the anti-c-Myc or anti-HA antibodies followed by the HRP-conjugated goat anti-mouse or HRP-conjugated goat anti-rat secondary antibodies, respectively. Then, the membrane was stripped off and subjected to another immunoblotting, using the anti-β-actin antibody followed by the HRP-conjugated goat anti-rabbit secondary antibody. In this system, it was observed that there was a dose-dependent down-regulation of chIRF7 protein level coordinated with the increasing amount of Meq expression (Figure 6.2.4). These data suggest Meq may act as a negative regulator for chIRF7 at the protein level and in a dose-dependent manner.

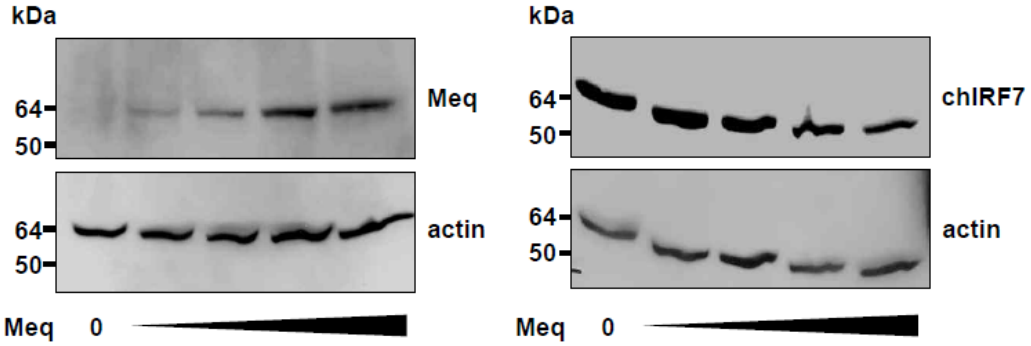



**Figure 6.2.4: Dose-dependent down-regulation of chIRF7 by Meq.** DF-1 cells were transfected with an HA-tagged chIRF7 expression plasmid in combination with increasing amounts of a Myc-tag Meq expression plasmid (0-ng, 250 ng, 500 ng, 750 ng or 1 µg). After 72 hrs, cells were lysed and the lysates were analysed by immunoblotting with either an anti-HA primary antibody (chIRF7), an anti-c-Myc (Meq) or an anti-β-actin primary antibody followed by HRP-conjugated secondary antibodies.

### 6.2.5 Meq affects chIRF7 protein stability

To further study the effect of Meq on chIRF7, we aimed to determine if Meq influenced the half-life of chIRF7 protein turnover. Another transfection experiment was carried out, in which protein synthesis was blocked by treating cells with the protein synthesis blocker (cycloheximide). DF-1 cells were seeded in 6 well plates with $8x10^5$ cells per well. After 24 hr, cells were transfected with 2 µg of total DNA in each well. The transfected DNA complex was composed of 1 µg of HA-tagged N-terminal chIRF7 expression plasmid and either 1 µg of Myc-tagged N-terminal Meq expression plasmid or 1 µg of eGFP control plasmid (see Section 2.2.2). After 72 hours, the cells were treated with 50 µg/ml cycloheximide. Thereafter, the cells were harvested at different time points. The protein concentrations of the cellular lysates were measured, and 10 µg of the total protein concentration from each sample was subjected to SDS-PAGE and immunoblotting. The immunoblotting was performed using the anti-HA antibody followed by the HRP-conjugated goat anti-rat secondary antibody. Then, the membrane was stripped off and subjected to another immunoblotting using the anti-β-actin antibody followed by the HRP-conjugated

goat anti-rabbit secondary antibody. Western blot films were quantified by using Quantity One software (Bio-Rad, Richmond, CA).

As shown in Figure 6.2.5, chIRF7 protein degraded gradually either in the presence or absence of Meq protein. In the presence of Meq, the half-life of the chIRF7 protein decreased from approximately 24 hr to approximately 4 hr.

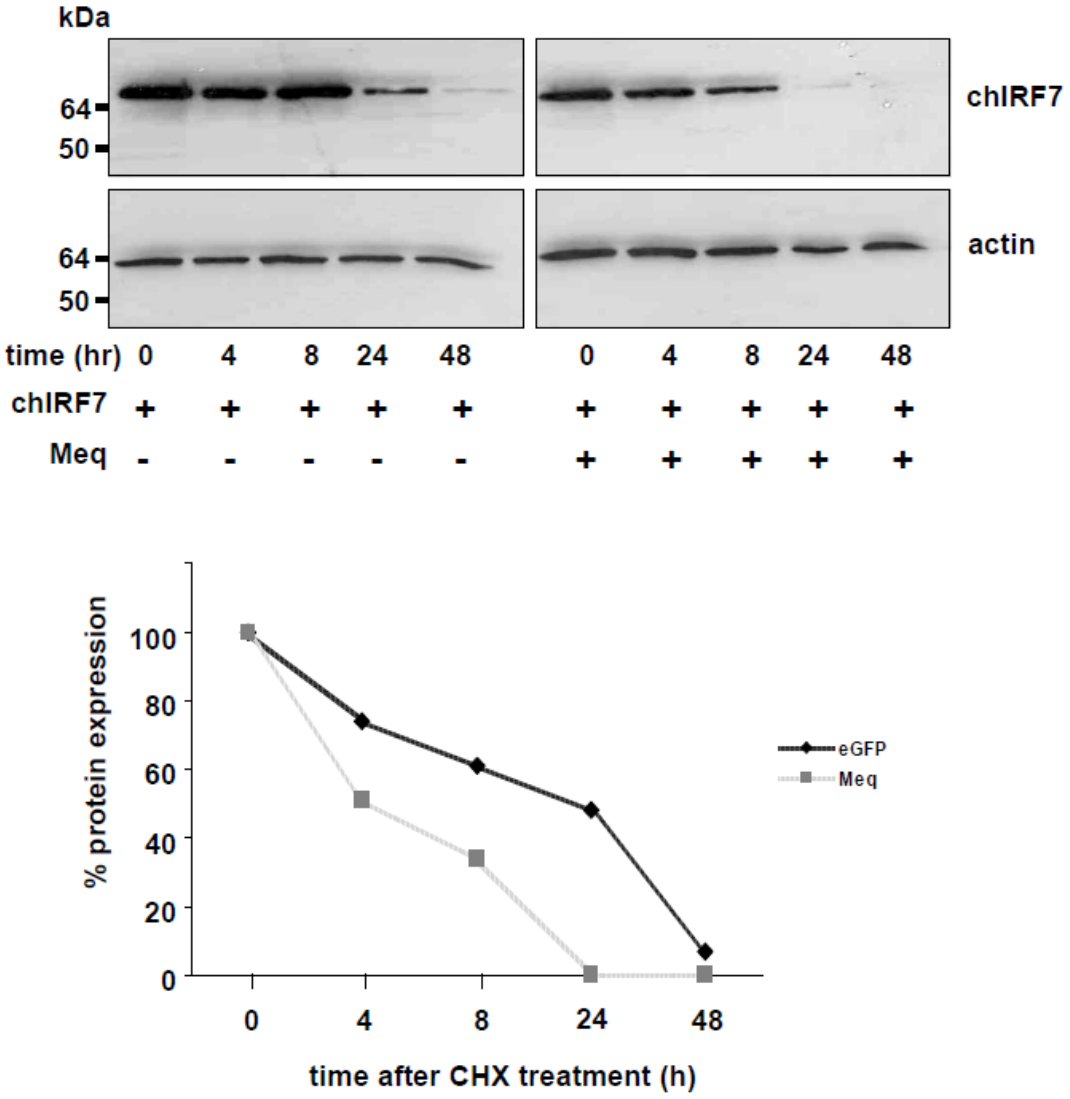



**Figure 6.2.5: Meq shortening chIRF7 protein half-life.** DF-1 cells were transfected with an HA-chIRF7 expression plasmid in combination with a Myc-Meq expression plasmid (+) or an eGFP control plasmid (-). After 72 hr, cells were treated with 50 µg/ml cycloheximide and harvested and lysed at different time points. The samples were analysed by immunoblotting using an anti-HA primary antibody (chIRF7) or an anti-β-actin primary antibody followed by HRP-conjugated secondary antibodies. Western blot films were quantified by using Quantity One software (Bio-Rad, Richmond, CA), and each sample was normalised using the actin signal.

### 6.2.6 Meq degrades chIRF7 in a proteasome-dependent pathway

To test if Meq-induced chIRF7 protein degradation is a proteasome-dependent, DF-1 cells were seeded in 6 well plate at seeding density $8x10^5$ cells per well. After 24 hr, cells were transfected with 2 μg of total DNA in each well (see Section 2.2.2). The transfected DNA complex was composed of 1 μg of HA-tagged N-terminal chIRF7 expression plasmid and either 1 μg of Myc-tagged N-terminal Meq expression plasmid or 1 μg of eGFP control plasmid. After 72 hours, the cells were treated with 50 μg/ml cycloheximide alone, 50 μg/ml cycloheximide and 100 μM MG132 or DMSO alone. Thereafter, the cells were harvested after 24 hr. The protein concentrations of the cellular lysates were measured and 15 μg of protein from each sample was subjected to SDS-PAGE and immunoblotting. The immunoblotting was performed using the anti-HA antibody followed by the HRP-conjugated goat anti-rat secondary antibody. Thereafter, the membrane was stripped off and subjected to another immunoblotting using the anti-β-actin antibody followed by the HRP-conjugated goat anti-rabbit secondary antibody. This investigation showed that by inhibiting proteasome activity, the chIRF7 protein level was restored, and chIRF7 degradation was inhibited inside the cells (Figure 6.2.6). This result suggested that Meq in fact mediates chIRF7 protein degradation via a proteasome-dependent pathway.

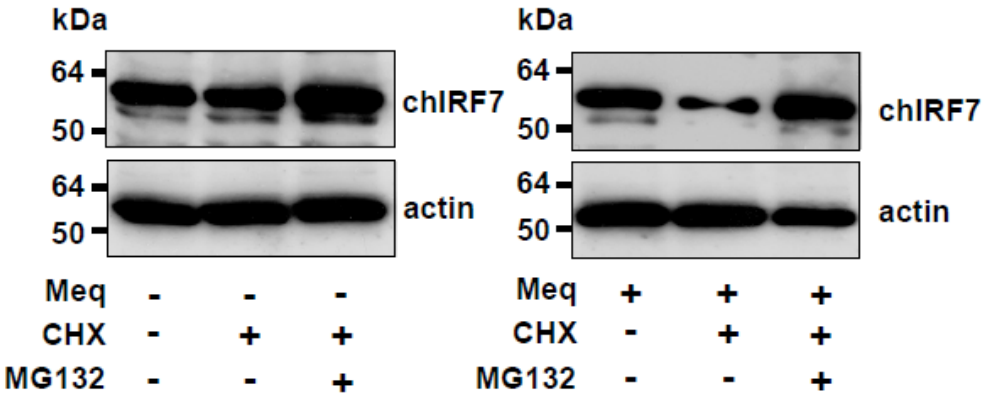



**Figure 6.2.6: Proteasome-dependent reduction of chIRF7 protein stability by Meq.** DF-1 cells were transfected with an HA-chIRF7 expression plasmid in combination with a Myc-Meq expression plasmid (+) or an eGFP control plasmid (-). After 72 hr, cells were treated with DMSO, 50 µg/ml cycloheximide or 50 µg/ml cycloheximide and 100 µM MG132. After 24 hr, cells were harvested, lysed and the samples were analysed by immunoblotting using an anti-HA primary antibody (chIRF7) or an anti-β-actin primary antibody followed by HRP-conjugated secondary antibodies.

### 6.2.7 Meq auto-regulates its protein level in the presence of chIRF7

The previous experiments indicated that Meq targets chIRF7 and consequently induces its degradation in a proteasome-dependent pathway. To investigate if the degradation of the both proteins is linked to each other, another transfection experiment was performed. In this experiment, DF-1 cells were seeded into 6 well plate with $8x10^5$ cells per well. After 24 hr, five wells were transfected with 2 µg of total DNA each, and the DNA transfection complex was composed of 1 µg of N-terminal Myc-tagged Meq expression plasmid (Myc-Meq) and increasing quantities of N-terminal HA-tagged chIRF7 expression plasmid (0 ng, 250 ng, 500 ng, 750 ng or 1 µg). eGFP control plasmid was used to fill up the total amount of DNA up to 2 µg. Seventy two hours later, total cell lysates were harvested, and the total protein concentrations were measured. The total amount of the loaded protein was 10 µg of each sample, and the samples were subjected to SDS-PAGE and immunoblotting. The immunoblotting was performed using anti-HA, anti-c-Myc and anti-β-actin

antibodies followed by HRP-conjugated goat anti-rat, HRP-conjugated goat anti-mouse and HRP-conjugated goat anti-rabbit secondary antibodies, respectively.

This experiment showed that there was a dose-dependent down-regulation in the Meq protein expression level coordinated with the increasing amount of chIRF7 expression (Figure 6.2.7). Therefore, the over-expression of chIRF7 in turn led to increase Meq degradation, indicating that the degradation of both proteins is linked to each other.

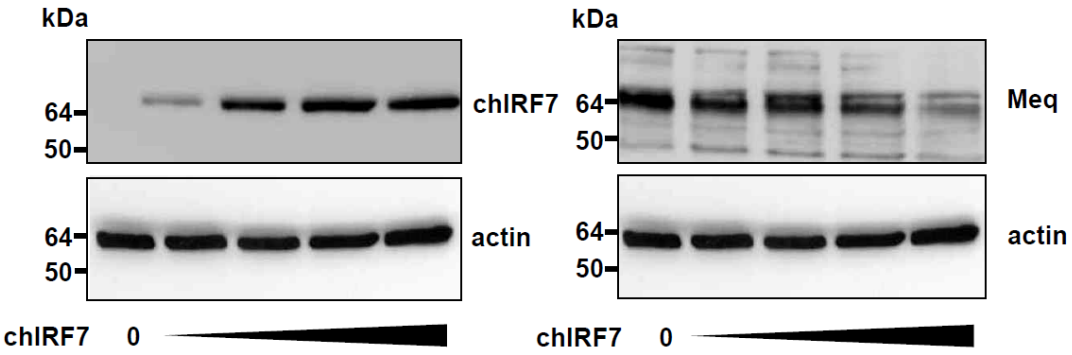



**Figure 6.2.7: Meq auto-regulates itself in the presence of chIRF7.** DF-1 cells were transfected with a Myc-Meq expression plasmid in combination with increasing amounts of an HA-chIRF7 expression plasmid (0 ng, 250 ng, 500 ng, 750 ng or 1 µg). After 72 hrs, cells were lysed and the lysates were analysed by immunoblotting with either an anti-HA primary antibody (chIRF7), an anti-c-Myc (Meq) or an anti-β-actin primary antibody followed by HRP-conjugated secondary antibodies.

### 6.2.8 Over-expression of chIRF7 leads to a cytoplasmic accumulation of Meq

Work described in the previous sections suggested that Meq-induced chIRF7 degradation is proteasome-dependent degradation. In fact, Meq is detected in the nucleus, when it is expressed alone. IRF7 is detected at the cytoplasm while its phophorylated version is detected in the nucleus, when it is expressed alone. Therefore, it was of interest to investigate microscopically the localisation of the two proteins when they are over-expressed together in the same cell. DF-1 cells grown on chamber glass slide were transfecetd with an HA-tagged chIRF7 expression plasmid

(HA-chIRF7), a Myc-tagged Meq expression plasmid (Myc-Meq) or both (see Section 2.2.1). After 72 hr from the transfection, cells were fixed and subjected to immunofluorescence staining (see Section 2.3.7). Cells were dually stained with anti-c-Myc, mouse monoclonal and anti-HA high affinity, rat monoclonal antibodies. Then, the fluorescent signals were detected using Alexa fluor® 488 goat anti-mouse IgG (green) and Alexa fluor® 594 goat anti-rat IgG (red) conjugated antibodies.

As presented in Figure 6.2.8, the upper panel of the images shows chIRF7 localised in the cytoplasm and nucleus. The middle panel of the images shows Meq localised in the nucleus only. If both proteins were coexpressed, the lower panel of the images, Meq could be detected both in the nucleus and in the cytoplasm, again suggesting that both proteins are targeted for proteasomal degradation if present simultaneously in the same cell.

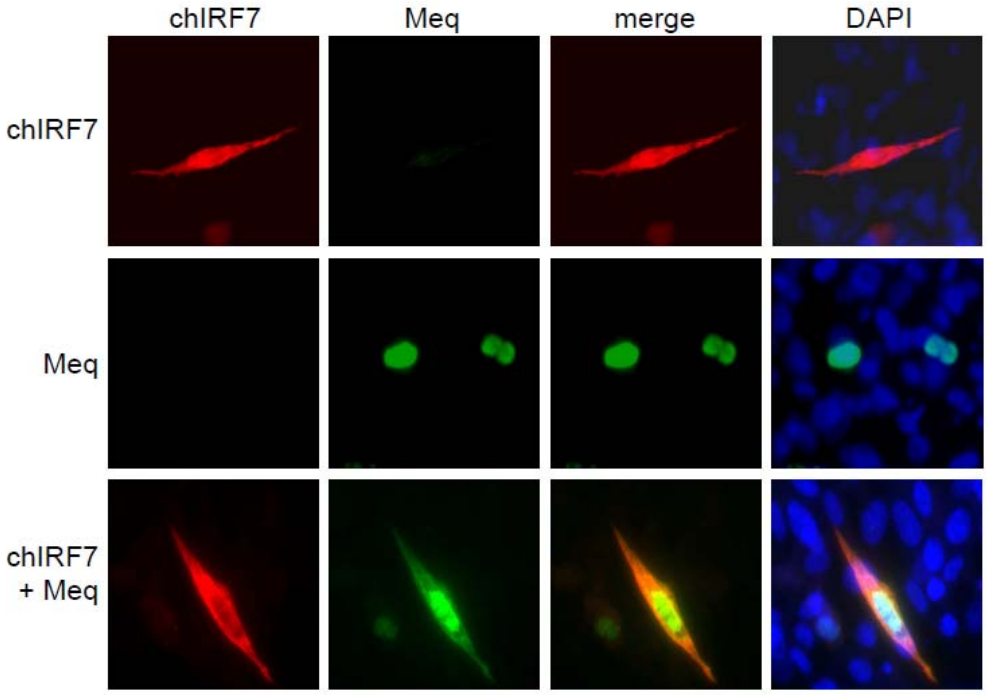



**Figure 6.2.8: Over-expression of chIRF7 leads to a cytoplasmic accumulation of Meq.** DF-1 cells were transfected with either an HA-tag chIRF7 expression plasmid (HA-chIRF7), a Myc-tag Meq expression plasmid (Myc-Meq) or both. After 72 hr, the slides were fixed and dually immuno-stained with anti-HA and anti-c-Myc primary antibodies. Then, the fluorescent signals were detected using Alexa fluor® 488 goat anti-mouse IgG (green) and Alexa fluor® 594 goat anti-rat IgG (red) conjugated secondary antibodies. The slides were examined using a LEICA DM LB2 fluorescent microscope.

