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Development of novel virus vectors for influenza vaccination

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Declaration

I declare that this thesis has been composed by myself and has not been submitted for any other degree. The work described herein is my own except where otherwise indicated and all work of other authors is duly acknowledged.

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Abstract

The influenza virus, a member of the *Orthomyxoviridae* family, causes regular, large-scale morbidity and mortality in birds and humans and significant human suffering and economic loss. The primary aim of this study was to develop a novel influenza vaccine. Vaccines are an essential tool for the control of influenza because they increase resistance to infection, prevent illness and death and help to limit virus transmission to other birds and mammals, including humans. By reducing the environmental contamination of influenza virus in global poultry stocks, the risk of a new pandemic virus being generated by the human-avian link is diminished.

Marek's Disease is a common lymphoproliferative disease of poultry that is readily controlled worldwide using the live attenuated vaccine, CVI988. The Marek's Disease Virus (MDV) CVI988 viral genome, available as a Bacterial Artificial Chromosome (BAC), forms viable infectious viral particles when transfected into Chicken Embryo Fibroblast (CEF) cells. Using BAC mutagenesis, two non-essential genes in the MDV CVI988 BAC (UL41 and US10), were identified and replaced by the low pathogenic influenza haemagglutinin 10 (H10) gene. These live recombinant MDV-H10 vectors will allow simultaneous vaccination against both pathogens. In addition, the non-essential genes were also replaced with GFP creating MDV-GFP constructs. Both genes were expressed initially using a CMV promoter, although this disrupted the MDV CVI988 BAC; a second promoter, PGK-1, proved more successful. A third MDV gene (UL50) was deleted, but severe attenuation prevented the incorporation of H10 into this open reading frame.

Future work to test the MDV-HA constructs *in vivo* will be carried out in collaboration with the Istituto Zooprofilattico Sperimentale delle Venezie in Italy. In addition, development of MDV constructs containing multiple HA genes (H10 and H5) linked by the 2A polyprotein can be developed with the goal of establishing heterosubtypic immunity.

Table of Contents

Abbreviations

AI	Avian influenza
AP	Antarctic phosphatase
BAC	Bacterial artificial chromosome
BHV-1	Bovine herpesvirus-1
BSA	Bovine serum albumin
BVDV	Bovine viral diarrhoea virus
CEF	Chicken embryo fibroblast
CRM1	Chromosome region maintenance protein 1
cDNA	Complementary deoxyribonucleic acid
cMGF	Chicken myelomonocytic growth factor
CMV	Cytomegalovirus
CPE	Cytopathic effect
cRNA	Complementary ribonucleic acid
DC	Dendritic cell
DEF	Duck embryo fibroblast
DIVA	Distinguish between infected and vaccinated animals
DMEM	Dulbecco’s Minimal Essential Medium
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EHV-1	Equine herpesvirus type 1
EtBr	Ethidium bromide
FCS	Foetal calf serum
FFE	Feather follicle epithelium

FSA	Flanking sequence A
FSB	Flanking sequence B
gB	Glycoprotein B
GFP	Green fluorescent protein
HA	Haemagglutinin
HPAI	Highly pathogenic avian influenza
HSV	Herpes simplex virus
HVT	Herpesvirus of Turkey
IE	Immediate early
IFN	Interferon
ILTV	Infectious laryngotracheitis virus
ISCOM	Immune stimulating complexes
LB	Luria bertani
LPAI	Low pathogenic avian influenza
MD	Marek’s disease
MDV	Marek’s disease virus
MHC	Major histocompatability complex
WNV	West nile virus
MuV	Mumps virus
M1	Matrix 1
M2	Matrix 2
mRNA	Messenger ribonucleic acid
NA	Neuraminidase
NaAc	Sodium acetate
NEP	Nuclear export protein

NK cells	Natural killer cells
NP	Nucleoprotein
NS1	Non-structural protein 1
NS2	Non-structural protein 2
NLS	Nuclear localisation signal
PA	Polymerase acidic
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PB1	Polymerase basic 1
PB1-F2	Polymerase basic 1 - Frame 2
PB2	Polymerase basic 2
PCR	Polymerase chain reaction
PRR	Pattern-recognition receptors
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RT	Room Temperature
S-OIV	Swine-origin influenza virus
TAE	Tris acetic acid EDTA
TLR	Toll-like receptor
VLP	Virus like particle
vRNA	Viral ribonucleic acid
VHS	Virion host shutoff
VZV	Varicella Zoster Virus