## 6.3 Discussion

This chapter has focused on study of the mechanism behind chIRF7 down-regulation by Meq. Meq is the main MDV oncoprotein, and it is highly expressed in MDV-transformed T-cells (Ross et al., 1997; Liu et al., 1998). It can independently transform Rat-2, NIH3T3 and DF-1 cell lines (Liu et al., 1998; Levy et al., 2005; Ajithdoss et al., 2009). Recombinant virulent MDV lacking Meq (rMd5ΔMeq) provided the birds with higher protection than that provided by the Rispens strain (Lee et al., 2008). The mechanism by which Meq expressed inside the latently or transformed cells has remained uncertain yet. However, it is clearly that Meq owns a mechanism to evade the cellular and the innate immune response. Here it was demonstrated that Meq and its homologue in the vaccine strain LMeq were the most potent down- regulators for the interferon-alpha responsiveness of the chicken ISRE promoter (see Chapter 5). Meq immunosuppressive function is exerted via its interference with the chIRF7 at the proteomic level.

### 6.3.1 Co-IP has not detected interaction between Meq and chIRF7

Many viral proteins evade the innate immune response via binding with the interferon regulatory factors (IRFs). This binding leads to interruption of the IRFs dimerisation and consequently their DNA binding activity. This was the case of KSHV-vIRF3 protein and bICP0 proteins of BoHV-1 (Joo et al., 2007; Saira et al., 2009). Co-IP was not able to detect any direct binding between Meq and chIRF7. This result could be attributed to many factors such as the follows. There is no existed interaction between Meq and chIRF7, or the interaction is existed but the buffering conditions of the experiment interfere with the interaction stability. However, it is also possible that the interaction occurs, and as a result of this interaction chIRF7 undergoes certain modifications. This modification leads to chIRF7 degradation and down-regulation. The latter case was reported before for bICP0 protein of bovine herpesvirus, as bICP0 degrades bovine IRF3 protein, and therefore the two proteins can not be co-precipitated together (Saira et al., 2007). The

interesting observation from this experiment was that the precipitated chIRF7 in the case of Meq absence was more abundant, compared with Meq presence.

### 6.3.2 Meq negatively regulates chIRF7 and its own protein stability

One of the possible mechanisms to evade the innate immune response is targeting the innate immune system components for protein degradation. This mechanism has been used by the NDV V protein, the BoHV-1 bICP0 protein and the KSHV RTA protein. NDV V protein targets the degradation of the STAT1 protein, and mediates interruption for interferon-alpha pathway at the up-stream level of the ISRE transcriptional activity (Huang et al., 2003). On the other hand, bICP0 targets IRF3 for a proteasome-dependent degradation, therefore it mediates down-stream effect (Saira et al., 2007). Moreover, KSHV RTA lytic protein encodes ubiquitin E3 ligase activity at its N-terminal, which targets IRF7 for degradation and down-regulation (Yu et al., 2005). The real-time PCR experiment showed that Meq and LMeq can not exert any down-regulation to the endogenous chIRF7 mRNA. The data presented here show that Meq down-regulates chIRF7 at the protein level and decreases its protein stability. Unlike the EBV LF2 protein, which is able to down-regulate IRF7 at transcriptional level (Wu et al., 2009b), Meq targets chIRF7 for a protein degradation. Similar to KSHV RTA, Meq auto-regulates its own protein level in the presence of chIRF7, possibly by creating a multiprotein complex with chIRF7 which is degraded together.

### 6.3.3 Meq enhances chIRF7 protein degradation via a proteasome-dependent pathway

Proteasome is cellular machinery that targets endogenous unneeded proteins for degradation. The targeted protein is mainly ligated with ubiquitin peptides via ubiquitin ligases and processed with the cellular proteasomes for degradation. Down-regulation of mammalians IRF7 protein stability and enhancing its degradation process is mainly via ubiquitin-dependent proteasomal degradation (Yu et al., 2005; Prakash and Levy, 2006). This study presented the first evidence that the chIRF7

protein undergoes proteasome-dependent degradation. It was demonstrated that the chIRF7 protein degradation was completely stopped after the cells were treated with the proteasomal-inhibitor drug MG132. This was the case in either the presence or absence of Meq. However, particularly in the presence of Meq, the high turnover of chIRF7 protein is totally reversed after inhibition of the cellular proteasomal activity. Therefore, during viral infection Meq may antagonise the interferon system via targeting chIRF7 for proteasome-dependent degradation. Meq may target chIRF7 either directly or indirectly. The direct effect could be via forming polyubiquitinated complex with chIRF7, which undergoes proteasomal degradation. The evidence for the previous hypothesis is that Meq also degrades in a dosage-dependent manner in the presence of chIRF7. However, we were not able to detect any direct physical binding between Meq and chIRF7, so Meq may also act indirectly through other cellular proteins that cause chIRF7 proteasomal degradation. Meq N-terminal domain contains three lysine residues at the amino acids 45, 84 and 101, which are predicted to be ubiquitin substrate residues (Tung and Ho, 2008), and therefore Meq is predicted to be a substrate for the ubiquitin protein modification, although this is not confirmed yet. Interestingly, a proteomic analysis of MDV infected spleen $CD4^+$ T-cells revealed that many proteins of the ubiquitin-proteasome protein degradation system were up- or down-regulated (Thanthrige-Don et al., 2009).

### 6.3.4 Over-expression of chIRF7 leads to a cytoplasmic accumulation of Meq

Most of viral proteins, which encode interferon antagonistic function, have nuclear localisation signals such as bICP0 of BoHV-1, ICP0 of HSV-1, LF2 of EBV and RTA of KSHV. Localisation of RTA protein inside the nucleus leads to IRF7 retaining in the cytoplasm and inhibition of IRF7 entrance into the nucleus (Yu et al., 2005). bICP0 interacts with IRF7 inside the nucleus, and interrupts IRF7 binding to the interferon-β promoter (Saira et al., 2009). On the other hand, LF2 expression inside the nucleus has no effect on IRF7 nuclear localisation. In our study, Meq presence inside the nucleus inhibits chIRF7 entrance to the nucleus. Furthermore, shift in Meq localisation to the cytoplasm was detected.

Overall and from all the above mentioned results, a model for the interferon-alpha antagonistic function displayed by Meq can be hypothesised (Figure 6.3). We have concluded that the presence of Meq inside the nucleus inhibits chIRF7 entrance to the nucleus and initiating the transcriptional activitation for type 1 interferons inducible promoter. Moreover, Meq acts as a suicide gene that targets the chicken IRF7 directly or indirectly. As a result of this targeting which mainly happens at nuclear rim or cytoplasm, the proteasomal degradation of Meq and chIRF7 started. It is also very likely that Meq acts as ubiquitin source for chIRF7 degradation, although there is no evidence for that.

From this study, it can be concluded that MDV utilises Meq, the main oncoprotein, to evade the host interferon-signalling. Thus, using of vaccine lacking Meq or LMeq will provide the birds with better immune response and better protection rate. Also, this study provides additional data for the role of the basic leucine zipper containing protein in the evading of interferon signalling. Thereby, Meq is considered as a very good hit for the drug designers to develop antiviral therapy.

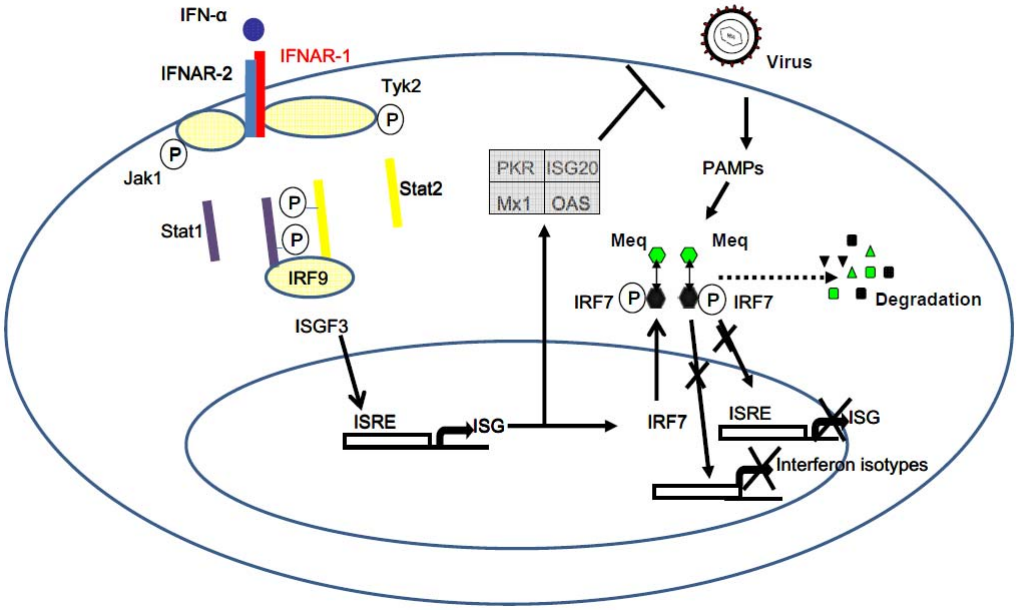



**Figure 6.3: Model illustrating Meq mediated proteasomal degradation of chIRF7.** Meq targets chIRF7 and itself for cytoplasmic proteasomal degradation. Therefore, it inhibits chIRF7 entrance to the nucleus, in order to initiate the transcriptional activity of type 1 interferon inducible promoters and type 1interferon promoters.

# Investigation of Meq-deletion MDV mutant sensitivity for chicken interferon-alpha treatment

## 7.1 Introduction

The cloning of RB-1B MDV as a bacterial artificial chromosome (pRB-1B5) has produced an extremely useful tool for the molecular analysis of the MDV genome (Petherbridge et al., 2004). *In vitro* and *in vivo* experiments revealed growth properties of pRB-1B5 which can cause visceral tumours similar to the wild-type RB-1B virus (Petherbridge et al., 2004). pRB-1B5 mutants have been generated to investigate the roles of various MDV genes such as UL13, RLORF4, RLORF5a and Meq genes (Jarosinski et al., 2005; Blondeau et al., 2007; Jarosinski et al., 2007b). The pRB-1B-D2 MDV mutant was engineered by Brown and colleagues by replacing the two copies of the Meq gene with a marker-less cassette (Brown et al., 2006). This pRB-1B5 Meq knock-out mutant virus has been used to investigate the role of Meq in viral life cycle and in tumour formation. As an example, this mutant highlighted the importance of the physical interaction between Meq and CtBP for tumour formation (Brown et al., 2006). Furthermore, pRB-1B-D2 MDV was used to identify the contribution of Meq's leucine zipper domain in MDV latency and transformation properties (Brown et al., 2009).

Work described here has used pRB-1B-D2 MDV to study the interplay between MDV infection and chIFN-α signalling pathway in CEF cells, particularly in the case of Meq absence.

### 7.1.1 Aim

To verify the role of Meq in the down-regulation of chIFN-α signalling in the context of MDV infection, the plaque formation efficiency of the wild-type pRB-1B5 and pRB-1B-D2 viruses were compared in the presence or absence of chIFN-α treatment.

Levels of mRNA encoding endogenous chIRF7 were measured after infection with the two viruses in the presence or absence of chIFN-α treatment.

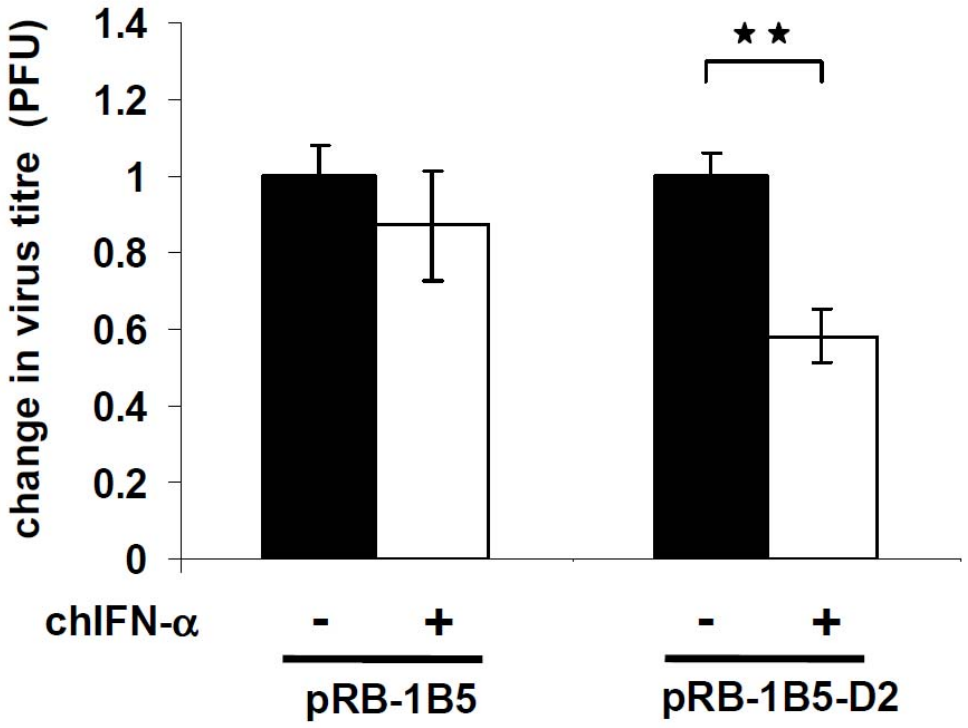
## 7.2 Results

### 7.2.1 Meq knock-out double deletion mutant is more sensitive to chIFN-α treatment compared to the wild-type virus

To study Meq's role in antagonising chIFN-α in the virus context, the plaque formation efficiency of pRB-1B5 and pRB-1B-D2 viruses was compared in the presence and absence of chIFN-α treatment.

Primary chicken embryo fibroblasts (CEF) cells were seeded into two 12 well plates, at a density of $4.1x10^5$ cells per well. After cells settled down, half of the wells in the two plates were treated with 500 U of chIFN-α per well in reduced serum conditions and the other half were mock treated in the same reduced serum condition. Twenty four hours later, one plate was infected with 50 PFU of pRB-1B-D2 virus and the other plate was infected with pRB-1B5 virus in normal 5% serum growth medium. Interferon treatment was renewed at 3 days post infection only. After five days, three wells from each treatment of the infected cells were fixed and stained with HB3 Ab as described in Section 2.10, and the plaques were counted using the ordinary light microscope.

As shown in Figure 7.2.1, both of pRB-1B-D2 and pRB-1B5 viruses were sensitive to chIFN-α treatment. Infection with 50 PFU of pRB-1B5 virus showed 10% reduction in the plaques number in case of chIFN-α treatment, compared to the untreated cells (Table 7.2.1). Whilst there was approximately a 40% reduction in the plaque number in case of infection with 50 PFU of pRB-1B-D2 and chIFN-α treatment, compared to the untreated cells (Table 7.2.1), the reduction in the plaques number was statistically significant only in the case of pRB-1B-D2 virus infection.




| Virus | chIFN-α | Average of plaque number | Normalisation |
|---|---|---|---|
| pRB-1B5 | - | 59.3 ±4.9 | 1.0 ±0.08 |
| | + | 51.7 ±8.5 | 0.9 ±0.14 |
| pRB-1B-D2 | - | 44.0 ±2.6 | 1.0 ±0.06 |
| | + | 25.7 ±3.1 | 0.6 ±0.07 |

**Figure 7.2.1: Effect of chIFN-α treatment on the plaque formation efficiency of pRB-1B-D2 MDV and pRB-1B5 MDV.** CEF cells were treated with 500U/ml of recombinant chIFN-α or kept untreated. Twenty four hours later, cells were infected with 50 PFU of wild-type pRB-1B5 MDV or pRB-1B-D2 MDV. The effectiveness of chIFN-α treatment on virus plaque formation was measured by normalisation of the chIFN-α-treated samples to the untreated samples. The black bars represent the non-interferon-treated samples, while the white bars represent the interferon-treated samples. The statistical analysis was done using Welch's T test (**P<0.01).

### 7.2.2 pRB-1B-D2 infected CEF cells have up-regulation of chIRF7 mRNA level

As shown in Section 7.2.1, pRB-1B-D2 MDV plaque formation efficiency was more sensitive to chIFN-α treatment compared to the wild-type virus. In order to investigate the effect of MDV infection on the endogenous chIRF7 mRNA expression level, in particular in case of Meq absence, quantitative real-time PCR experiment was performed.

In this experiment, chicken embryo fibroblasts (CEF) cells were seeded into two 12 well plates, at a density of $4.1x10^5$ cells per well. After the cells settling down, half of the wells in the two plates were treated with 500 U of chIFN-α per well in a reduced serum condition and the other half were mock treated under the same reduced serum conditions. Twenty four hours later, one plate was infected with 50 PFU of pRB-1B-D2 virus and the other plate was infected with pRB-1B5 virus in normal 5% serum growth medium. Interferon treatment was renewed 3 days post infection only. After five days, RNA was extracted from the infected cells, and real-time PCR was performed. Differences in the mRNA expression levels of chIRF7 were determined in the different samples, using β-actin as an internal control.