Chapter 1: Introduction

1.1. Influenza virus

1.1.1. Influenza classification

Influenza viruses A, B and C belong to the *Orthomyxoviridae* family, a group of enveloped viruses that possess segmented, negative-sense, single-stranded RNA genomes (Palese and Shaw, 2007). The name *Orthomyxoviridae* derives from the Greek *orthos*, meaning 'standard, correct' and *myxa*, meaning 'mucus' (Cheung and Poon, 2007). Influenza A has the broadest host range and infects a variety of animals that includes humans, pigs, birds, horses and sea mammals. Aquatic birds are the source of all influenza A viruses in other species (Webster *et al*, 1992; Noda and Kawaoka, 2010; Taubenberger and Morens, 2010). Influenza B viruses naturally infect humans and occasionally seals (Baigent and McCauley, 2003; Osterhaus *et al*, 2000; Noda and Kawaoka, 2010), while influenza C viruses benignly infect humans, pigs and dogs (Guo *et al*, 1983; Webster *et al*, 1992; Noda and Kawaoka, 2010). Only influenza A viruses have been responsible for all influenza pandemics (Cheung and Poon, 2007). In addition to the influenza viruses, this family also contains the genera *Thogotovirus*, which contains two different species, Dhori and Thogoto virus, both isolated from ticks; and *Isavirus*, which includes the species Infectious salmon anaemia virus (Cheung and Poon, 2007; Palese and Shaw, 2007; Wright *et al*, 2007).

The influenza viruses are classified according to antigenic differences exhibited by two of the internal structural proteins, the nucleocapsid and the matrix proteins (Webster *et al*, 1992; Cheung and Poon, 2007; Noda and Kawaoka, 2010). In addition, antigenic variations in the surface glycoproteins, Haemagglutinin (HA) and Neuraminidase (NA), are used to subtype the influenza A viruses (Fouchier *et al*, 2005; Palese and Shaw, 2007; Wright *et al*, 2007; Cheung and Poon, 2007; Bouvier and Palese, 2008; Noda and Kawaoka, 2010). Antigenic subtypes have not been identified for influenza B and C (Palese and Shaw, 2007). With regards to influenza A, there are now 16 different haemagglutinin subtypes (H1-H16) (Fouchier *et al*, 2005) and 9 different neuraminidase subtypes known (N1-N9) (Laver *et al*, 1984; Cheung and Poon, 2007; Palese and Shaw, 2007; Bouvier and Palese, 2008;

Taubenberger and Morens, 2010). Only three HA (H1, H2 and H3) and two NA (N1 and N2) subtypes have caused human epidemics, defined by sustained and widespread person-to-person transmission (Palese and Shaw, 2007; Bouvier and Palese, 2008). However, the avian influenza strains H5N1, H7N7 and H9N2, have also been transmitted to humans (Baigent and McCauley, 2003; Cheung and Poon, 2007; Wright *et al*, 2007; Taubenberger and Morens, 2010).

Different influenza strains are named according to their genus, the species from which the virus was isolated (omitted in the case of humans), the location of the isolate, the number of the isolate, the year of isolation, and in the case of influenza A viruses, the haemagglutinin and the neuraminidase subtypes. For example, an influenza strain isolated by Shope (Shope, 1931) was given the designation A/Swine/Iowa/15/30 (H1N1) virus, meaning that it was the 15^{th} isolate of an H1N1 subtype virus isolated from pigs in Iowa in 1930 (Palese and Shaw, 2007; Wright *et al*, 2007; Bouvier and Palese, 2008; Taubenberger and Morens, 2010).

1.1.2. Structure of influenza A virus

The influenza virus is an enveloped virus, surrounded by a lipid bilayer that is derived from the host's cell membrane during the viral budding process (Figure 1). The virions are pleomorphic, displaying shapes that range from spherical to filamentous (Cheung and Poon, 2007; Lee and Saif, 2009; Noda and Kawaoka, 2010; Rossman and Lamb, 2011). Laboratory isolated strains of influenza are roughly spherical with a diameter of 80-120 nm (Stanley, 1944; Palese and Shaw, 2007; Bouvier and Palese, 2008; Noda and Kawaoka, 2010). The filamentous form was first observed in 1949 (Chu and Dawson, 1949) and it has been determined that this shape occurs in newly isolated strains. Recently, electron microscopic analysis of autopsied lung tissue, acquired from a patient who died as a result of the Swine-Origin H1N1 2009 pandemic influenza strain, revealed filamentous viral particles (Nakajima *et al*, 2010; Rossman and Lamb, 2011).

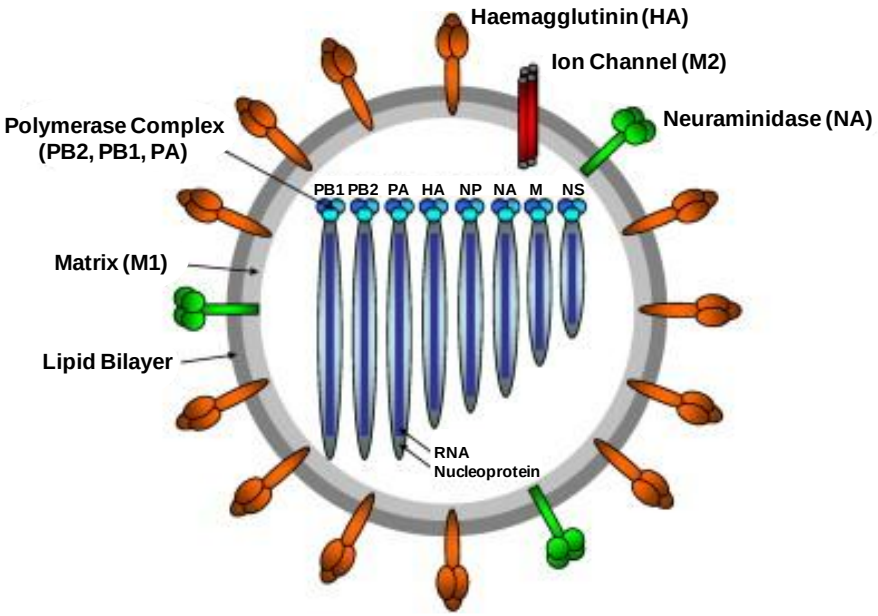


Figure 1. A diagram of the structure of the influenza A virus. Presented with permission (Lee and Saif, 2009).

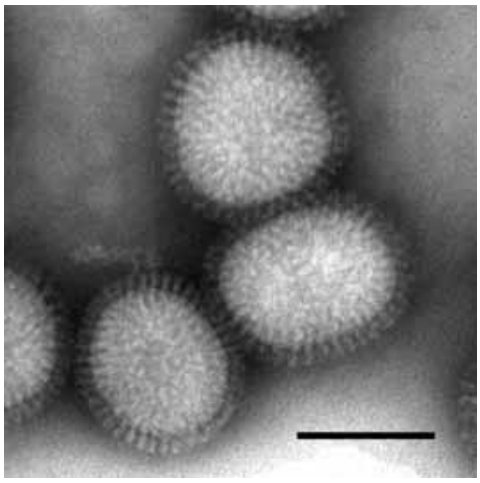


Figure 2. Negative staining electron microscopy showing purified influenza virions (Bar; 100 nm). Presented with permission (Noda and Kawaoka, 2010).

The influenza virion is coated with the surface glycoproteins HA and NA, which are anchored in the lipid bilayer, in a ratio of approximately four to one. Laver and Valentine (1969) were the first to demonstrate that the surface projections, visible on electron micrographs, were the HA and NA proteins (Figure 2). The envelope, which also contains the matrix 2 (M2) protein, overlays the matrix 1 (M1) proteins that enclose the virion core. This core comprises of the nuclear export protein (NEP), also referred to as the non-structural protein 2 (NS2), as well as the ribonucleoprotein (RNP) complex (Cheung and Poon, 2007; Palese and Shaw, 2007; Bouvier and Palese, 2008; Noda and Kawaoka, 2010). The eight RNP complexes consist of a genomic viral RNA (vRNA) segment coated with nucleoprotein (NP) and the RNA polymerase, which is composed of two polymerase basic (PB1 and PB2) and one polymerase acidic (PA) subunit. The ends of each vRNA segment form a helical hairpin, which is bound by the heterotrimeric RNA polymerase complex. The M1 protein provides structural support by interacting with both the cytoplasmic tails of HA and NA in the plasma membrane, as well as the NP of the RNP complex (Rossman and Lamb, 2011). The NP, which provides a net positive charge that binds the negatively charged, phosphate backbone of the vRNA, is the major determinant of the rod-like structure of the RNP complex (Cheung and Poon, 2007; Palese and Shaw, 2007; Bouvier and Palese, 2008; Noda and Kawaoka, 2010).