As shown in Figure 7.2.2, CEF cells infected with either pRB-1B5 or pRB-1B-D2 MDV showed significant up-regulation in chIRF7 mRNA expression level, compared to the mock treated cells infected with the same viruses. Particularly in case of pRB-1B-D2 MDV infection, the up-regulation of chIRF7 mRNA expression levels was more pronounced and highly significant in either interferon-treated or untreated samples.

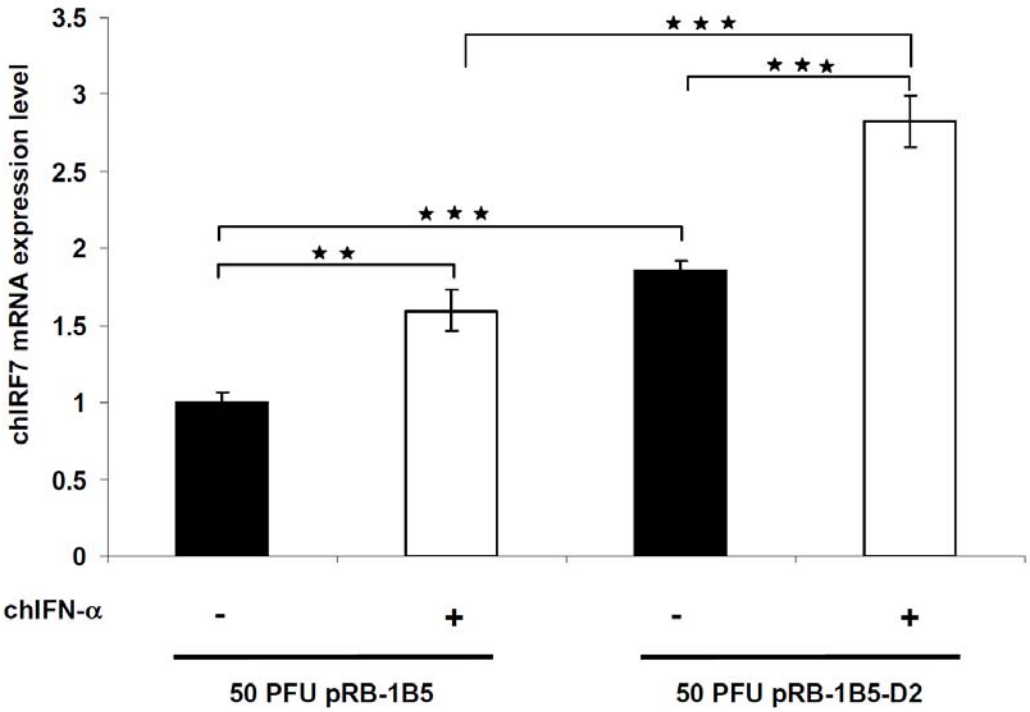



**Figure 7.2.2: Infection with Meq knock-out mutant up-regulates IRF7 mRNA after chIFN-α treatment.** CEF cells were infected with 50 PFU of either wild-type pRB-1B5 or pRB-1B-D2 MDV. The mRNA was extracted at the 5th day post infection. All the samples were normalised according to the non-interferon-treated samples which infected with pRB-1B5 MDV. The interferon-treated cells and infected with pRB-1B5 or pRB-1B-D2 MDV showed significant up-regulation in chIRF7 mRNA level compared to the non-treated cells infected with same viruses. The up-regulation was more significant in case of infection with pRB-1B-D2 MDV. The statistical analysis was done using Welch's T test (**P<0.01) and (***P<0.001).

## 7.3 Discussion

MDV infection is associated with the development of the innate immune response (Abdul-Careem et al., 2009a), nevertheless MDV infections progress and virus's genome load increases in infected birds. Many studies suggest that MDV has evolved several mechanisms to evade the host immune response (Thanthrige-Don et al., 2010).

In this study, evidences are provided for Meq down-regulation of chIFN-α by inducing a proteasome-dependent degradation of chIRF7 protein. Although in our screen Meq was not the only MDV gene that restricts chIFN-α response, it was of curiosity to test the effect of Meq absence on virus growth, in the presence of chIFN-α.

### 7.3.1 Meq knock-out double deletion mutant is more sensitive to chIFN-α treatment compared with the wild-type virus

Viral mutagenesis is considered a beneficial tool for functional analysis of viral proteins. Indeed, genetic modification of the herpesvirus genome was the reason for discovering many of its interferon antagonistic encoded hits. By testing the growth of the deletion mutant compared with the wild type virus in the presence or absence of exogenous antiviral cytokines, the contribution of the deleted gene in the expression of innate immunity can be investigated. For instance, the growth of ICP0 knock-out HSV-1 was severely impaired in the presence of IFN-α compared with the wild type virus (Mossman et al., 2000). Another example, the growth of VZV mutant lacking ORF63 was severely impaired in the presence of IFN-α compared with its absence (Ambagala and Cohen, 2007).

Since Meq is the main MDV oncogene, in recent years various Meq-deletion MDV mutants have been constructed and studied (Brown et al., 2006; Lee et al., 2008). These mutants were tested in vitro and in vivo for their ability to replicate and induce tumours. However, it was found that vaccination with rMd5V lacking Meq, provided

birds with 100% protection against challenge with a very virulent plus MDV strain (Lee et al., 2008; Lee et al., 2010). Furthermore, the protection exerted by this mutant was superior to the protection exerted by the Rispens vaccine strain. Thus indicating that the lack of Meq correlates with improving the host immune response and that Meq may encode some immunomodulatory functions. The data presented here demonstrated that Meq expression correlates with the modulation of the chicken's innate immune response during MDV infection. Although the Meq-deletion mutant was able to grow and form plaques in the presence of chIFN-α, there was a significant difference in the plaque formation efficiency of RB-1B-D2 MDV in the presence of chIFN-α, compared to the wild-type virus. This confirms the contribution of Meq in antagonising IFN-α signalling in the virus context.

### 7.3.2 Analysis of chIRF7 mRNA levels following infection with pRB-1B5 and pRB-1B-D2

After virus infection, cells produced IFN-α and IFN-β which generate waves of signals to create an antiviral state inside the cells. These signals lead to secretion of group of antiviral cytokines and transcription factors such as Mx1 and IRF7, respectively (Muller-Doblies et al., 2002). The existence of the lytic infection of the gammaherpesvirus, EBV, is associated with marked down-regulation of IRF3 and IRF7 at both the RNA and protein levels (Bentz et al., 2010). MDV also induces lytic infection in CEF, but no previous studies have recorded the modulation of the chIRF7 during MDV infection. Furthermore, there is no commercially available antibody with specificity for chIRF7 protein, and therefore the estimation of the endogenous chIRF7 protein level seems to be impossible. The availability of the chIRF7 sequence makes it easier to analyse chIRF7 mRNA expression levels, using quantitative real-time PCR technology. This study quantified the change in the chIRF7 mRNA expression level after infection with either pRB-1B5 or pRB-1B-D2 MDV, in the presence or absence of chIFN-α. There was no change in chIRF7 mRNA level in Meq transfected cells (see Section 6.2.1), however this investigation aimed to detect the indirect effect which results from the positive feedback loop

between chIFN-α and chIRF7. Both pRB-1B5 and pRB-1B-D2 MDV infected cells have up-regulation of the chIRF7 mRNA levels in case of IFN-α treatment. This up-regulation was more pronounced and more significant in case of Meq-deletion MDV (pRB-1B-D2) compared with wild-type virus (pRB-1B5). This finding confirmed that the expression of Meq in the virus context counteracts chIRF7 level, subsequently negatively regulating chIFN-α levels and the innate immune response. As mentioned above, it is very likely that the difference in the mRNA level in the virus context is due to a positive feedback loop, especially since chIRF7 mRNA levels were measured at 5 dpi. Hence, the down-regulation of chIRF7 protein stability leads to decreased chIFN-α levels and subsequently decreases the transcriptional activity of the ISRE promoter including chIRF7 mRNA levels.

The presence of Meq in the MDV genome has been confirmed and implicated in the down-regulation of chicken innate immune responses, namely chIFN-α signalling and production. Moreover, this role was clear in the virus context by modulation of chIRF7 levels during CEF lytic infection. Perhaps, the role of Meq becomes clearer during MDV latent infection and tumour formation, especially as recent findings suggested that IRF7 protein encodes anti-tumour activity (Solis et al., 2006; Goubau et al., 2009).

# Final discussion and future work

Marek's disease virus was originally described in 1907, and since then the virus has developed a smart evolution strategy to replicate inside the host cells and increase its virulence. Moreover, MDV can exist and remain latent in chicken host cells masking itself from the chicken's immune response. MDV is also considered a virus of biomedical importance. It has a genomic structure similar to HSV-1 and VZV of Alphaherpesvirinae (Cebrian et al., 1982; Buckmaster et al., 1988; Osterrieder et al., 2006), and it exerts biological properties similar to EBV of Gammaherpesvirinae. Using molecular approaches to understand the role played by each of the MDV genes, on a genome-wide scale, becomes necessary. Better understanding of MDV's molecular nature and composition will lead to the identification of essential components of the MDV genome required for viral replication and/or interaction with host cells. These components can be used by drug designers as targets to knock-down virus replication or infection. Additionally, it can help to develop an innovative MDV vaccine that can overcome the disadvantages of the already existing MDV vaccine. As example, the deletion of either vIL8 or Meq gene from the MDV genome led to engineering of new vaccine constructs, which provide the birds with a protection similar to or sometimes better than established MDV vaccines (Cui et al., 2005b; Lee et al., 2008; Lee et al., 2010).

This study involved construction of the first genome-wide clone collection library for the MDV genome. The MDV ORFeome library was created in order to perform the first systematic functional analysis of MDV genes. By testing their interactions with themselves or the chicken interferon-alpha pathway, very useful information was obtained regarding the role played by each of the MDV genes. Recent studies suggested that the MDV genome encodes more than 400 ORFs (Spatz et al., 2007c), however not all of them are truly functional and are expressed during the course of MDV infection. Only the proteins which are more likely to be expressed during the viral infection have been studied (Lee et al., 2000a; Tulman et al., 2000). The MDV

ORFeome library is composed of 149 MDV clone constructs. These clones represent 114 functional genes cloned as full length, 4 functional genes cloned as fragments (10 fragments) and 25 extra and intra-cytoplasmic domains for the membrane-associated proteins. Two MDV strains which are different in their virulence level were used to clone the different genes from the two genomes. This library was cloned into pDONR 207 using the Gateway® cloning system and confirmed by sequencing. The cloned library was later subcloned into different destination vectors to be used for different purposes (discussed in the previous chapters).

Y2H assay is a technique used to analyse protein-protein interactions, especially on a genome-wide scale. It was used previously to investigate interactions between proteins encoded by many members of the Herpesviridae family (Calderwood et al., 2007; Rozen et al., 2008; Fossum et al., 2009), and it led to the identification of many biological facts of herpesviridae. One example is the formation of the nuclear egress complex between UL31 and UL34 in many members of the herpesviridae (Fuchs et al., 2002c; Lake and Hutt-Fletcher, 2004). In this study, Y2H assay has been used to analyse the intraviral protein interactions of MDV (Chapter 4). The Y2H screen led to the identification of 435 interactions among the MDV proteins. Although the Y2H system is sometimes associated with some false positive and false negative interactions, The MDV interactome has remarkably good reliability, reaching 81%. This was enabled by confirmation of some of the identified interactions using biochemical assays (Co-IP and LUMIER). Interestingly, many of the identified interactions were found to represent the roles of these proteins in the different replication steps of MDV, including DNA replication, nucleic acid egress, nucleocapsid tegumentation, virus envelopment and finally virus release. A large number of interactions were reproducible and proved to have roles in the assembly and biology of other herpesviridae family members. For example, the screen identified the MDV large tegument protein, UL36, to be interacted with several proteins representing different parts of MDV virion. These interactions highlighted the central role of the MDV UL36 in virion assembly via interacting with various capsid, tegument and membrane-associated proteins. Additionally, four biologically

important proteins form a dense network together (Meq, PP38, PP24 and vIL8), indicating a relationship between these proteins in the regulation of MDV infection. However, performing Y2H assay to study the interactions between MDV proteins and chicken proteins will provide more information regarding the roles of the mentioned genes in the regulation of MDV infection.

MDV infection is characterised by immediate development of innate-immune responses. These immune responses are composed of activation of Macrophages and NK cells, which subsequently mediate the secretion of different antiviral cytokines (Garcia-Camacho et al., 2003; Abdul-Careem et al., 2008a; Abdul-Careem et al., 2009a). Although MDV infection is associated with up-regulation of interferon-gamma (Abdul-Careem et al., 2007; Heidari et al., 2008b), there is no evidence for up-regulation of interferon-alpha during MDV infection (Xing and Schat, 2000a; Heidari et al., 2008b). Furthermore, MDV was able to block interferon-alpha responses of chicken cells (Quere et al., 2005). The work presented in Chapter 5 described the discovery of proteins encoded by MDV which are responsible for antagonising interferon-alpha response. The MDV library, cloned into the pCR3 expression vector, was used to study the effect of MDV protein expression on chicken interferon-alpha signalling. The chicken Mx1 promoter contains an ISRE motif, which is induced by chIFN-α treatment (Schumacher et al., 1994). Therefore a luciferase reporter assay was designed that was based on using this ISRE motif upstream of Firefly luciferase as a reporter for chIFN-α secretion. Using a functional reporter assay, several viral proteins that can antagonise chIFN-α secretion were identified. These genes were identified as Meq, LMeq, UL50, UL26 and UL12, which encode MDV's leucine zipper protein, dUTPase, capsid cleavage protein and DNase, respectively. Most of these genes proved to have an immunoregulatory function in other herpesviruses, particularly EBV (Hippenmeyer et al., 1997; Hahn et al., 2005; Wu et al., 2009b). Thus, EBV and MDV, which belong to two distinct herpesvirus subfamilies, use two structurally related proteins pairs, two bZIP-containing proteins (BZLF-1 and Meq) and two dUTPase homologs (LF2 and UL50), to evade the innate immune response. In addition, these newly discovered

antagonists have mainly immediate-early or early kinetics, as their early expression during the viral life cycle can facilitate the establishment of viral infection.

Meq is a leucine zipper protein that has transcriptional activity similar to the cellular Jun/Fos family. Meq is the main MDV oncoprotein and its expression alone was enough to induce transformation in rat and chicken cell lines (Liu et al., 1998; Levy et al., 2005; Ajithdoss et al., 2009). Meq has an anti-apoptotic activity which is important for the protection of transformed cells from apoptosis (cell death) (Liu et al., 1998). It is expressed in both cytolytic and latent stages of MDV infection (Heidari et al., 2008a), and MDV transformed T lymphocytes are considered $CD30^+$ $CD4^+$ $Meq^+$ cells (Gimeno et al., 2005). Indeed, Meq expression during latency and tumour formation is strategically good for these stages of infection; it is very likely that Meq expression exerts a certain immunomodulatory function. This function may allow the virus to exist latently inside the host cell without activation of the chicken immune response. Meq and LMeq were the only MDV proteins that robustly antagonise chIFN-α secretion in a dosage-dependent manner (Chapter 5). For the former, this study focused on the mechanism behind Meq's role in interferon-alpha down regulation. The N-terminal half of Meq encodes bZIP domain is required for both DNA binding and nuclear localisation (Qian et al., 1996; Liu et al., 1997; Lee et al., 2003; Ajithdoss et al., 2009). The C-terminal, proline-rich domain (129 to 339) has transactivating activity, particularly in the last 33 aminoacids (Qian et al., 1995). In this study, the sequence between aminoacids 25 to 151 was found to be essential for inhibiting chIFN-α signalling (Chapter 5), while the transregulatory C-terminal motif was not required, suggesting that the chIFN-α inhibitory function either requires nuclear localisation, DNA binding or, most likely dimerisation. This study indicates that both Meq, which is derived from the pathogenic RB1B strain, as well as its isoform LMeq derived from the CVI988 vaccine strain down-regulate the chIFN-α response for the chicken Mx1 promoter, indicating that the inhibition of chIFN-α signalling through Meq does not contribute to the difference in pathogenicity in these strains. Meq was not the only basic leucine zipper protein belonging to the herpesvirus involved in interferon-alpha down regulation, but also

the EBV leucine zipper protein BZLF-1 has also been reported to down-regulate the interferon-alpha response (Hahn et al., 2005).

Interferon regulatory factor 7 (IRF7) is a transcriptional factor and is responsible for type 1 interferon secretion after binding with their promoters. Chicken interferon regulatory factor 7 has the ability to bind efficiently to the ISRE motif in the chicken Mx1 promoter and acts as a transcriptional activator (Grant et al., 1995; Grant et al., 2000). Thus, it is involved in the chIFN-α signalling process and plays a similar role to the mammalian ISGF3. Herpesviruses generally interfere with the production of type 1 interferon via modulation of IRF7 at different levels. Herpesviruses encoded interferon-antagonist proteins interfer with IRF7 at the transcriptional, translation or post-translational level. Work described in Chapter 6 showed that Meq inhibits chIFN-α through degradation of chIRF7. The over-expression of Meq protein alone did not affect chIRF7 mRNA level. On the other hand, it does affect chIRF7 at the protein level. Meq over-expression in chicken cells resulted in chIRF7 protein degradation in a dose-dependent manner. Meq negatively regulates chIRF7 protein stability and leads to shortening of its half-life. Moreover, this negative effect on chIRF7 protein stability is through proteasomal-dependent degradation, as chIRF7 degradation by Meq was completely inhibited by inhibition of the proteasome. Similar to KSHV RTA (Yu et al., 2005), Meq auto-regulates its own protein stability in the presence of chIRF7, possibly by creating a multiprotein complex with IRF7 then degraded together. Since the direct physical binding between Meq and IRF7 was not confirmed in Co-IP assay, Meq may act indirectly through other cellular proteins that cause proteasomal degradation. It became clear that Meq targets chIRF7 for proteasomal degradation, and probably acts as E3 ubiquitine ligase for chIRF7, although evidence for this is lacking. A proteomic analysis of MDV infected spleen cells revealed that many proteins of the ubiquitin-proteasome protein degradation system were up- or down-regulated (Thanthrige-Don et al., 2009). Immunofluorescent study revealed that Meq inhibits chIRF7 from entering into the nucleus by localising with it in the cytoplasm and targeting it for proteasomal degradation.