The genome of the influenza A virus contains eight vRNA segments, which can be observed as an ordered 7 + 1 configuration in budding virions (Figure 3) (Noda and Kawaoka, 2010). In total, from eight vRNA segments, twelve proteins are produced (Cheung and Poon, 2007; Palese and Shaw, 2007; Bouvier and Palese, 2008, Wise *et al*, 2009). Segments 1 (PB2), 3 (PA), 4 (HA), 5 (NP) and 6 (NA) are monocistronic, encoding just one protein per segment. Viral mRNA from segment 2 contains an alternate +1 open reading frame; one frame encodes the protein PB1, while the alternative open reading frame encodes the PB1-F2 protein. In addition, a third major polypeptide, PB1-N40, is synthesised from PB1 mRNA via differential AUG codon usage, although its function is unknown (Wise *et al*, 2009). Viral mRNA derived from segments 7 and 8 can undergo alternative splicing for protein expression. Segment 7 encodes for the proteins M1 and M2, while segment 8 encodes for the

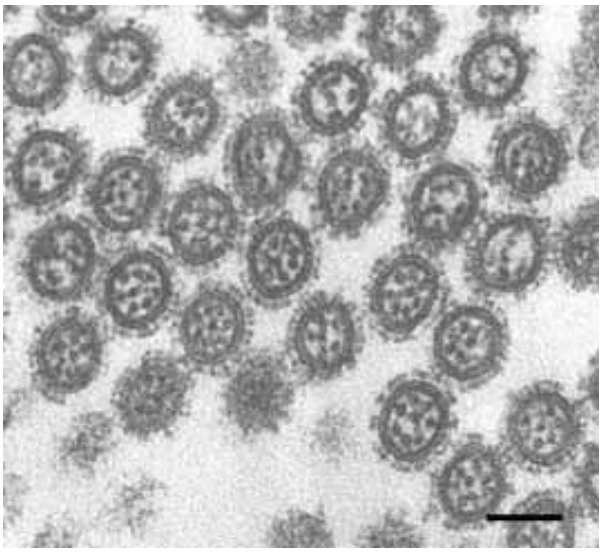


Figure 3. Thin-section electron microscopy showing transversely sectioned budding influenza virions containing eight RNPs (Bar; 100 nm). Presented with permission (Noda and Kawaoka, 2010).

Table 1: The genomic segments of influenza A and their encoded proteins

RNA Segment	Encoded Protein	Protein Function
1	PB2	Polymerase Subunit, mRNA cap recognition
2	PB1	Polymerase Subunit; RNA elongation, endonuclease activity
	PB1-F2	Pro-apoptopic activity
	PB1-N40	Unknown
3	PA	Polymerase Subunit, Endonuclease activity
4	HA	Surface glycoprotein, major antigen, receptor binding and fusion activity
5	NP	RNA binding protein; Nuclear Import regulation
6	NA	Surface glycoprotein; sialidase activity; virus release
7	M1	Matrix protein; vRNP interaction; RNA nuclear export regulation; viral budding
	M2	Ion channel; virus uncoating and assembly
8	NS1	Interferon antagonist protein; regulation of host gene expression
	NEP/NS2	Nuclear Export of RNP

(Palese and Shaw, 2007; Bouvier and Palese, 2008; Wise *et al*, 2009)

non-structural protein 1 (NS1) and the NEP/NS2 (Table 1). The NS1 protein possesses regulatory functions, controlling both mRNA splicing and translation, as well as acting as an interferon antagonist to help the influenza virus evade the host immune response (Palese and Shaw, 2007; Wright *et al*, 2007; Lee and Saif, 2009; Neumann *et al*, 2009; Rossman and Lamb, 2011). The PB1-F2 protein can trigger apoptosis in immune-related cells such as monocytes (Chen *et al*, 2001; Palese and Shaw, 2007; Lee and Saif, 2009; Neumann *et al*, 2009; Rossman and Lamb, 2011).

1.1.3. Replication of Influenza A virus

Virus Attachment

The HA glycoprotein of influenza is a type I integral membrane protein that is responsible for receptor binding and membrane fusion. It is a rod-shaped homotrimer with two structurally distinct regions: a triple-stranded coiled-coil of alpha-helices that form a stem and a globular head of antiparallel beta-sheets on top that contains the sialic acid receptor binding domain (Figure 4) (Cheung and Poon, 2007; Palese and Shaw, 2007; Bouvier and Palese, 2008). The COOH-terminus is inserted into the viral membrane and the hydrophilic end projects away from the viral surface forming the spike (Palese and Shaw, 2007).

The HA recognises and binds to the N-acetylneuraminic (sialic) acid on the surface of the host cell (Figure 5), binding preferentially to either α-2,3- or α-2,6-linkages that are formed by the association of carbon-2 with either the carbon-3 or carbon-6 of galactose (Baigent and McCauley, 2003; Cheung and Poon, 2007; Bouvier and Palese, 2008). In ducks and other avian species, α-2,3-linkages predominate in the gut epithelium, whereas in human epithelial cells sialic acids with α-2,6-linkages are more common. α-2,3-linkages are present in human epithelial cells especially in the lower respiratory tract and, although they are much less common than the α-2,6-linkages, this does mean that avian influenza viruses can infect humans and cause severe infection (Matrosovich *et al*, 2004; Palese and Shaw, 2007; Wright *et al*,

2007; Bouvier and Palese, 2008; Lee and Saif, 2009; Neumann *et al*, 2009). In addition, Ito *et al* (1998) demonstrated that the trachea of pigs contained both α-2,3 and α-2,6-linked sialic acids, indicating that pigs could readily be infected by both human and avian influenza virus strains, potentially resulting in reassortant pandemic viruses (Ito *et al*, 1998; Wright *et al*, 2007; Neumann *et al*, 2009).

Virus Entry

Following attachment of the HA protein to the sialic acid, the influenza virus is taken into the cell by receptor-mediated endocytosis (Rust *et al*, 2003; Cheung and Poon, 2007; Palese and Shaw, 2007; Bouvier and Palese, 2008; Rossman and Lamb, 2011). The low pH environment of the acidic endosome triggers two key events. In the first, a conformational change in the HA occurs, exposing the fusion peptide and aligning it antiparallel to the membrane anchor of the HA2. This subsequently mediates the merging of the viral envelope with the endosomal membrane, opening a pore and releasing the viral RNPs into the cytoplasm. In the second, hydrogen ions from the endosome are pumped into the virion causing disruption of the internal protein-protein interactions, allowing the viral RNPs to be released (Bui *et al*, 1996; Cheung and Poon, 2007; Palese and Shaw, 2007; Bouvier and Palese, 2008). This is mediated by the M2 protein, a highly conserved, tetrameric, type III integral membrane protein which functions as an ion channel for the acidification of the interior of the viral particle during viral infection (Pinto *et al*, 1992; Cros and Palese, 2003; Boulo *et al*, 2007; Cheung and Poon, 2007; Palese and Shaw, 2007; Bouvier and Palese, 2008; Rossman and Lamb, 2011).

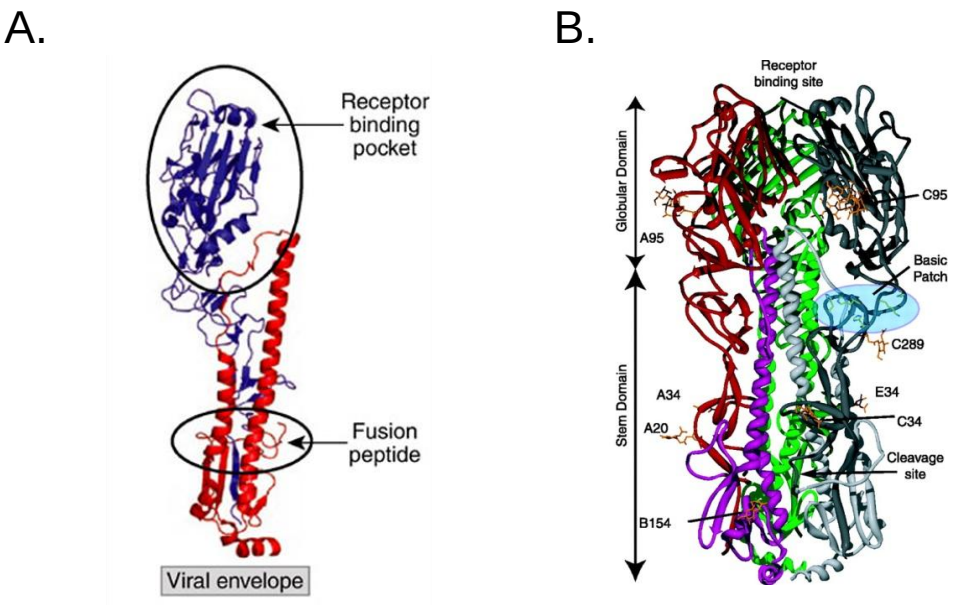


Figure 4. A. Structure of a haemagglutinin monomer: HA1 (blue) and HA2 (red). Adapted with permission (Wang & Palese, 2009) B. Structure of the haemagglutinin trimer. Adapted with permission (Stevens *et al*, 2004).