Experiments described in Chapter 7 showed that the Meq-deletion RB-1B MDV strain is more sensitive for chIFN-α treatment at an infection dose of 50 PFU. The difference between Meq-deletion MDV and wild-type MDV is clearer at chIRF7 mRNA expression level. DF-1 cells have a significantly higher level of chIRF7 mRNA after infection with Meq-lacking MDV compared with Meq-encoding MDV. This up-regulation at the chIRF7 mRNA level is perhaps due to a positive feedback mechanism for the changes which occur earlier at the protein level. These findings confirm Meq's antagonisitic effect on chIFN-α response, and it can be concluded that MDV utilises Meq, the main oncoprotein, as a suicidal protein to evade the host interferon signalling cascade. Therefore, Meq expression by MDV negatively regulates the antiviral state induced by the interferon system. However, it is not clear from our study if this novel function displayed by Meq is important for establishing either the lytic or the latent stage of MDV infection.

These observations lead to the recommendation that the use of a vaccine lacking Meq or LMeq will provide birds with a better immune response and protection. Furthermore, Meq is considered as a potential drug target for treating birds from MDV infection.

## Concluding remarks

In this study, a new tool, the MDV gene library, to be used in MDV research was generated with a suitable flexible cloning system. MDV ORFeome was used in two functional assays Y2H assay and chIFN-α signalling reporter assay. Both the genome-wide interactome and the chIFN-α signalling investigation gave us useful novel data for the structurally and the regulatory essential MDV genes. Since the number of MDV intraviral interactome studies is very limited, MDV interactome data were mainly correlated to other studies of other herpesviruses. However, the presence of many conserved interactions with the other herpesviruses and the formation of Meq homodimers make it reasonable to expect that MDV interactome may present many other truly novel data.

Genome-wide analysis of MDV proteins which inhibit chIFN-α signalling identified many chIFN-α antagonists encoded by the MDV genome. Meq is a potent interferon antagonist, according to the chIFN-α luciferase reporter assay. It negatively regulates chIFN-α signalling via inhibition of the chIRF7 protein entrance to the nucleus and by targeting it for proteasomal-dependent degradation. Meq deletion MDV was more sensitive for chIFN-α treatment.

## Future work

Taking all the previous data together, it is therefore crucial to test the essential interactions via generation of mutant viruses which express tagged viral proteins using virus genomes cloned into BAC vectors. These mutants can be tested *in vitro* and *in vivo* for their ability to replicate and produce a disease.

Furthermore, it is essential to undertake microarray analysis of cellular gene expression after infection with wild-type or Meq knock-out MDV. Performing such a comparison will be informative and will give more insight into the difference between the responses of interferon-related genes after infection with the two viruses. In addition, it is crucial to study the novel function displayed by Meq *in vivo*. Comparing the antiviral cytokine levels in birds after infection with either the Meq knock-out or the wild-type MDV in the future will help in understanding the importance of the immunosuppressive function displayed by Meq. Hopefully, this will help in controlling the world-wide problem of endemic MDV infection.

# Appendices

## Appendix A

**Table 1.9.2: Orthologues of MDV genes present in HSV-1, VZV, murine cytomegalovirus (mCMV), Epstein-Barr virus (EBV) and Kaposi's sarcoma virus (KSHV)***

| α-Herpesvirus | | | β–Herpesvirus | ϒ-Herpesvirus | | Function in MDV |
|---|---|---|---|---|---|---|
| **MDV** | **HSV-1** | **VZV** | **mCMV** | **EBV** | **KSHV** | |
| **UL1** | UL1 | ORF60 | M115 | BKRF2 | ORF47 | Glycoprotein L |
| **UL2** | UL2 | ORF59 | M114 | BKRF3 | ORF46 | Uracil –DNA glycosylase |
| **UL3** | UL3 | ORF58 | - | - | - | Nuclear phosphoprotein protein |
| **UL4** | UL4 | ORF56 | - | - | - | Nuclear protein |
| **Ul5** | UL5 | ORF55 | M105 | BBLF4 | ORF44 | DNA helicase primase-associated protein |
| **UL6** | UL6 | ORF54 | M104 | BBRF1 | ORF43 | Minor capsid protein |
| **UL7** | UL7 | ORF53 | M103 | BBRF2 | ORF42 | Capsid protein |
| **UL8** | UL8 | ORF52 | M102 | BBLF2 | ORF40 | DNA helicase-primase associated protein |
| **UL9** | UL9 | ORF51 | - | - | - | Origin binding protein |

| α-Herpesvirus | | | β–Herpesvirus | ϒ-Herpesvirus | | Function in MDV |
|---|---|---|---|---|---|---|
| **MDV** | **HSV-1** | **VZV** | **mCMV** | **EBV** | **KSHV** | |
| **UL10** | UL10 | ORF50 | M100 | BBRF3 | ORF39 | Glycoprotein M |
| **UL11** | UL11 | ORF49 | M99 | BBLF1 | ORF38 | Myristylated tegument protein |
| **UL12** | UL12 | ORF48 | M98 | BGLF5 | ORF37 | DNase |
| **UL13** | UL13 | ORF47 | M97 | BGLF4 | ORF36 | Serine/Threonine protein kinase |
| **UL14** | UL14 | ORF46 | M95 | BGLF3 | ORF34 | Minor tegument protein |
| **UL15** | UL15 | ORF42 | M89 | BGRF1 | ORF29a | (DNA packaging protein) Terminase |
| **UL16** | UL16 | ORF44 | M94 | BGLF2 | ORF33 | Tegument protein |
| **UL17** | UL17 | ORF43 | M93 | BGLF1 | ORF32 | Tegument protein, DNA packaging |
| **UL18** | UL18 | ORF41 | M85 | BDLF1 | ORF26 | Capsid protein |
| **UL19** | UL19 | ORF40 | M86 | BcLF1 | ORF25 | Major capsid protein |
| **UL20** | UL20 | ORF39 | - | - | - | Membrane protein, virus egress |
| **UL21** | UL21 | ORF38 | M88 | BTRF1 | ORF23 | Tegument protein |
| **UL22** | UL22 | ORF37 | M75 | BXLF2 | ORF22 | Glycoprotein H |

| α-Herpesvirus | | | β–Herpesvirus | ϒ-Herpesvirus | | Function in MDV |
|---|---|---|---|---|---|---|
| **MDV** | **HSV-1** | **VZV** | **mCMV** | **EBV** | **KSHV** | |
| **Ul23** | UL23 | ORF36 | - | BXLF1 | ORF21 | Thymidine kinase |
| **Ul24** | UL24 | ORF35 | M76 | BXRF1 | ORF20 | Non-glycosylated membrane associated protein |
| **UL25** | UL25 | ORF34 | M77 | BVRF1 | ORF19 | DNA packaging |
| **UL26** | UL26 | ORF33 | M80 | BVRF2 | ORF17 | Protease |
| **UL26.5** | UL26.5 | ORF33.5 | M80.5 | BdRF1 | ORF17.5 | Minor capsid scaffold protein |
| **UL27** | UL27 | ORF31 | M55 | BALF4 | ORF8 | Glycoprotein B |
| **UL28** | UL28 | ORF30 | M56 | BALF3 | ORF7 | DNA packaging protein |
| **UL 29** | UL29 | ORF29 | M57 | BALF2 | ORF6 | Single stranded DNA binding protein |
| **UL30** | UL30 | ORF28 | M54 | BALF5 | ORF9 | DNA polymerase |
| **UL31** | UL31 | ORF27 | M53 | BFLF2 | ORF69 | Nuclear phosphoprotein |
| **UL32** | UL32 | ORF26 | M52 | BFLF1 | ORF68 | DNA packaging protein |
| **UL33** | UL33 | ORF25 | M51 | BFRF4 | ORF67.5 | DNA packaging protein |

| α-Herpesvirus | | | β–Herpesvirus | ϒ-Herpesvirus | | Function in MDV |
|---|---|---|---|---|---|---|
| **MDV** | **HSV-1** | **VZV** | **mCMV** | **EBV** | **KSHV** | |
| **UL34** | UL34 | ORF24 | M50 | BFRF1 | ORF67 | Membrane phosphoprotein |
| **UL35** | UL35 | ORF23 | M48.2 | BFRF3 | ORF65 | Capsid protein VP26 |
| **UL36** | UL36 | ORF22 | M48 | BPLF1 | ORF64 | Large tegument protein |
| **UL37** | UL37 | ORF21 | M47 | BOLF1 | ORF63 | Tegument protein |
| **UL38** | UL38 | ORF20 | M46 | BORF1 | ORF62 | Capsid protein |
| **UL39** | UL39 | ORF19 | M45 | BORF2 | ORF61 | Ribonucleotide reductase, large subunit |
| **UL40** | UL40 | ORF18 | - | BaRF1 | ORF60 | Ribonucleotide reductase, small subunit |
| **UL41** | UL41 | ORF17 | - | - | - | Tegument, virion shut off protein |
| **UL42** | UL42 | ORF16 | M44 | BMRF1 | ORF59 | DNA polymerase |
| **UL43** | UL43 | ORF15 | - | - | - | Membrane-associated protein |
| **UL44** | UL44 | ORF14 | - | - | - | Glycoprotein C |
| **UL46** | UL46 | ORF12 | - | - | - | Tegument phosphoprotein |

| α-Herpesvirus | | | β–Herpesvirus | ϒ-Herpesvirus | | Function in MDV |
|---|---|---|---|---|---|---|
| **MDV** | **HSV-1** | **VZV** | **mCMV** | **EBV** | **KSHV** | |
| **UL47** | UL47 | ORF11 | - | - | - | Tegument phosphoprotein |
| **UL48** | UL48 | ORF10 | - | - | - | Tegument immediate early protein |
| **UL49** | UL49 | ORF9 | - | - | - | Tegument phosphorprotein |
| **UL50** | UL50 | ORF8 | M72 | BLLF3 | ORF54 | dUTPase |
| **UL51** | UL51 | ORF7 | M71 | BSRF1 | ORF55 | Virion phosphoprotein |
| **UL52** | UL52 | ORF6 | M70 | BSLF1 | ORF56 | DNA helicase primase associated protein |
| **UL53** | UL53 | ORF5 | - | - | - | Glycoprotein K |
| **UL54** | UL54 (ICP27) | ORF4 | M69 | BSLF2 | ORF57 | Post translational gene regulation |
| **UL55** | UL55 | ORF3 | - | - | - | Nuclear matrix associated protein |
| **LORF10** | | ORF2 | | | | |
| **US1** | ICP22 | ORF63 | - | - | - | Immediate- early protein |
| **ICP4** | RS1 | ORF62 | - | - | - | Immediate-early protein |
| **US2** | US2 | | | | | |

| α-Herpesvirus | | | β–Herpesvirus | ϒ-Herpesvirus | | Function in MDV |
|---|---|---|---|---|---|---|
| **MDV** | **HSV-1** | **VZV** | **mCMV** | **EBV** | **KSHV** | |
| **US3** | US3 | ORF66 | - | - | - | Serine/threonine protein kinase |
| **US6** | US6 | | | | | Glycoprotein D |
| **US7** | US7 | ORF67 | - | - | - | Glycoprotein I |
| **US8** | US8 | ORF68 | - | - | - | Glycoprotein E |
| **US10** | US10 | ORF64 | - | - | - | |

**** Modified from (Davison, 2004).***

## Appendix B

**Table 2.5.1: The forward and the reverse primers used to amplify MDV genes and chicken interferon regulatory factor 7 (chIRF7) gene**

| ORF | FWD Primer | Rev Primer | Role |
|---|---|---|---|
| **RLORF1** | AAAAAGCAGGCTCCG CC ATG ACC CGG GGG CAT CGC AC | AGAAAGCTGGGTC CTA GCG AGG GGT CCT CTC GCC | HSV-1 ICP0-like protein |
| **RLORF2** | AAAAAGCAGGCTCCG CC ATG CAG GCG TTG TTG CTA GTA TTG | AGAAAGCTGGGTC CTA AAG ACA GAT ATG GGA ACC AAT AG | vIL8, CxC chemokine |
| **RLORF2-cyt** | AAAAAGCAGGCTCCG CC ATG GAG AGT CTC GCT GTC GAC AAG | AGAAAGCTGGGTC CTA ATT GTG TTT TTT ACC GGT CCC ACG G | |
| **RLORF3** | AAAAAGCAGGCTCCG CC ATG ACA ACC CCA TAT TTT GGC C | AGAAAGCTGGGTC CTA TAC TGA TGT AAC CAG TAC TCG | Hypothetical protein |
| **RLORF3-extra** | AAAAAGCAGGCTCCG CC ATG CCC AAG CCA GTA GGC CGA TTA G | AGAAAGCTGGGTC CTA TAC TGA TGT AAC CAG TAC TCG | |
| **RLORF4** | AAAAAGCAGGCTCCG CC ATG CGC CAG TTA TGC ATG ACG C | AGAAAGCTGGGTC CTA TAG CAT CGA AAC ACT AAA ATG | Hypothetical protein |
| **RLORF4-cyt** | AAAAAGCAGGCTCCG CC ATG CGC CAG TTA TGC ATG ACG C | AGAAAGCTGGGTC CTA TTT TGC AGT ACG CAT GCG CCC TC | |
| **RLORF4-extra** | AAAAAGCAGGCTCCG CC ATG AAG GGT CCT CAT AGT CTT GTG TTC | AGAAAGCTGGGTC CTA TTT TGC AGT ACG CAT GCG CCC TC | |
| **RLORF5** | AAAAAGCAGGCTCCG CC ATG CGC GTC ATG CAT AAC TGG CG | AGAAAGCTGGGTC CTA ACG GTT CGT GGT TTT TTT TTT TAC | Hypothetical protein |
| **RLORF5b(R)** | AAAAAGCAGGCTCCG CC ATG CGC GTC ATG CAT AAC TGG CG | AGAAAGCTGGGTC CTA ACG GTT CGT GGT TTT TTT TTT TTT | Hypothetical protein |
| **L1** | AAAAAGCAGGCTCCG CC ATG TCG ACA TTA ACG AAT ATA CAG GC | AGAAAGCTGGGTC CTA CAC GTG GCA CTG CCG CGT AAA C | Hypothetical protein, transcript identified in MD lymphoblastoid |
| **23KDa** | AAAAAGCAGGCTCCG CC ATG TGG GGG AGA TGG GGT AAA AAG | AGAAAGCTGGGTC CTA CCG GAT GAA CCT AAC GCT CC | 23kD nuclear protein, binds to binds alpha-enolase |
| **RLORF7** | AAAAAGCAGGCTCCG CC ATG TCT CAG GAG CCA GAG CCG | AGAAAGCTGGGTC CTA GGG TCT CCC GTC ACC TGG | Oncogene, anti-apoptotic |
| **RLORF6** | AAAAAGCAGGCTCCG CC ATG AAC CTA ACG CTC CAC ATT G | AGAAAGCTGGGTC CTA TAT ATA ACT AGG GGA GAA GAA | Hypothetical protein |
| **RLORF8** | AAAAAGCAGGCTCCG CC ATG ACT ATC GTC TTT ATA TCA CCG | AGAAAGCTGGGTC CTA AGT TGT ATG TAG TGT ATC GGT CTT | Binds to C1q-binding protein |
| **RLORF9** | AAAAAGCAGGCTCCG CC ATG TGG AAC ACG ATT GGC CGT TG | AGAAAGCTGGGTC CTA TCG ATA ATC GGC TCC GAT CCC | 14 kDa lytic protein C |

| ORF | FWD Primer | Rev Primer | Role |
|---|---|---|---|
| **RLORF10** | AAAAAGCAGGCTCCG CC ATG TGC AAA CAG CGA CTC ATT ATC | AGAAAGCTGGGTC CTA CAG CTG TGA TAT CGT TGC AGA | Binds to MHC class-II invariant chain |
| **14KDA** | AAAAAGCAGGCTCCG CC ATG CTC TCT CAG AAT GGC ACG | AGAAAGCTGGGTC CTA GAG GGT CGA TTC CAC TTC TTT CTC | 14 kDa lytic protein A |
| **14KD B** | AAAAAGCAGGCTCCG CC ATG ATA AAT ACT ACG GCA GAA AG | AGAAAGCTGGGTC CTA GAG GGT CGA TTC CAC TTC TTT C | 14 kDa lytic protein B |
| **RLORF11** | AAAAAGCAGGCTCCG CC ATG TTA CAT ATC TAT AAC TTG ATT | AGAAAGCTGGGTC CTA TAC TTT ACC TGC TTT TGC TGC GTA | Hypothetical protein |
| **B6b8** | AAAAAGCAGGCTCCG CC ATG GCG ATT TTT TAT TTA CGG CGA | AGAAAGCTGGGTC CTA GCC CAT CCT TTC TAC ATT GCA CG | VZV transinducing protein |
| **B68b-cyt** | AAAAAGCAGGCTCCG CC ATG GCG ATT TTT TAT TTA CGG CGA | AGAAAGCTGGGTC CTA GCT CAT CCC GGG TCG ATG TTG | |
| **RLORF12b** | AAAAAGCAGGCTCCG CC ATG TGT ATG CAA ATG AGC AGT GCG | AGAAAGCTGGGTC CTA CTT ATT TGA TGA AGG GAG AAA TTC | Binds to growth-related translational controlled tumor protein |
| **RLORF12b(R)** | AAAAAGCAGGCTCCG CC ATG TGT ATG CAA ATG AGC AGT GCG | AGAAAGCTGGGTC CTA TAC ACA AGA GCC GAG CCG CC | Binds to growth-related translational controlled tumor protein |
| **LORF1** | AAAAAGCAGGCTCCG CC ATG GTG AAT AGA CGC AAC TAT AAT | AGAAAGCTGGGTC CTA TTG GTT CGC AGT GCG AAC GC | Hypothetical protein |
| **LORF1-cyt** | AAAAAGCAGGCTCCG CC ATG GTG AAT AGA CGC AAC TAT AAT | AGAAAGCTGGGTC CTA TCT GGC CTT TCT TGC GGG ATT TC | |
| **LORF1-cyt** | AAAAAGCAGGCTCCG CC ATG TCC CGT TCA GCT TCG ACA AG | AGAAAGCTGGGTC CTA TCT GGC CTT TCT TGC GGG ATT TC | |
| **RLORF13b** | AAAAAGCAGGCTCCG CC ATG ACC CAC AAC CGC GAT TTG | AGAAAGCTGGGTC CTA TCC CCA TGG TCC CTC TGG | binds with C1q-binding protein |
| **RLORF14b** | AAAAAGCAGGCTCCG CC ATG GAA TTC GAA GCA GAA CAC GAA G | AGAAAGCTGGGTC CTA AAC ATG TCG AGT CAG AAA TCC | Early 24kDa phosphoprotein, PP24 |
| **LORF2** | AAAAAGCAGGCTCCG CC ATG TGG ACT GAT ATC AAT CCT CCA TC | AGAAAGCTGGGTC CTA TAC CCG CGA AGT TGT GAG AG | Hypothetical protein |
| **LIPASE** | AAAAAGCAGGCTCCG CC ATG CCG AGT AAA AGT ATT GCG GAT C | AGAAAGCTGGGTC CTA TAC CCG CGA AGT TGT GAG AG | |
| **LORF3** | AAAAAGCAGGCTCCG CC ATG TTT ACC GGA GGA GGA ACT ATT GC | AGAAAGCTGGGTC CTA TTC ATC ATC TGA ACT CGA CAT | Hypothetical protein |
| **UL1** | AAAAAGCAGGCTCCG CCATG AAA ATT TAT AGA GTA CTC GTG | AGAAAGCTGGGTC CTA GGC ATT GGC TCG TCG GCT | Virion surface glycoprotein L |
| **LORF4** | AAAAAGCAGGCTCCG CC ATG CAA CCC GAT CCG CGA TTT C | AGAAAGCTGGGTC CTA TAG AGT ACT CGT GCA TCT TTC CTT | binds to MHC class II beta chain |