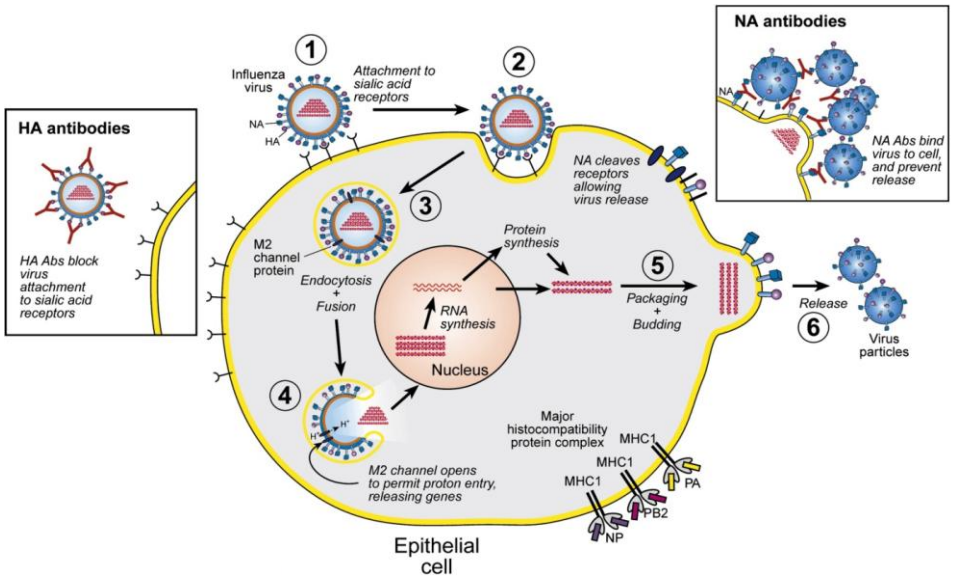


Figure 5. Life cycle of the influenza virus. Adapted with permission (Subbarao *et al*, 2006).

Synthesis of Viral RNA

Unlike some other negative-sense, single-stranded RNA viruses, influenza virus RNA synthesis takes place in the nucleus of infected cells (Amorim and Digard, 2006; Cheung and Poon, 2007; Palese and Shaw, 2007; Noda and Kawaoka, 2010). This is possibly due to the fact that the influenza virus requires components of the host's cellular mRNA splicing machinery present in the nucleus (Amorim and Digard, 2006; Boulo *et al*, 2007). Once released, the RNPs are transported to the nucleus as a result of nuclear localisation signals (NLSs) present on all proteins of the RNP complex (Cros and Palese, 2003; O'Neill *et al*, 1995; Boulo *et al*, 2007; Palese and Shaw, 2007). Signals on the NP, however, are sufficient and necessary for the transport of viral RNA (O'Neill *et al*, 1995; Palese and Shaw, 2007).

Three polymerase subunits, PB1, PB2 and PA, along with NP, are required for both viral transcription and replication (Huang *et al*, 1990; Cheung and Poon, 2007). NP mediates binding and packaging of the influenza viral genome. PB1 plays a key role in the assembly of the RNP complex and the catalytic function of RNA polymerisation, with the sequential addition of nucleotides during RNA chain elongation. The initiation of transcription begins with the binding of the 5' end of the negative RNA strand (vRNA) to the PB1 subunit which, in turn, allows the PB2 protein to recognise and bind the 5' cap structure on host pre-mRNAs. The influenza RNP complex binds cellular mRNAs and, in a process known as 'cap snatching', the PA endonuclease cleaves approximately 10-15 bases from host pre-mRNA transcripts to initiate viral mRNA synthesis (Amorim and Digard, 2006; Cheung and Poon, 2007; Palese and Shaw, 2007; Bouvier and Palese, 2008; Dias *et al*, 2009). PA is also essential for viral transcription and replication (Cheung and Poon, 2007; Palese and Shaw, 2007) and for the efficient accumulation of the PB1 subunit in the nucleus (Fodor and Smith, 2004; Boulo *et al*, 2007; Cheung and Poon, 2007).

The viral RNA-dependent RNA polymerase uses the negative-sense vRNA as a template to synthesise two positive-sense RNA species: capped and polyadenylated mRNA templates for viral protein synthesis in the cytoplasm, and complementary RNA (cRNA) intermediates that are used for the transcription of more copies of

vRNA in the nucleus to form the genomes of progeny virus (Cheung and Poon, 2007; Palese and Shaw, 2007; Bouvier and Palese, 2008; Lee and Saif, 2009). Following virus replication, the nuclear export of RNP complexes to the cell surface is mediated by the viral proteins M1 and NEP/NS2 (Palese and Shaw, 2007; Bouvier and Palese, 2008). The M1 protein binds to vRNP, promoting nuclear export and inhibiting nuclear import (Martin and Helenius, 1991; Cheung and Poon, 2007); while the NEP/NS2 is responsible for directing the export of the RNP complex (Cros and Palese, 2003; Boulo *et al*, 2007; Palese and Shaw, 2007; Lee and Saif, 2009; Noda and Kawaoka, 2010).

Synthesis of viral membrane proteins

The integral membrane proteins, HA, NA and M2, are synthesised from viral mRNA on membrane-bound ribosomes within the endoplasmic reticulum. Once folded the proteins are transported to the golgi apparatus for post-translational modification and the HA and NA become glycosylated. The HA glycoprotein is synthesised as the precursor polypeptide, HA0, which is post-translationally cleaved by serine proteases into the disulphide-linked subunits, HA1 and HA2 (Cheung and Poon, 2007; Palese and Shaw, 2007; Bouvier and Palese, 2008). The cleavage of HA0 is necessary for the infectivity of the influenza virus as it liberates the fusion peptide which is important for membrane fusion (Baigent and McCauley, 2003; Cheung and Poon, 2007). The HA cleavage site of low pathogenic avian influenza (LPAI) viruses contains a single arginine so only extracellular trypsin-like proteases, normally found in the respiratory and intestinal tract of the host, can cause cleavage. Highly pathogenic avian influenza (HPAI) viruses possess a HA with a multi-basic cleavage site, containing arginine and lysine, meaning the HA0 protein is cleavable by endogenous proteases, such as furin, throughout the body (Horimoto and Kawaoka, 1994; Cheung and Poon, 2007; Palese and Shaw, 2007; Lee and Saif, 2009). HA1 contains the receptor binding and antigenic sites; HA2 mediates the fusion of the virus envelope with cell membranes. Apical sorting signals subsequently direct the HA, NA and M2 proteins to the cell membrane for virion assembly, where M1 is

thought to play a key role in combining the RNP-NEP complex with the envelope bound HA, NA and M2 (Cheung and Poon, 2007; Palese and Shaw, 2007; Bouvier and Palese, 2008). The HA and NA selectively accumulate at and are incorporated into lipid rafts; non-ionic, detergent-resistant lipid microdomains within the plasma membrane that are rich in cholesterol and sphingolipids. Raft association of HA is essential for efficient virus replication and is mediated by the transmembrane domain (Takeda *et al*, 2003; Palese and Shaw, 2007; Rossman and Lamb, 2011).

Packaging, assembly and virus budding

Budding of the influenza virus occurs at the cell membrane and is likely to be initiated by the accumulation of HA and M1 protein which stimulates the outward curvature of the plasma membrane (Palese and Shaw, 2007; Rossman and Lamb, 2011). Packaging of the eight vRNA segments occurs selectively as a result of discreet packaging signals on all vRNA segments (Fujii *et al*, 2003; Hutchinson *et al*, 2010), resulting in the 7 + 1 configuration (Noda and Kawaoka, 2010). This ensures a full genome is incorporated into the majority of virus particles, essential for a fully infectious virion. The release of the virion, though membrane scission, is mediated by the M2 protein in a cholesterol-dependent manner (Rossman *et al*, 2010). Once budded, HA continues to bind the virion to sialic acids until released by the sialidase activity of the NA protein. The NA spike is a mushroom-shaped, type II integral membrane homotetramer which acts to release the virus progeny by destroying the receptors through hydrolysing sialic acid groups from glycoproteins (Cheung and Poon, 2007; Palese and Shaw, 2007; Bouvier and Palese, 2008). Host antibodies to NA, as well as therapeutic inhibitors of NA, prevent virus release from infected cells, inhibiting virus replication (Palese and Shaw, 2007; Bouvier and Palese, 2008).

1.1.4. Influenza – Antigenic Drift and Shift

Antigenic Drift

Influenza viruses seem to be in evolutionary stasis in their natural host reservoir, the aquatic birds, however in other hosts they evolve rapidly in response to immune selection (Webster *et al*, 1992; Wright *et al*, 2007; Lee and Saif, 2009). As strains evolve to evade detection by host antibodies directed against the surface glycoproteins, frequent amino acid changes occur at the antigenic sites within HA1, NA or both (Treanor, 2004; Carrat and Flahault, 2007; Wright *et al*, 2007; Bouvier and Palese, 2008). The accumulation of these changes is known as antigenic drift. Antigenic drift occurs because the influenza's RNA polymerase complexes have no proof reading ability; therefore, high mutation rates can lead to point mutations in the antigenic sites (Taubenberger and Morens, 2010). Mutations that include deletions, substitutions, and insertions can affect the antigenic binding sites and are among the most important mechanisms for producing antigenic variation in influenza viruses (Webster *et al*, 1992 Carrat and Flahault, 2007). Eventually, antigenic sites mutate to the point that they are no longer recognised and neutralised by the host immune system (Treanor, 2004; Carrat and Flahault, 2007; Wright *et al*, 2007; Bouvier and Palese, 2008). Genetic drift of the haemagglutinin protein in poultry occurs at a similar rate to those observed in mammals (Suarez, 2000; Lee and Saif, 2009). In humans, antigenic drift means that current seasonal influenza vaccines containing H3N2 and H1N1 subtypes, and an influenza B subtype, must be updated every 1-3 years to account for the changes to HA and NA proteins in circulating viruses (Neumann *et al*, 2009).