| ORF | FWD Primer | Rev Primer | Role |
|---|---|---|---|
| **LORF4 extra** | AAAAAGCAGGCTCCG CC ATG CAA CCC GAT CCG CGA TTT C | AGAAAGCTGGGTC CTA CGT TTT GAA AAG CGG ATT GGA | |
| **LORF4-cyt** | AAAAAGCAGGCTCCG CC ATG AGA ATG AAT TCG GGT ATG CTG | AGAAAGCTGGGTC CTA CGT TTT GAA AAG CGG ATT GGA | |
| **UL2** | AAAAAGCAGGCTCCG CC ATG GCT CAA CTA GAC TTG ACA GG | AGAAAGCTGGGTC CTA CGC GGT AAG GCT CCA GTC | Uracil-DNA glycosylase |
| **UL3** | AAAAAGCAGGCTCCG CC ATG TAC AAA AGA CCG GGT CAC AAA C | AGAAAGCTGGGTC CTA CGA ACC AGA AGA ATC TAC TAT | Nuclear phosphoprotein |
| **UL3.5** | AAAAAGCAGGCTCCG CC ATG TTT GTA GTC TCC GCG TCT TTG | AGAAAGCTGGGTC CTA TGT ACT AGC CGT TTT CGA AAT | Tegument protein, virus egress |
| **UL4** | AAAAAGCAGGCTCCG CC ATG GGA TCT ACC TTT ATC GCG TAT AC | AGAAAGCTGGGTC CTA GGG ATA AAT TTT CAC AGA TTC | Nuclear protein |
| **UL5** | AAAAAGCAGGCTCCG CC ATG TCA CAG GAA TCG AAT GAC TTA TTT | AGAAAGCTGGGTC CTA ATA GAC AAT ATG AAC CTT AGG | DNA helicase-primase associated protein" |
| **UL6** | AAAAAGCAGGCTCCG CC ATG GAT GGC TAT GAT CGA AGA GCG | AGAAAGCTGGGTC CTA ATC TCT CTG ACT TCC ATC ATC ATC CT | Minor capsid protein, DNA packaging |
| **UL7** | AAAAAGCAGGCTCCG CC ATG GAA GAA GAA ATG ACT TCC ATT CTC | AGAAAGCTGGGTC CTA TTT TTG GTA TGT GTG AAA TAA ACA | Capsid protein, DNA packaging/cleavage |
| **UL8** | AAAAAGCAGGCTCCG CC ATG CGT CAA ACC ATA TCA ACG ATG | AGAAAGCTGGGTC CTA TTT ATT ATT AAT AAA CAT AAA CCT | DNA helicase-primase associated |
| **UL8.5** | AAAAAGCAGGCTCCG CC ATG TTT GCA TAC ATC AAG CCT ACG | AGAAAGCTGGGTC CTA TAA ACG ATG AAA ATC TAC AGC | Origin binding protein |
| **UL9** | AAAAAGCAGGCTCCG CC ATG ATA GAC TAT GCA TCC AGC GCC | AGAAAGCTGGGTC CTA TAA ACG ATG AAA ATC TAC AGC | Origin binding protein |
| **UL10** | AAAAAGCAGGCTCCG CC ATG GCC AGT CGA GCA CGA ATG G | AGAAAGCTGGGTC CTA ATC ATC CCA TTC GCT CTC AGA TCC | Virion membrane glycoprotein M |
| **UL10-cyt1** | AAAAAGCAGGCTCCG CC ATG GCC AGT CGA GCA CGA ATG G | AGAAAGCTGGGTC CTA CCA CAT CTT TTT GTG CAC GTA GTC | |
| **UL10-cyt2** | AAAAAGCAGGCTCCG CC ATG CGG GCC AGT TTA TAT CAT CGT CG | AGAAAGCTGGGTC CTA CCA CAT CTT TTT GTG CAC GTA GTC | |
| **UL11** | AAAAAGCAGGCTCCG CC ATG GGC CAA GCA GTG TCG TAT TTA | AGAAAGCTGGGTC CTA TTC TTT ATT AAA CAT CAT AAC ATA | Myristylated tegument protein |
| **UL12** | AAAAAGCAGGCTCCG CC ATG GAA CTA GGA ACC AAG TTT CCA C | AGAAAGCTGGGTC CTA AAT ACG ACA CTG CTT GGC CCA | DNAse |
| **UL13** | AAAAAGCAGGCTCCG CC ATG GAT ACT GAA TCA AAA AAC AAA | AGAAAGCTGGGTC CTA GTT CCA TAA CAA CAA ATC AGC | Serine/threonine protein kinase |

| ORF | FWD Primer | Rev Primer | Role |
|---|---|---|---|
| **UL13A(R)** | AAAAAGCAGGCTCCG CC ATG GAT ACT GAA TCA AAA AAC AAA | AGAAAGCTGGGTC CTA AAA CAT TCC AAT ACT TTT TTT GAC | |
| **UL13B(R)** | AAAAAGCAGGCTCCG CC ATG ACA TTG ATA GCC GGT GAA TGT G | AGAAAGCTGGGTC CTA GTT CCA TAA CAA CAA ATC AGC | |
| **UL14** | AAAAAGCAGGCTCCG CC ATG TTT GCA GTG AGC GCA ATG CG | AGAAAGCTGGGTC CTA TAC ACA GCT GTC TGA GAC ATC G | Minor tegument protein |
| **UL15(Fr)1** | AAAAAGCAGGCTCCG CC ATG TTT GGT GGT TTA CTT GGC GAA G | TCC ACG AAT GCT GTT CGT GTT ATG GCT AGA TGC | DNA packaging protein (terminase) |
| **UL15(Fr)2** | TAA CAC GAA CAG CAT TCG TGG ACA AGA TTT C | AGAAAGCTGGGTC CTA TAC TCT AAT GGG GGC AAA TGC | DNA packaging protein (terminase) |
| **UL16** | AAAAAGCAGGCTCCG CC ATG ACT ACG CAG AGA CTG AAG ATA CC | AGAAAGCTGGGTC CTA TAC GAC GTT GGG CTG TCT GTC | Tegument protein |
| **UL17** | AAAAAGCAGGCTCCG CC ATG GAG GCG CAT ATA GAA AGC G | AGAAAGCTGGGTC CTA CAT ATA CAC CTC TGA AAC GTA | Tegument protein, DNA packaging |
| **UL18** | AAAAAGCAGGCTCCG CC ATG AGT ACT TCC AAC GGC ACG ATA G | AGAAAGCTGGGTC CTA ATA CCA GTT TGC AAT AAC AGG | Capsid protein |
| **UL19** | AAAAAGCAGGCTCCG CC ATG GCC GGA TGC CAT TGT CCC | AGAAAGCTGGGTC CTA GCA TAT GGG CAA ACA TCC ACC AA | Major capsid protein |
| **UL20** | AAAAAGCAGGCTCCG CC ATG TCG AAG CAT GGA TTT GGA TAC | AGAAAGCTGGGTC CTA GAA TGG CAC ACA CGA TTT AAG | Membrane protein, virus egress |
| **UL20-extra** | AAAAAGCAGGCTCCG CC ATG TCG AAG CAT GGA TTT GGA TAC | AGAAAGCTGGGTC CTA AAC ATA CCG AGT AAA TTT GGG | |
| **UL21** | AAAAAGCAGGCTCCG CC ATG GAT ATT AAA TAC GAA CAT GTT | AGAAAGCTGGGTC CTA AGA ACA TAT TGT AAT ACA CCC | Tegument protein |
| **UL22** | AAAAAGCAGGCTCCG CC ATG GGT CTT CCC GGT AGT ATA G | AGAAAGCTGGGTC CTA AAG ATC GTC GTA CAG GCT CAA | Glycoprotein H |
| **UL22-extra** | AAAAAGCAGGCTCCG CC ATG GGT CTT CCC GGT AGT ATA G | AGAAAGCTGGGTC CTA AGT AGG GTT AAA ATA CCG AAT | |
| **UL23** | AAAAAGCAGGCTCCG CC ATG TCG GAG CCA CAA TCG TGG TC | AGAAAGCTGGGTC CTA CAT AGC CAT CTC TTT ATT ATA GGC | Thymidine kinase |
| **UL24** | AAAAAGCAGGCTCCG CC ATG TCA TCT GAG ATG CCA TTA CCG | AGAAAGCTGGGTC CTA CGG TCT CTG AAC AAG ACG GG | Non-glycosylated membrane-associated |
| **UL25** | AAAAAGCAGGCTCCG CC ATG GCA AAC TTT ATT TGG GAC GCG | AGAAAGCTGGGTC CTA CAA CGT AGA TAT TAA TTT CGG | DNA packaging |
| **UL26** | AAAAAGCAGGCTCCG CC ATG AAT CCG GCC GAC CAT CCA TC | AGAAAGCTGGGTC CTA TTG ATG CGC CAT CAT TTG ATT AAT | Protease, minor capsid scaffold protein |

| ORF | FWD Primer | Rev Primer | Role |
|---|---|---|---|
| **UL26.5** | AAAAAGCAGGCTCCG CC ATG AAC ACT CAA TCT TCT CGC CC | AGAAAGCTGGGTC CTA TTG ATG CGC CAT CAT TTG ATT AAT | Minor capsid scaffold protein |
| **LORF5** | AAAAAGCAGGCTCCG CC ATG AGT AAA CCA TAT CAT GCT CTA TGC | AGAAAGCTGGGTC CTA TCC TAT GAC AAC AGA AGT GCT | Hypothetical protein |
| **UL27** | AAAAAGCAGGCTCCG CC ATG CAC TAT TTT AGG CGG AAT TGC | AGAAAGCTGGGTC CTA CAC AGC ATC ATC TTC TGA GTC | Virion membrane glycoprotein B |
| **UL27-cyt** | AAAAAGCAGGCTCCG CC ATG AAC AAG CTT AAA AGC AAT CCA | AGAAAGCTGGGTC CTA CAC AGC ATC ATC TTC TGA GTC | |
| **UL27-extra** | AAAAAGCAGGCTCCG CC ATG AAC AAG CTT AAA AGC AAT CCA | AGAAAGCTGGGTC CTA GGA TTT GAC ATG AAA GCA GAG | |
| **UL28** | AAAAAGCAGGCTCCG CC ATG TTG GGA ATG TCT CAT AAC CGG | AGAAAGCTGGGTC CTA GAT GGG GGC GTG GCT GTT G | DNA packaging protein |
| **UL29(Fr)1** | AAAAAGCAGGCTCCG CC ATG GAT GGT GTA GGA AAA AGC GTG | AGAAAGCTGGGTC CTA TGA TCG CTT GAG GGT CGA AAA | Single-stranded DNA binding protein |
| **UL29(Fr)2** | AAAAAGCAGGCTCCG CC ATG GAA GGC GAG GCA AGC CGT | AGAAAGCTGGGTC CTA CAA CAT ATC TAC AGA TAA CAC | Single-stranded DNA binding protein |
| **UL30** | AAAAAGCAGGCTCCG CC ATG TCA GTA GAC GGA ACT AAA ACA | AGAAAGCTGGGTC CTA ATA TCG ATG GGG AGT TGC TGT | DNA polymerase |
| **UL31** | AAAAAGCAGGCTCCG CC ATG ACT GGT CAT ACC TTA GTG AGA C | AGAAAGCTGGGTC CTA ACG AGG AGG AAG AAA CTC GTC | Nuclear phosphoprotein |
| **UL32** | AAAAAGCAGGCTCCG CC ATG GCC AAC CGC CCT ACA GAG | AGAAAGCTGGGTC CTA CAC GTA GAC TCC TAA TGT ATG CTC | DNA packaging |
| **UL33** | AAAAAGCAGGCTCCG CC ATG GCC GGT GAA AAT ACC TCT CG | AGAAAGCTGGGTC CTA ACA GTC TTT CAA AAT TTG ATG | DNA packaging |
| **UL34** | AAAAAGCAGGCTCCG CC ATG GAA GTC ATT CCG AAC ATA AAT | AGAAAGCTGGGTC CTA AGA GTA CAA ACG ATA GCT GCC C | Membrane phosphoprotein |
| **UL34-extra** | AAAAAGCAGGCTCCG CC ATG GAA GTC ATT CCG AAC ATA AAT | AGAAAGCTGGGTC CTA GCG CGG TAT ACG TAT CGT CGT C | |
| **UL35** | AAAAAGCAGGCTCCG CC ATG TCT CGT GCA TCA TCC CAA CAG | AGAAAGCTGGGTC CTA TGA CGT CGA TAT ATC ATC ATC | Capsid protein, VP26 |
| **UL36(Fr)1** | AAAAAGCAGGCTCCG CC ATG ACA GAT TCT ACT GAC AGC AGA | AGAAAGCTGGGTC CTA CCT TTC TAT AAG GTC TAT AGA TTC AG | Large tegument protein |
| **UL36(Fr)2** | AAAAAGCAGGCTCCG CC ATG GTT CTT GCG GAG AAT TCC AAG | AGAAAGCTGGGTC CTA TTT TGA AAG TTC AGC TAT CTT GCG | Large tegument protein |
| **UL36(Fr)3** | AAAAAGCAGGCTCCG CC ATG CTA ATA AGG TCT GCA AAT CAG | AGAAAGCTGGGTC CTA AAC CGT TCG AAA ACA GAG AGA | Large tegument protein |

| ORF | FWD Primer | Rev Primer | Role |
|---|---|---|---|
| **UL36(Fr)4** | AAAAAGCAGGCTCCG CC ATG GGG GAA AAA TTA GCT GCT TGT TTG | AGAAAGCTGGGTC CTA TCC AGT CAG GAT CAT TTT AAT TTT | Large tegument protein |
| **LORF6** | AAAAAGCAGGCTCCG CC ATG ACT GTA TCT AAT CCA TAC GCA | AGAAAGCTGGGTC CTA AAT ATC CGA ATT TAA CTT CAG | Hypothetical protein |
| **LORF6-cyt** | AAAAAGCAGGCTCCG CC ATG ACT GTA TCT AAT CCA TAC GCA | AGAAAGCTGGGTC CTA GGA CCT AAG TCC TGC GTC TTC AG | |
| **LORF6-extra** | AAAAAGCAGGCTCCG CC ATG CAT TCA TCT AGA ATT ACC TCA GG | AGAAAGCTGGGTC CTA TTC AAG AAC GAC ATC GAA CGC AC | |
| **LORF6-cyt** | AAAAAGCAGGCTCCG CC ATG ATT GAA AAT ATG CGT CGA AAC | AGAAAGCTGGGTC CTA TTC AAG AAC GAC ATC GAA CGC AC | |
| **UL37** | AAAAAGCAGGCTCCG CC ATG TCT GCC GTA ACG ACC GAT G | AGAAAGCTGGGTC CTA TGC ATT ATC ACC GTT TGC CCT CC | Small tegument protein |
| **UL37(R)** | AAAAAGCAGGCTCCG CC ATG TCT GTC GTA | AGAAAGCTGGGTC CTA TGC ATT ATC ACC GTT TGC CCT CC | Small tegument protein |
| **LORF7** | AAAAAGCAGGCTCCG CC ATG CGC CAG AGA ATT AAA CCG ACG | AGAAAGCTGGGTC CTA CAT TTT ATA GAT CTT TCT AAT ATC GAT | Hypothetical protein |
| **UL38** | AAAAAGCAGGCTCCG CC ATG AAA CCA CTC TTA CGA TCG C | AGAAAGCTGGGTC CTA ATA ACA TTC GAT CCA TGT ACC | Capsid protein |
| **UL39** | AAAAAGCAGGCTCCG CCATG GAC CAT TCA GAT GTC TAC GAA C | AGAAAGCTGGGTC CTA CAA GAC GCA CGA CGA GCA G | Ribonucleotide reductase large subunit |
| **UL40** | AAAAAGCAGGCTCCG CC ATG AGC GGC CCT CCG TCA CAT | AGAAAGCTGGGTC CTA AAG ATC GTT TGA CAC GCT TCC | Ribonucleotide reductase small subunit |
| **UL41** | AAAAAGCAGGCTCCG CC ATG GGA GTA TAT GGA TGT ATG AAT | AGAAAGCTGGGTC CTA GTC ATT AGA TCG TGT TGT CTT TAA | UL41 virion host shutoff protein |
| **UL42** | AAAAAGCAGGCTCCG CC ATG GCA GGA ATA ACT ATG GGC AG | AGAAAGCTGGGTC CTA TTG GTT GAT AAC TTT GGC AAG | UL42 DNA polymerase processivity subunit |
| **UL43** | AAAAAGCAGGCTCCG CC ATG GAT TCT GTC AAC AAC TCA TCA | AGAAAGCTGGGTC CTA AGA TAG CGC TAC TAA TAT ACA | Membrane-associated protein |
| **UL43-extra** | AAAAAGCAGGCTCCG CC ATG GAT TCT GTC AAC AAC TCA TCA | AGAAAGCTGGGTC CTA CAT GGT TAC TAC GAT ACA TTT | |
| **UL44** | AAAAAGCAGGCTCCG CC ATG CTC ACG CCG CGT GTG TTA C | AGAAAGCTGGGTC CTA TAA TCG AAT ATT TTT TCG TGT GGA | Glycoprotein C |
| **UL44-extra** | AAAAAGCAGGCTCCG CC ATG CTC ACG CCG CGT GTG TTA C | AGAAAGCTGGGTC CTA CGT AAT AAC CAT GGG TGT TCC TCT | |
| **UL44A(R)** | AAAAAGCAGGCTCCG CC ATG CTC ACG CCG CGT GTG TTA C | AGAAAGCTGGGTC CTA AGG CTG GCT CCT AGG AC | |

| ORF | FWD Primer | Rev Primer | Role |
|---|---|---|---|
| **UL44B(R)** | AAAAAGCAGGCTCCG CC ATG GGC GTG GAA ATT AGA AAT GTA G | AGAAAGCTGGGTC CTA TAA TCG AAT ATT TTT TCG TGT GGA | |
| **UL44B(R) extra** | AAAAAGCAGGCTCCG CC ATG GGC GTG GAA ATT AGA AAT GTA G | AGAAAGCTGGGTC CTA CGT AAT AAC CAT GGG TGT TCC | |
| **Md57.4** | AAAAAGCAGGCTCCG CC ATG ATC GTG CCA GTC ACC AC | AGAAAGCTGGGTC CTA TAC AAT CAG CCC GAC GGA GAT | |
| **Md57.4 (R)** | AAAAAGCAGGCTCCG CC ATG ATC GTG TCA GTC ACC ACA ATA | AGAAAGCTGGGTC CTA TAC AAT CAG CCC GAC GGA GAT | |
| **LORF8** | AAAAAGCAGGCTCCG CC ATG GTG GGT AGT ATA CAG GTA G | AGAAAGCTGGGTC CTA TGA CAA AAG AGT TGC ACG GAT CC | 23 kDa protein |
| **UL45** | AAAAAGCAGGCTCCG CC ATG ATG TCG CCT ACA CCC GAG G | AGAAAGCTGGGTC CTA TTT CAT AAT TGC GTT CGA GAG AAT | Cell fusion protein |
| **UL46** | AAAAAGCAGGCTCCG CC ATG AAG CGG CTC AGC TCT TCT G | AGAAAGCTGGGTC CTA ATC GGT AGC CAC CCT CAA CCT AC | VP11/12, tegument protein |
| **UL47** | AAAAAGCAGGCTCCG CC ATG CAA ATG CCT TCT ATG CAT CGG | AGAAAGCTGGGTC CTA ATT CGC CCG TTG TGG CTC ACG | VP13/14, tegument protein |
| **UL48** | AAAAAGCAGGCTCCG CC ATG GAG GCA AAT ATG AG | AGAAAGCTGGGTC CTA TAA AGT ACT GAT AGT AG | VP16, tegument protein |
| **UL49** | AAAAAGCAGGCTCCG CC ATG GGG GAT TCT GAA AGG CGG | AGAAAGCTGGGTC CTA TTC GCT ATC ACT GCT ACG ATA | VP22, tegument protein |
| **UL49.5** | AAAAAGCAGGCTCCG CC ATG GGA CTC ATG GAC ATC CAT AAT G | AGAAAGCTGGGTC CTA CCA CTC CTC TTT AAA CAT ATC TGC | Glycoprotein N |
| **UL49.5(R)** | AAAAAGCAGGCTCCG CC ATG GGA CTC ATG GAC ATT CAT AAT GC | AGAAAGCTGGGTC CTA CCA CTC CTC TTT AAA CAT ATC TGC | Glycoprotein N |
| **UL50** | AAAAAGCAGGCTCCG CC ATG GAT GTC CAT GAG TCC CAT CCT C | AGAAAGCTGGGTC CTA TAT ACC GGT GGA TCC AAA CCC GG | dUTPase-like protein |
| **UL50(R)** | AAAAAGCAGGCTCCG CC ATG AAT GTC CAT GAG TCC CAT CCT | AGAAAGCTGGGTC CTA TAT ACC GGT GGA TCC AAA CCC GG | dUTPase-like protein |
| **UL51** | AAAAAGCAGGCTCCG CC ATG CAA ACC AGC TCA AGA ACA TAC G | AGAAAGCTGGGTC CTA TAA TTC GGT AAT GAG ATT ATT | UL51 virion phosphoprotein-like protein |
| **UL52** | AAAAAGCAGGCTCCG CC ATG GCG AGA TTT TCG TCT ATA TCC G | AGAAAGCTGGGTC CTA TTG ATG TTC TAA TCG ACA TTT CGA | DNA helicase-primase-like protein |
| **UL53** | AAAAAGCAGGCTCCG CC ATG TCG ATT AGA ACA TCA ATA GCT | AGAAAGCTGGGTC CTA AGG CAA ATA GGC ACG CCC AAA C | Glycoprotein K |
| **UL54** | AAAAAGCAGGCTCCG CC ATG TCT GTA GAT GCA TTC TCT CGC | AGAAAGCTGGGTC CTA CAT ACC AAA CAG AGT ATT GCA | ICP27 |

| ORF | FWD Primer | Rev Primer | Role |
|---|---|---|---|
| **LORF9** | AAAAAGCAGGCTCCG CC ATG TCT GCT AAC GGT ACA GAC C | AGAAAGCTGGGTC CTA TAT ATC ACC GAC TTT AGA TAC | Hypothetical protein |
| **UL55** | AAAAAGCAGGCTCCG CC ATG GCA GCA GGG GCG ATG TC | AGAAAGCTGGGTC CTA ATG ACA GTA CAT TCG CAG ATC | Nuclear matrix-associated protein |
| **LORF10** | AAAAAGCAGGCTCCG CC ATG GGC ATT ATT TTT TCC AAC CCC | AGAAAGCTGGGTC CTA ATC TAC TGT TGT TGG TCC AAA C | Similar to VZV ORF2 and to EHV-4 gene 3, Hypothetical protein |
| **LORF11** | AAAAAGCAGGCTCCG CC ATG TCT TGC TTC GCT TCT TCT ATA | AGAAAGCTGGGTC CTA ATT GTA AAT GGA TAT ATT CTG | Hypothetical protein |
| **LORF12** | AAAAAGCAGGCTCCG CC ATG GAA AAT GGA CAG CTG CAG C | AGAAAGCTGGGTC CTA TGT TTC TGT GAT AAT AGT TAC AAG | Hypothetical protein |
| **LORF12-cyt** | AAAAAGCAGGCTCCG CC ATG GAA AAT GGA CAG CTG CAG C | AGAAAGCTGGGTC CTA ATC ATG TCG AAT GAT AAA ACG | |
| **RLORF14a** | AAAAAGCAGGCTCCG CC ATG GAA TTC GAA GCA GAA CAC GAA GG | AGAAAGCTGGGTC CTA ATT TGA TTC AGA TTT TGT TTC TCC | Early 38-kDa phosphoprotein, PP38 |
| **RLORF13a** | AAAAAGCAGGCTCCG CC ATG ACC CAC AAC CGC GAT TTG TTT TTC | AGAAAGCTGGGTC CTA CCC TTT ATT GGA ATA GCC CCC | Binds to C1q-binding protein |
| **B68a** | AAAAAGCAGGCTCCG CC ATG ATG AAG CGG TTC GTC GGT CAG | AGAAAGCTGGGTC CTA GCC CAT CCT TTC TAC ATT GCA CG | VZV transinducing protein |
| **RLORF5a(R)** | AAAAAGCAGGCTCCG CC ATG CGC GTC ATG CAT AAC TGG CG | AGAAAGCTGGGTC CTA AAA ACT AAC GGT TCG TGG TTT TTT | Hypothetical protein |
| **RSORF1** | AAAAAGCAGGCTCCG CC ATG TCG GGA ATA TCC CTT ACT CC | AGAAAGCTGGGTC CTA CTG TGT TCT CGG TCC GGA GAT C | Hypothetical protein |
| **ANTISENSE** | AAAAAGCAGGCTCCG CC ATG TAC CCG ATT GTC CGG CTA GC | AGAAAGCTGGGTC CTA GCA CAT CGA AAT TTC CGC ACT TG | Antisense RNA protein |
| **ICP4(Fr)1** | AAAAAGCAGGCTCCG CC ATG GAC AAC CCG CCT GAT TTC GAC | AGAAAGCTGGGTC CTA TAT AAT ATA TGC ATA CTT GGG | Immediate-early protein |
| **ICP4(Fr)2** | AAAAAGCAGGCTCCG CC ATG TCA GCT TTT CTT CGA TTT CTG GG | AGAAAGCTGGGTC CTA ACG GCA TTT CTC CCA TTG TTG | Immediate-early protein |
| **ICP4(Fr)3** | AAAAAGCAGGCTCCG CC ATG ATT CTC CTC GTC TCC TGG TAC | AGAAAGCTGGGTC CTA GTC TGT AAA GGT CGT GTC CCG | Immediate-early protein |
| **SORF1** | AAAAAGCAGGCTCCG CC ATG CAT ATG TCG CAA CAT CAA TAT | AGAAAGCTGGGTC CTA AAC TGA CAC GGT CCA AGC GAA | Hypothetical protein |
| **SORF1(R)** | AAAAAGCAGGCTCCG CC ATG CAT ATG TCG CAA CAT CAA TAT | AGAAAGCTGGGTC CTA GGG GAT ATG AGA ACA GCT GC | Hypothetical protein |
| **SORF2** | AAAAAGCAGGCTCCG CC ATG CAG CGC CAA ACC GGA CAT ATG | AGAAAGCTGGGTC CTA ATG TAC TAC TTG CTC TAT ATA TTC | Binds to growth hormone |

| ORF | FWD Primer | Rev Primer | Role |
|---|---|---|---|
| **US1** | AAAAAGCAGGCTCCG CC ATG AGT CGT GAT CGA GAT CGA GCC | AGAAAGCTGGGTC CTA ATG CAA TTT ACT GTC TAC CGA | immediate early phosphoprotein, ICP22, binds to retinoblastoma- |
| **US10** | AAAAAGCAGGCTCCG CC ATG GCC ATG TGG TCT CTA CGG C | AGAAAGCTGGGTC CTA TAA GTA GGA TTC CCC GTC TCC TG | Virion protein binds to stem cell antigen 25 lymphocyte antigen 6 |
| **SORF3** | AAAAAGCAGGCTCCG CC ATG AGC AGA GTC AAT GCT ACA ATG | AGAAAGCTGGGTC CTA TAT GGG GTA TGC ATG AGG ATT AC | |
| **SORF4** | AAAAAGCAGGCTCCG CC ATG GCA CCT TCG GGA CCT ACG | AGAAAGCTGGGTC CTA GAA AAA TGA GAA TGA AAT TTT | |
| **US2** | AAAAAGCAGGCTCCG CC ATG GGT GTG TCC ATG ATA ACT ATA GTC | AGAAAGCTGGGTC CTA ATG ACT ACC GGC TCT ACA TTT TTT CTG | |
| **US3** | AAAAAGCAGGCTCCG CC ATG TCT TCG AGT CCG GAG GCA G | AGAAAGCTGGGTC CTA CAT ATG AGC GGC AGT TAT CGT | serine/threonine protein kinase |
| **US6** | AAAAAGCAGGCTCCG CC ATG AAT AGA TAC AGA TAT GAA AGT | AGAAAGCTGGGTC CTA TAG GCG GGA ATA TGC CCG TCT TG | Glycoprotein D |
| **US6-extra** | AAAAAGCAGGCTCCG CC ATG GGA CTT AAG AAA GAC AAT TCT | AGAAAGCTGGGTC CTA TTG CGT TCG GGA TTT TTT TTC TGT | |
| **US7** | AAAAAGCAGGCTCCG CC ATG TAT CTA CTA CAA TTA TTA TTT TGG | AGAAAGCTGGGTC CTA TAC ACA TTC TTC TCT TTC CAA CAA TTC | Glycoprotein I |
| **US7-extra** | AAAAAGCAGGCTCCG CC ATG TAT CTA CTA CAA TTA TTA TTT TGG | AGAAAGCTGGGTC CTA CCT TGG AAG GGT ATA TTT TAA | |
| **US7-cyt** | AAAAAGCAGGCTCCG CC ATG TGT GAA AGG TGC CGC TCT CC | AGAAAGCTGGGTC CTA TAC ACA TTC TTC TCT TTC CAA CAA TTC | |
| **US8** | AAAAAGCAGGCTCCG CC ATG TGT GTT TTC CAA ATC CTG ATA | AGAAAGCTGGGTC CTA GTG GTA TAA ATC TAA GCG TTT CCT | Glycoprotein E |
| **US8-extra** | AAAAAGCAGGCTCCG CC ATG TGT GTT TTC CAA ATC CTG ATA | AGAAAGCTGGGTC CTA TAA AAT ATC ATA AAT GAT AGT | |
| **US8-cyt** | AAAAAGCAGGCTCCG CC ATG AGG CGT AGG AGA CGA CGT CG | AGAAAGCTGGGTC CTA GTG GTA TAA ATC TAA GCG TTT CCT | |
| **vTR** | AAAAAGCAGGCTCCG CC ATG AAT GAC CGC GGA GTT CCA A | AGAAAGCTGGGTC CTA CTC ACA GAG CCC CGC G | MDV encoded telomerase |
| **chIRF7** | AAAAAGCAGGCTCCG CC ATG GCA GCG CTG GAC AGC G | AGAAAGCTGGGTC CTA GTC TGT CTG CAT GTG GTA TTG CTC | Chicken interferon regulatory factor 7 |

***Cyt*** *cytoplasmic domain;* ***Extra*** *extracellular domain;* ***R*** *RB-1B;* ***Fr*** *fragment.*

## Appendix C

**Table 3.2: The amplified and cloned ORFs of the MDV library**

| No. | Name | ORF | Template | PCR | Entry vector | Sequence |
|---|---|---|---|---|---|---|
| 1 | RLORF1 | MDV 002 | CVI988-RB-1B | - | - | - |
| 2 | RLORF2* | MDV 003 | RB-1B RNA | + | + | + |
| 3 | RLORF3* | MDV 003.2 | RB-1B BAC | + | + | + |
| 4 | RLORF4* | MDV 003.4 | CVI988 BAC | + | + | + |
| 5 | RLORF5 | MDV 003.6 | CVI988 BAC | + | + | + |
| 6 | RLORF5b | MDV 003.6 | RB-1B BAC | + | + | + |
| 7 | L1 | MDV 003.8 | CVI988 BAC | + | + | + |
| 8 | 23KDa | MDV 004 | CVI988 BAC | + | + | + |
| 9 | 23KDa | MDV 004 | RB-1B BAC | + | + | + |
| 10 | RLORF7 | MDV 005 | CVI988 BAC | + | + | + |
| 11 | RLORF7 | MDV 005 | RB-1B BAC | + | + | + |
| 12 | RLORF6 | MDV005.1 | CVI988 BAC | + | + | + |
| 13 | RLORF6 | MDV005.1 | RB-1B BAC | + | + | + |
| 14 | RLORF8 | MDV 005.4 | RB-1B BAC | + | + | + |
| 15 | RLORF9 | MDV 005.7 | RB-1B BAC | + | + | + |
| 16 | RLORF10 | MDV 005.8 | CVI988 BAC | + | + | + |
| 17 | 14KDA | MDV 006 | RB-1B RNA | + | + | + |
| 18 | 14KDB | MDV 006.1 | RB-1B RNA | + | + | + |
| 19 | RLORF11 | MDV 006.4 | CVI988 BAC | + | + | + |
| 20 | B68b* | MDV 006.6 | CVI988 BAC | + | + | + |
| 21 | RLORF12 | MDV 007 | CVI988 BAC | + | + | + |
| 22 | LORF1* | MDV 009 | CVI988-RB-1B | - | - | - |
| 23 | RLORF13b | MDV 009.5 | RB-1B BAC | + | + | + |
| 24 | RLORF14b | MDV 008 | CVI988 BAC | + | + | + |
| 25 | LORF2 | MDV 008.4 | CVI988 BAC | + | + | + |
| 26 | Lipase | MDV 010 | RB-1B RNA and cDNA library | + | + | Intron present |
| 27 | LORF3 | MDV 012 | CVI988 BAC | + | + | + |
| 28 | UL1 | MDV 013 | RB-1B BAC | + | + | + |
| 29 | LORF4* | MDV 013.5 | CVI988 BAC | + | + | + |
| 30 | UL2 | MDV 014 | CVI988 BAC | + | + | + |
| 31 | UL3 | MDV 015 | RB-1B BAC | + | + | + |

| No. | Name | ORF | Template | PCR | Entry vector | Sequence |
|---|---|---|---|---|---|---|
| **32** | **UL3.5** | **MDV 015.5** | CVI988 BAC | + | + | + |
| **33** | **UL4** | **MDV 016** | CVI988 BAC | + | + | + |
| **34** | **UL5** | **MDV 017** | RB-1B BAC | + | + | + |
| **35** | **UL6** | **MDV 018** | CVI988 BAC | + | + | + |
| **36** | **UL7** | **MDV 019** | CVI988 BAC | + | + | + |
| **37** | **UL8** | **MDV 020** | CVI988 BAC | + | + | + |
| **38** | **UL8.5** | **MDV 020.5** | CVI988 BAC | + | + | + |
| **39** | **UL9** | **MDV 021** | RB-1B BAC | + | + | + |
| **40** | **UL10*** | **MDV 022** | CVI988 BAC | + | + | + |
| **41** | **UL11** | **MDV 023** | CVI988 BAC | + | + | + |
| **42** | **UL12** | **MDV 024** | RB-1B BAC | + | + | + |
| **43** | **U13** | **MDV 025** | CVI988 BAC | + | + | + |
| **44** | **U13B** | **MDV 025.2** | RB-1B BAC | + | + | + |
| **45** | **U13A** | **MDV 025.1** | RB-1B BAC | + | + | + |
| **46** | **UL14** | **MDV 026** | CVI988 BAC | + | + | + |
| **47** | **UL15** | **MDV 027** | RB-1B BAC | + | + | + |
| **48** | **UL16** | **MDV 028** | CVI988 BAC | + | + | + |
| **49** | **UL17** | **MDV 029** | RB-1B BAC | + | + | + |
| **50** | **UL18** | **MDV 030** | CVI988 BAC | + | + | + |
| **51** | **UL19** | **MDV 031** | RB-1B BAC | + | + | + |
| **52** | **UL20*** | **MDV 032** | CVI988 BAC | + | + | + |
| **53** | **UL21** | **MDV 033** | CVI988 BAC | + | + | + |
| **54** | **UL22*** | **MDV 034** | CVI988 BAC | + | + | + |
| **55** | **UL23** | **MDV 036** | CVI988 BAC | + | + | + |
| **56** | **UL24** | **MDV 035** | CVI988 BAC | + | + | + |
| **57** | **UL25** | **MDV 037** | CVI988 BAC | + | + | + |
| **58** | **UL26** | **MDV 038** | RB-1B BAC | + | + | + |
| **59** | **UL26.5** | **MDV 039** | CVI988 BAC | + | + | + |
| **60** | **LORF5** | **MDV 039.5** | CVI988 BAC | + | + | + |
| **61** | **UL27*** | **MDV 040** | CVI988 BAC | + | + | + |
| **62** | **UL28** | **MDV 041** | RB-1B BAC | + | - | - |
| **63** | **UL29** | **MDV 042** | RB-1B BAC | + 2 fragments | + | + |
| **64** | **UL30** | **MDV 043** | CVI988 BAC | + | + | + |
| **65** | **UL31** | **MDV 044** | CVI988 BAC | + | + | + |
| **66** | **UL32** | **MDV 046** | CVI988 BAC | + | + | + |

| No. | Name | ORF | Template | PCR | Entry vector | Sequence |
|---|---|---|---|---|---|---|
| **67** | **UL33** | **MDV 045** | CVI988 BAC | + | + | + |
| **68** | **UL34*** | **MDV 047** | CVI988 BAC | + | + | + |
| **69** | **UL35** | **MDV 048** | CVI988 BAC | + | + | + |
| **70** | **UL36** | **MDV 049** | RB-1B BAC | + 4 fragments | + | + |
| **71** | **LORF6*** | **MDV 049.5** | CVI988 BAC | + | + | + |
| **72** | **UL37** | **MDV 050** | CVI988 BAC | + | + | + |
| **73** | **UL37** | **MDV 050** | RB-1B BAC | + | + | + |
| **74** | **LORF7** | **MDV 050.5** | CVI988 BAC | + | + | + |
| **75** | **UL38** | **MDV 051** | CVI988 BAC | + | + | + |
| **76** | **UL39** | **MDV 052** | RB-1B BAC | + | - | - |
| **77** | **UL40** | **MDV 053** | RB-1B BAC | + | + | + |
| **78** | **UL41** | **MDV 054** | RB-1B BAC | + | + | + |
| **79** | **UL42** | **MDV 055** | CVI988 BAC | + | + | + |
| **80** | **UL43*** | **MDV 056** | CVI988 BAC | + | + | + |
| **81** | **UL44*** | **MDV 057** | CVI988 BAC | + | + | + |
| **82** | **UL44a** | **MDV 057** | RB-1B BAC | + | + | + |
| **83** | **UL44b** | **MDV 057.1** | RB-1B BAC | + | + | + |
| **84** | **MDV57.4** | **MDV 057.4** | CVI988 BAC | + | + | + |
| **85** | **MDV57.4** | **MDV 057.4** | RB-1B BAC | + | + | + |
| **86** | **LORF8** | **MDV 057.8** | CVI988 BAC | + | + | + |
| **87** | **UL45** | **MDV 058** | CVI988 BAC | + | + | + |
| **88** | **UL46** | **MDV 059** | CVI988 BAC | + | + | + |
| **89** | **UL47** | **MDV 060** | RB-1B BAC | + | + | + |
| **90** | **UL48** | **MDV 061** | RB-1B BAC | + | + | + |
| **91** | **UL49** | **MDV 062** | RB-1B BAC | + | + | + |
| **92** | **UL49.5** | **MDV 064** | CVI988 BAC | + | + | + |
| **93** | **UL49.5** | **MDV 064** | RB-1B BAC | + | + | + |
| **94** | **UL50** | **MDV 063** | CVI988 BAC | + | + | + |
| **95** | **UL50** | **MDV 063** | RB-1B BAC | + | + | + |
| **96** | **UL51** | **MDV 065** | CVI988 BAC | + | + | + |
| **97** | **UL52** | **MDV 066** | CVI988 BAC | + | + | + |
| **98** | **UL53*** | **MDV 067** | CVI988 BAC | + | + | + |
| **99** | **UL54** | **MDV 068** | CVI988 BAC | + | + | + |
| **100** | **LORF9** | **MDV 069** | RB-1B BAC | + | + | + |

| No. | Name | ORF | Template | PCR | Entry vector | Sequence |
|---|---|---|---|---|---|---|
| **101** | **UL55** | **MDV 070** | CVI988 BAC | + | + | + |
| **102** | **LORF10** | **MDV 071** | CVI988 BAC | + | + | + |
| **103** | **LORF11** | **MDV 072** | RB-1B BAC | + | - | - |
| **104** | **LORF12** | **MDV072.8** | CVI988BAC | + | + | + |
| **105** | **RLORF14a** | **MDV 073** | RB-1B BAC | + | + | + |
| **106** | **RLORF13a** | **MDV 073.4** | RB-1B BAC | + | + | + |
| **107** | **RLORF12** | **MDV 074** | RB-1B BAC | + | + | + |
| **108** | **B68a*** | **MDV 075.1** | RB-1B BAC | + | + | + |
| **109** | **RLORF5a** | **MDV 078.2** | RB-1B BAC | + | + | + |
| **110** | **RSORF1** | **MDV 082** | CVI988 BAC | + | + | + |
| **111** | **Antisense** | **MDV 083** | CVI988 BAC | + | + | + |
| **112** | **ICP4** | **MDV 084** | RB-1B BAC | + 2 fragments | + | + |
| **113** | **SORF1** | **MDV 086.6** | CVI988 BAC | + | + | + |
| **114** | **SORF1** | **MDV 086.6** | RB-1B BAC | + | + | + |
| **115** | **SORF2** | **MDV 087** | RB-1B BAC | + | + | + |
| **116** | **US1** | **MDV 088** | CVI988 BAC | + | + | + |
| **117** | **US10** | **MDV 089** | CVI988 BAC | + | + | + |
| **118** | **SORF3** | **MDV 090** | RB-1B BAC | + | + | + |
| **119** | **US2** | **MDV 091** | RB-1B cDNA Library | + | + | + |
| **120** | **US3** | **MDV 092** | RB-1B BAC | + | + | + |
| **121** | **SORF4** | **MDV 093** | RB-1B BAC | + | + | + |
| **122** | **US6*** | **MDV 094** | RB-1B BAC | + | + | + |
| **123** | **US7*** | **MDV 095** | RB-1B BAC | + 2 fragments | + | + |
| **124** | **US8*** | **MDV 096** | RB-1B BAC | + | + | + |
| **125** | **vTR** | **MDV001a** | RB-1B BAC | - | - | - |

**** Represent the proteins which have transmembrane domain(s), and they were cloned as domains as well as full length.***

## Appendix D

**Table 4.2.1: Identification of Marek's disease virus protein-protein interaction using Yeast two-hybrid (Y2H)**

| Interaction | | Interaction | | Interaction | |
|---|---|---|---|---|---|
| **Bait** | **Prey** | **Bait** | **Prey** | **Bait** | **Prey** |
| vIL8 | Meq | RLORF5bRB-1B | UL20 | LMeq | SORF2 |
| vIL8 | UL27 | L1 | LORF4 | LMeq | UL51 |
| vIL8 | UL8.5 | 23KDaCVI | RLORF13a | LMeq | UL36 |
| RLORF3 | Md57.4CVI | 23KDaCVI | RLORF13b | LMeq | UL36 |
| RLORF3 | RLORF12aCVI | 23KDaCVI | UL25 | LMeq | US10 |
| RLORF3 | LORF6 | 23KDaRB-1B | 14KDb | RLORF6CVI | Md57.4CVI |
| RLORF3 | RLORF5bRB-1B | 23KDaRB-1B | B68b | RLORF6CVI | RLORF12aCVI |
| RLORF3 | UL27 | 23KDaRB-1B | Md57.4CVI | RLORF6RB-1B | 14KDb |
| RLORF3 | UL36 | 23KDaRB-1B | Md57.4RB-1B | RLORF8 | UL27 |
| RLORF3 | US6 | 23KDaRB-1B | RLORF12aCVI | RLORF9 | Md57.4CVI |
| RLORF3 | vIL8 | 23KDaRB-1B | SORF1CVI | RLORF9 | PP24 |
| RLORF4 | RLORF13a | 23KDaRB-1B | UL3.5 | RLORF9 | RLORF13a |
| RLORF4 | RLORF13b | 23KDaRB-1B | US7 | RLORF9 | RLORF13b |
| RLORF4 | Md57.4CVI | 23KDaRB-1B | US8 | RLORF9 | UL27 |
| RLORF4 | RLORF11 | 23KDaRB-1B | vIL8 | RLORF10 | UL27 |
| RLORF5CVI | UL27 | Meq | Meq | RLORF11 | LORF5 |
| RLORF5CVI | UL32 | Meq | PP24 | RLORF11 | US3 |
| RLORF5ARB-1B | UL25 | Meq | UL20 | RLORF12aCVI | PP38 |
| RLORF5bRB-1B | Meq | LMeq | LORF10 | RLORF12aCVI | vIL8 |
| RLORF12RB-1B | 14KDb | 14KDb | vIL8 | LORF4 | UL13 |
| RLORF12RB-1B | B68b | B68a | Meq | LORF4 | UL38 |

| Interaction | | Interaction | | Interaction | |
|---|---|---|---|---|---|
| **Bait** | **Prey** | **Bait** | **Prey** | **Bait** | **Prey** |
| RLORF12RB-1B | Md57.4CVI | B68a | PP24 | LORF4 | UL10 |
| RLORF12RB-1B | Md57.4RB-1B | B68a | UL20 | LORF4 | UL2 |
| RLORF12RB-1B | PP38 | B68a | UL30 | LORF4 | UL3.5 |
| RLORF12RB-1B | RLORF12aCVI | PP24 | Md57.4CVI | LORF4 | UL36 |
| RLORF12RB-1B | SORF1CVI | PP24 | Meq | LORF4 | UL36 |
| RLORF12RB-1B | UL3.5 | PP24 | PP24 | LORF4 | UL44CVI |
| RLORF12RB-1B | US8 | PP24 | PP38 | LORF4 | UL5 |
| RLORF12RB-1B | vIL8 | PP24 | UL20 | LORF4 | UL52 |
| RLORF13a | RLORF13b | PP24 | US8 | LORF4 | US10 |
| RLORF13a | UL47 | PP24 | vIL8 | LORF4 | UL27 |
| RLORF13b | 14KDb | PP38 | Meq | UL2 | SORF3 |
| RLORF13b | Md57.4CVI | PP38 | PP24 | UL2 | UL30 |
| RLORF13b | RLORF13a | PP38 | UL20 | UL3.5 | UL48 |
| RLORF13b | RLORF13b | UL1 | UL27 | UL4 | SORF3 |
| 14KDa | Meq | LORF4 | UL50RB-1B | UL4 | UL51 |
| 14KDa | PP24 | LORF4 | 14KDb | UL4 | UL27 |
| 14KDa | UL20 | LORF4 | B68a | UL4 | US10 |
| 14KDb | Meq | LORF4 | Md57.4CVI | UL4 | US8 |
| 14KDb | PP24 | LORF4 | Md57.4RB-1B | UL5 | UL52 |
| 14KDb | PP38 | LORF4 | Meq | UL5 | US10 |
| 14KDb | UL20 | LORF4 | RLORF12aCVI | UL6 | B68b |
| 14KDb | UL22 | LORF4 | SORF1CVI | UL6 | LORF7 |
| UL6 | UL29 | UL10 | B68b | UL12 | UL50CVI |
| UL6 | UL30 | UL10 | PP24 | UL12 | UL51 |

| Interaction | | Interaction | | Interaction | |
|---|---|---|---|---|---|
| **Bait** | **Prey** | **Bait** | **Prey** | **Bait** | **Prey** |
| UL6 | UL51 | UL10 | vIL8 | UL12 | UL20 |
| UL6 | UL9 | UL10 | Md57.4CVI | UL12 | UL36 |
| UL6 | UL3.5 | UL10 | Md57.4RB-1B | UL12 | UL40 |
| UL6 | UL4 | UL10 | RLORF12aCVI | UL12 | UL55 |
| UL6 | UL45 | UL10 | SORF1CVI | UL12 | US1 |
| UL6 | UL52 | UL10 | 23KDaCVI | UL12 | US10 |
| UL6 | UL6 | UL10 | UL14 | UL12 | US7 |
| UL7 | UL51 | UL10 | UL54 | UL13 | UL55 |
| UL8 | LORF10 | UL10 | UL22 | UL13ARB-1B | UL25 |
| UL8 | SORF2 | UL11 | UL16 | UL13ARB-1B | UL27 |
| UL8 | UL51 | UL11 | UL8.5 | UL13ARB-1B | UL37CVI |
| UL8 | UL36 | UL11 | vIL8 | UL13ARB-1B | UL37RB-1B |
| UL8 | UL48 | UL12 | PP24 | UL13ARB-1B | SORF4 |
| UL8 | UL52 | UL12 | RLORF12aCVI | UL13ARB-1B | B68a |
| UL8 | UL55 | UL12 | RLORF13a | UL13BRB-1B | UL27 |
| UL8 | US10 | UL12 | RLORF13b | UL14 | LORF5 |
| UL8.5 | UL6 | UL12 | SORF2 | UL14 | UL51 |
| UL9 | UL27 | UL12 | SORF3 | UL14 | US10 |
| UL9 | UL34 | UL12 | UL14 | UL15 | Md57.4CVI |
| UL9 | UL36 | UL12 | UL20 | UL16 | Md57.4CVI |
| UL9 | UL9 | UL12 | UL25 | UL17 | SORF3 |
| UL9 | US10 | UL12 | UL42 | UL17 | UL25 |
| UL17 | US10 | UL26 | B68b | UL30 | UL42 |
| UL18 | UL27 | UL26 | Md57.4CVI | UL30 | US10 |

| Interaction | | Interaction | | Interaction | |
|---|---|---|---|---|---|
| **Bait** | **Prey** | **Bait** | **Prey** | **Bait** | **Prey** |
| UL20 | Meq | UL26 | Md57.4RB-1B | UL32 | RLORF5bRB-1B |
| UL20 | PP24 | UL26 | PP38 | UL32 | RLORF5CVI |
| UL20 | UL20 | UL26 | RLORF12aCVI | UL32 | UL14 |
| UL20 | UL41 | UL26 | SORF1CVI | UL32 | UL32 |
| UL20 | LORF12 | UL26 | UL3.5 | UL32 | UL34 |
| UL20 | UL32 | UL26 | UL48 | UL32 | US6 |
| UL20 | vIL8 | UL26 | US8 | UL33 | Meq |
| UL22 | UL27 | UL26 | vIL8 | UL33 | UL16 |
| UL22 | UL36 | LORF5 | SORF1CVI | UL33 | UL26 |
| UL22 | 23KDaRB-1B | UL27 | as | UL33 | UL31 |
| UL22 | RLORF13a | UL27 | LORF1 | UL33 | vIL8 |
| UL22 | UL27 | UL27 | UL32 | UL34 | SORF1CVI |
| UL23 | UL23 | UL27 | UL53 | UL34 | UL36 |
| UL24 | UL13 | UL27 | PP24 | UL34 | 14KDb |
| UL25 | UL25 | UL27 | SORF3 | UL34 | Md57.4CVI |
| UL25 | UL29 | UL27 | UL27 | UL34 | Md57.4RB-1B |
| UL25 | UL54 | UL29 | UL27 | UL34 | PP38 |
| UL25 | UL9 | UL30 | Md57.4CVI | UL34 | RLORF12aCVI |
| UL25 | UL10 | UL30 | RLORF12aCVI | UL34 | SORF1CVI |
| UL25 | UL36 | UL30 | UL20 | UL34 | UL3.5 |
| UL25 | UL44CVI | UL30 | UL26 | UL34 | US8 |
| UL26 | 14KDb | UL30 | UL27 | UL34 | vIL8 |
| UL35 | Md57.4CVI | LORF6 | UL3.5 | UL44CVI | PP38 |
| UL35 | UL54 | LORF6 | US8 | UL44CVI | RLORF12aCVI |

| Interaction | | Interaction | | Interaction | |
|---|---|---|---|---|---|
| **Bait** | **Prey** | **Bait** | **Prey** | **Bait** | **Prey** |
| UL36 | LORF10 | LORF6 | vIL8 | UL44CVI | RLORF5CVI |
| UL36 | Md57.4CVI | LORF6 | UL32 | UL44CVI | UL51 |
| UL36 | RLORF12aCVI | UL38 | UL36 | UL44CVI | UL40 |
| UL36 | SORF2 | UL40 | UL40 | UL44CVI | US7 |
| UL36 | SORF3 | UL40 | US10 | UL44ARB-1B | UL27 |
| UL36 | UL25 | UL42 | Meq | UL44ARB-1B | PP24 |
| UL36 | UL51 | UL42 | PP24 | UL44BRB-1B | UL27 |
| UL36 | UL36 | UL42 | UL42 | UL44BRB-1B | PP24 |
| UL36 | UL36 | UL43 | PP24 | Md57.4CVI | 14KDb |
| UL36 | US10 | UL43 | 14KDb | Md57.4CVI | Md57.4CVI |
| UL37CVI | UL25 | UL43 | B68b | Md57.4CVI | RLORF13a |
| UL37CVI | UL51 | UL43 | Md57.4CVI | Md57.4CVI | RLORF13b |
| UL37CVI | UL36 | UL43 | Md57.4RB-1B | Md57.4CVI | UL41 |
| UL37CVI | US10 | UL43 | PP38 | Md57.4RB-1B | Md57.4CVI |
| UL37RB-1B | UL25 | UL43 | RLORF12aCVI | LORF8 | UL4 |
| UL37RB-1B | UL26 | UL43 | RLORF4 | LORF8 | 23KDaRB-1B |
| UL37RB-1B | UL27 | UL43 | SORF1CVI | LORF8 | LORF6 |
| UL37RB-1B | UL51 | UL43 | UL3.5 | LORF8 | RLORF12aCVI |
| UL37RB-1B | UL54 | UL43 | UL36 | LORF8 | RLORF5bRB-1B |
| UL37RB-1B | US10 | UL43 | US8 | LORF8 | UL14 |
| LORF6 | 14KDb | UL43 | vIL8 | LORF8 | UL27 |
| LORF6 | PP38 | UL44CVI | Md57.4CVI | LORF8 | UL36 |
| UL45 | Meq | UL49.5CVI | Meq | UL53 | Meq |
| UL45 | PP24 | UL49.5CVI | PP24 | UL53 | PP24 |

| Interaction | | Interaction | | Interaction | |
|---|---|---|---|---|---|
| **Bait** | **Prey** | **Bait** | **Prey** | **Bait** | **Prey** |
| UL45 | UL48 | UL49.5CVI | UL20 | UL53 | UL18 |
| UL46 | RLORF13b | UL49.5RB-1B | PP24 | UL53 | UL20 |
| UL46 | UL47 | UL49.5RB-1B | UL20 | UL53 | UL26 |
| UL47 | 14KDa | UL50CVI | UL50CVI | UL53 | UL26.5 |
| UL47 | RLORF13a | UL50RB-1B | Md57.4CVI | UL53 | UL29 |
| UL47 | RLORF13b | UL50RB-1B | UL50CVI | UL53 | UL45 |
| UL47 | UL5 | UL51 | UL51 | UL54 | UL14 |
| UL47 | US2 | UL51 | UL5 | UL54 | UL54 |
| UL45 | Meq | UL51 | UL7 | UL54 | US8 |
| UL45 | PP24 | UL52 | 14KDb | LORF9 | RLORF11 |
| UL45 | UL48 | UL52 | ICP4 | LORF9 | RLORF12aCVI |
| UL46 | RLORF13b | UL52 | LORF6 | LORF9 | RLORF13a |
| UL46 | UL47 | UL52 | RLORF3 | LORF9 | UL27 |
| UL47 | 14KDa | UL52 | UL22 | LORF9 | UL4 |
| UL47 | RLORF13a | UL52 | UL8 | LORF9 | US2 |
| UL47 | RLORF13b | UL52 | UL10 | UL55 | 14KDb |
| UL47 | UL5 | UL52 | UL36 | UL55 | Md57.4CVI |
| UL47 | US2 | UL52 | UL4 | UL55 | PP38 |
| UL49 | PP38 | UL52 | UL43 | UL55 | RLORF12aCVI |
| UL49 | SORF1CVI | UL52 | UL5 | UL55 | UL3.5 |
| UL49 | UL48 | UL52 | US10 | UL55 | UL55 |
| UL49.5RB-1B | Meq | UL53 | B68b | UL55 | US8 |
| UL55 | vIL8 | RSORF1 | US8 | US7 | vIL8 |
| LORF10 | LORF10 | RSORF1 | vIL8 | US8 | UL27 |

| Interaction | | Interaction | | Interaction | |
|---|---|---|---|---|---|
| **Bait** | **Prey** | **Bait** | **Prey** | **Bait** | **Prey** |
| LORF10 | Md57.4CVI | as | Md57.4CVI | US8 | US8 |
| LORF10 | SORF2 | as | RLORF12aCVI | | |
| LORF10 | UL11 | as | SORF1CVI | | |
| LORF10 | UL25 | as | UL27 | | |
| LORF10 | UL8 | ICP4 | 14KDb | | |
| LORF10 | UL9 | ICP4 | Md57.4CVI | | |
| LORF10 | UL10 | ICP4 | ICP4 | | |
| LORF10 | UL36 | SORF1CVI | B68b | | |
| LORF10 | UL43 | SORF1CVI | Md57.4CVI | | |
| LORF10 | US10 | US1 | UL13 | | |
| LORF10 | US2 | US1 | UL51 | | |
| LORF12 | RLORF12aCVI | US1 | UL36 | | |
| LORF12 | RLORF5CVI | US1 | UL40 | | |
| LORF12 | UL26 | US1 | US10 | | |
| LORF12 | UL13 | US1 | US8 | | |
| LORF12 | UL4 | US10 | SORF2 | | |
| RSORF1 | 14KDb | US10 | UL13 | | |
| RSORF1 | Md57.4CVI | US10 | UL51 | | |
| RSORF1 | Md57.4RB-1B | US10 | UL36 | | |
| RSORF1 | RLORF12aCVI | US10 | UL40 | | |
| RSORF1 | SORF1CVI | US10 | US10 | | |
| RSORF1 | UL3.5 | US7 | PP38 | | |

## Appendix E

**Table 5.2.2: The numerical values of the effect of MDV proteins on the interferon-alpha inducibility of the chickens ISRE motif**

| ORF+IFN | Fold change | Standard deviation | ORF | Fold change | Standard deviation |
|---|---|---|---|---|---|
| eGFP+IFN | 1.0 | 0.16534 | eGFP | 0.069823 | 0.062675 |
| vIL8+IFN | 1.49209 | 0.262507 | vIL8 | 0.041966 | 0.004871 |
| RLORF3+IFN | 1.328954 | 0.487856 | RLORF3 | 0.237044 | 0.017722 |
| RLORF4+IFN | 1.037674 | 0.334241 | RLORF4 | 0.011612 | 0.003654 |
| RLORF5RB-1B+IFN | 1.808734 | 0.704699 | RLORF5bRB-1B | 0.068819 | 0.027594 |
| RLORF5CVI+IFN | 1.765183 | 0.293051 | RLORF5CVI | 0.063423 | 0.033825 |
| L1+IFN | 1.25489 | 0.126396 | L1 | 0.056665 | 0.010278 |
| 23kda_CVI+IFN | 1.162766 | 0.202582 | 23KDaCVI | 0.072864 | 0.03851 |
| 23kda_RB-1B+IFN | 1.069637 | 0.184628 | 23KDaRB-1B | 0.078587 | 0.0358 |
| LMeq+IFN | 0.31064 | 0.026773 | LMeq | 0.024027 | 0.008082 |
| Meq+IFN | 0.449744 | 0.114416 | Meq | 0.015069 | 0.012314 |
| RLORF6CVI+IFN | 1.445779 | 0.166815 | RLORF6CVI | 0.087652 | 0.00747 |
| RLORF6RB-1B+IFN | 2.1089 | 0.886282 | RLORF6RB-1B | 0.110289 | 0.080382 |
| RLORF8+IFN | 1.329707 | 0.215092 | RLORF8 | 0.084709 | 0.048104 |
| RLORF9+IFN | 1.35835 | 0.140026 | RLORF9 | 0.054757 | 0.022971 |
| RLORF10+IFN | 1.58485 | 0.447502 | RLORF10 | 0.10022 | 0.065212 |
| 14KDa+IFN | 1.237381 | 0.088084 | 14KDa | 0.046228 | 0.00709 |
| 14KDb+IFN | 0.994252 | 0.094758 | 14KDb | 0.045296 | 0.026059 |
| RLORF11+IFN | 0.522039 | 0.052994 | RLORF11 | 0.020802 | 0.007716 |
| B68b+IFN | 1.116021 | 0.121436 | B68b | 0.029428 | 0.002101 |
| RLORF12bCVI+IFN | 0.832019 | 0.130909 | RLORF12bCVI | 0.016175 | 0.00413 |
| RLORF13b+IFN | 0.942246 | 0.201298 | RLORF13b | 0.037771 | 0.012397 |
| PP24+IFN | 1.341103 | 0.335274 | PP24 | 0.049424 | 0.033697 |
| LORF2+IFN | 1.682861 | 0.108702 | LORF2 | 0.049625 | 0.006588 |
| LORF3+IFN | 0.723325 | 0.136411 | LORF3 | 0.032662 | 0.006508 |
| UL1+IFN | 1.499119 | 0.348279 | UL1 | 0.081774 | 0.072394 |
| LORF4+IFN | 1.183722 | 0.095527 | LORF4 | 0.045067 | 0.01455 |
| UL2+IFN | 1.196484 | 0.262369 | UL2 | 0.316391 | 0.252665 |
| UL3+IFN | 1.26982 | 0.137849 | UL3 | 0.085913 | 0.016591 |
| UL3.5+IFN | 1.734225 | 0.841201 | UL3.5 | 0.508306 | 0.24926 |
| UL4+IFN | 0.594858 | 0.210887 | UL4 | 0.090546 | 0.017355 |
| UL5+IFN | 1.909871 | 0.062944 | UL5 | 0.255532 | 0.033011 |
| UL6+IFN | 1.988312 | 0.902447 | UL6 | 0.107 | 0.046826 |
| UL7+IFN | 0.919352 | 0.283584 | UL7 | 0.058762 | 0.003335 |
| UL8+IFN | 1.257369 | 0.760573 | UL8 | 0.186898 | 0.0968 |
| UL9+IFN | 1.040604 | 0.111186 | UL9 | 0.069287 | 0.025947 |
| UL10+IFN | 1.682967 | 0.934403 | UL10 | 0.121739 | 0.002928 |
| UL11+IFN | 0.916876 | 0.293177 | UL11 | 0.045984 | 0.008159 |
| UL12+IFN | 0.246524 | 0.014665 | UL12 | 0.071167 | 0.009674 |

| ORF+IFN | Fold change | Standard deviation | ORF | Fold change | Standard deviation |
|---|---|---|---|---|---|
| UL13+IFN | 1.596626 | 0.183903 | UL13 | 0.149715 | 0.047171 |
| UL13A+IFN | 0.87577 | 0.365039 | UL13aRB-1B | 0.24768 | 0.118737 |
| UL13b+IFN | 1.511026 | 0.264313 | UL13bRB-1B | 0.079071 | 0.008417 |
| UL14+IFN | 1.235135 | 0.116192 | UL14 | 0.056171 | 0.001616 |
| UL15+IFN | 1.038761 | 0.018748 | UL15 | 0.018748 | 0.003049 |
| UL16+IFN | 1.187254 | 0.28919 | UL16 | 0.085952 | 0.016884 |
| UL17+IFN | 1.718816 | 0.412036 | UL17 | 0.066694 | 0.016937 |
| UL18+IFN | 1.112079 | 0.149059 | UL18 | 0.600161 | 0.139604 |
| UL19+IFN | 1.694555 | 0.200181 | UL19 | 0.466247 | 0.051126 |
| UL20+IFN | 0.695007 | 0.135863 | UL20 | 0.423045 | 0.214537 |
| UL21+IFN | 1.068993 | 0.101595 | UL21 | 0.062631 | 0.034783 |
| UL22+IFN | 0.66958 | 0.07006 | UL22 | 0.012675 | 0.000623 |
| UL23+IFN | 1.12328 | 0.029275 | UL23 | 0.038805 | 0.016874 |
| UL24+IFN | 1.091794 | 0.120934 | UL24 | 0.016113 | 0.00532 |
| UL25+IFN | 0.888225 | 0.129982 | UL25 | 0.014994 | 0.002952 |
| UL26+IFN | 0.412425 | 0.08982 | UL26 | 0.015136 | 0.002819 |
| UL26.5+IFN | 0.551151 | 0.032191 | UL26.5 | 0.016616 | 0.003921 |
| LORF5+IFN | 1.661143 | 0.065003 | LORF5 | 0.075038 | 0.045707 |
| UL27+IFN | 1.444679 | 0.245428 | UL27 | 0.045212 | 0.02314 |
| UL30+IFN | 0.627062 | 0.017542 | UL30 | 0.014746 | 0.001789 |
| UL31+IFN | 1.169132 | 0.287987 | UL31 | 0.050541 | 0.000178 |
| UL32+IFN | 0.583084 | 0.070248 | UL32 | 0.010007 | 0.001644 |
| UL33+IFN | 0.793754 | 0.065273 | UL33 | 0.023201 | 0.007881 |
| UL34+IFN | 1.619251 | 0.308477 | UL34 | 0.030592 | 0.031362 |
| UL35+IFN | 1.455501 | 0.240831 | UL35 | 0.035731 | 0.000717 |
| LORF6+IFN | 1.265154 | 0.133452 | LORF6 | 0.024812 | 0.006617 |
| UL37_CVI+IFN | 0.865137 | 0.044159 | UL37CVI | 0.118946 | 0.044562 |
| UL37_RB-1B+IFN | 1.128124 | 0.104866 | UL37RB-1B | 0.420194 | 0.156932 |
| LORF7+IFN | 0.634582 | 0.366021 | LORF7 | 0.035517 | 0.000621 |
| UL38+IFN | 1.805827 | 0.12199 | UL38 | 0.086299 | 0.020257 |
| UL40+IFN | 2.673375 | 1.090528 | UL40 | 0.099089 | 0.008966 |
| UL41+IFN | 0.898225 | 0.006341 | UL41 | 0.139678 | 0.028961 |
| UL42+IFN | 0.660176 | 0.062826 | UL42 | 0.040624 | 0.005444 |
| UL43+IFN | 1.219835 | 0.255028 | UL43 | 0.246007 | 0.023494 |
| UL44CVI+IFN | 1.166072 | 0.462613 | UL44CVI | 0.040617 | 0.037494 |
| UL44a+IFN | 1.028272 | 0.09567 | UL44aRB-1B | 0.035279 | 0.003596 |
| UL44b+IFN | 0.821422 | 0.233449 | UL44bRB-1B | 0.022262 | 0.009716 |
| MD57.4CVI+IFN | 1.799864 | 0.528962 | MD57.4 | 0.065463 | 0.045611 |
| LORF8+IFN | 0.883265 | 0.077757 | LORF8 | 0.03832 | 0.017787 |
| UL45+IFN | 0.929565 | 0.169686 | UL45 | 0.102615 | 0.028908 |
| UL46+IFN | 0.663386 | 0.125843 | UL46 | 0.106084 | 0.101098 |
| UL48+IFN | 1.302712 | 0.07279 | UL48 | 0.095594 | 0.010654 |
| UL49.5_CVI+IFN | 1.447516 | 0.102125 | UL49.5CVI | 0.091263 | 0.007836 |
| UL49.5_RB-1B+IFN | 1.234157 | 0.115882 | UL49.5RB-1B | 0.093556 | 0.005454 |

| ORF+IFN | Fold change | Standard deviation | ORF | Fold change | Standard deviation |
|---|---|---|---|---|---|
| UL50CVI+IFN | 0.455546 | 0.053802 | UL50CVI | 0.006681 | 0.011572 |
| UL50RB-1B+IFN | 0.450693 | 0.140661 | UL50RB-1B | 0.032085 | 0.008965 |
| UL51+IFN | 1.028272 | 0.09567 | UL51 | 0.035279 | 0.003596 |
| UL52+IFN | 1.718816 | 0.412036 | UL52 | 0.066694 | 0.016937 |
| UL53+IFN | 1.431731 | 0.052063 | UL53 | 0.082695 | 0.011428 |
| UL54+IFN | 0.698898 | 0.1102 | UL54 | 0.044294 | 0.022974 |
| LORF9+IFN | 0.97934 | 0.095722 | LORF9 | 0.024011 | 0.003606 |
| UL55+IFN | 0.904668 | 0.143916 | UL55 | 0.546908 | 0.300884 |
| LORF10+IFN | 1.788456 | 0.134222 | LORF10 | 0.035228 | 0.004813 |
| LORF12+IFN | 1.093384 | 0.095898 | LORF12 | 0.039548 | 0.018336 |
| PP38+IFN | 1.141776 | 0.065059 | PP38 | 0.209004 | 0.064627 |
| RLORF13a+IFN | 0.791402 | 0.196789 | RLORF13a | 0.021756 | 0.005287 |
| RLORF12a+IFN | 1.040934 | 0.207575 | RLORF12a | 0.049289 | 0.018054 |
| B68a+IFN | 1.008997 | 0.071038 | B68a | 0.027764 | 0.004686 |
| RSORF1+IFN | 1.306082 | 0.295115 | RSORF1 | 0.04113 | 0.013423 |
| AS+IFN | 1.15371 | 0.210209 | AS | 0.032659 | 0.006458 |
| SORF1CVI+IFN | 0.850416 | 0.144585 | SORF1CVI | 0.03617 | 0.01415 |
| SORF1RB-1B+IFN | 1.264532 | 0.184965 | SORF1RB-1B | 0.042009 | 0.003105 |
| SORF2+IFN | 1.660094 | 0.529852 | SORF2 | 0.059386 | 0.016165 |
| US1+iIFN | 0.69414 | 0.051642 | US1 | 0.031017 | 0.013478 |
| US10+IFN | 1.046616 | 0.395184 | US10 | 0.052247 | 0.014336 |
| SORF3+IFN | 0.883528 | 0.041291 | SORF3 | 0.050312 | 0.014624 |
| US2+IFN | 1.293782 | 0.186567 | US2 | 0.039032 | 0.013763 |
| US3+IFN | 0.77547 | 0.158048 | US3 | 0.178141 | 0.02916 |
| SORF4+IFN | 1.431731 | 0.052063 | SORF4 | 0.082695 | 0.011428 |
| US6+IFN | 1.348856 | 0.511236 | US6 | 0.051167 | 0.002772 |
| US8+IFN | 1.296726 | 0.402383 | US8 | 0.078969 | 0 |

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