Antigenic Shift

The segmented nature of the genome means that a reassortment event known as antigenic shift can occur, where the influenza A strain acquires the HA segment, and sometimes the NA segment, from another influenza A subtype. Reassortment can

produce genetic diversity rapidly, thus providing an evolutionary advantage, and can occur in cells simultaneously infected with different strains of human and animal viruses. Mixtures of parental gene segments may be assembled into virions and the resulting virus may encode antigenic proteins for which the human population has no pre-existing immunity. The result can be a pandemic or worldwide epidemic (Treanor, 2004; Webster *et al*, 1992; Carrat and Flahault, 2007; Wright *et al*, 2007; Bouvier and Palese, 2008; Hutchinson *et al*, 2010). Antigenic shift triggered the 1957 and 1968 pandemic outbreaks as well as the most recent swine-origin H1N1 pandemic virus (Hutchinson *et al*, 2010). And, in addition, it likely produced the extremely virulent influenza A (H1N1) virus that caused the 1918-1919 'Spanish flu', where the HA, NA and PB1 genes all contributed to the high pathogenicity in an immunologically naive global population (Pappas *et al*, 2008; Bouvier and Palese, 2008).

1.1.5. Pandemics

Pandemics are defined as outbreaks that affect large populations, typically on more than one continent, in a short space of time. Typically, they occur at intervals of between ten and forty years, although reliable records only date back to the 1918 pandemic (Wright *et al*, 2007). To date, there have now been five pandemics, including the 1918 Spanish influenza.

The Pandemic of 1918 – Spanish Influenza (H1N1)

Worldwide, this strain killed as many as 50 million people, and the outbreak remains unprecedented in its severity (Johnson and Mueller, 2002; Neumann *et al*, 2009; Taubenberger and Morens, 2010). Kansas, in the spring of 1918, saw the first case which was attributed to a soldier who had been cleaning pig pens. The first wave was highly contagious, moving along rail lines and via troopships to Europe, but caused few deaths. In August, however, a virulent form emerged that caused a significant increase in the death toll, mostly due to secondary bacterial pneumonia (no

antibiotics were available) and respiratory failure, although some showed massive acute pulmonary haemorrhage or edema indicating just how virulent the virus was. In addition, many of the dead were young adults, with a mortality rate for that age group twenty times higher than in previous years (Wright *et al*, 2007; Taubenberger and Morens, 2010). Recovered viral RNA from an Inuit female, exhumed from a mass grave in Alaska's permafrost, confirmed the causative agent to be an H1N1 virus (Reid *et al*, 1999; Wright *et al*, 2007). All eight gene segments were avian in origin (Taubenberger and Morens, 2010) and, as previously stated, Pappas *et al* (2008) determined that the 1918 HA, NA and PB1 genes all contributed to the high pathogenicity of the virus. Recombinant influenza viruses that express the 1918 HA protein induce a strong, macrophage-derived cytokine inflammatory response, causing haemorrhaging that was typical of the pandemic strain (Kobasa *et al*, 2004; Wright *et al*, 2007). However, despite its high pathogenicity, the HA of the H1N1 virus lacked a multibasic cleavage site, indicative of current highly pathogenic strains (Wright *et al*, 2007; Neumann *et al*, 2009).

The Pandemic of 1957 – Asian Influenza (H2N2)

Originating in the Southern Chinese province of Guizhou in February 1957, this strain caused more than one million deaths worldwide, with infection rates highest in the 5-19 year old group. Isolated in May 1957, it was found to have originated by reassortment between human and avian viruses and contained HA, NA and PB1 genes of avian virus origin (Wright *et al*, 2007; Neumann *et al*, 2009; Taubenberger and Morens, 2010). Although the virus itself was not particularly pathogenic, a lack of existing immunity led to increased mortality (Wright *et al*, 2007).

The Pandemic of 1968 – Hong Kong Influenza (H3N2)

This strain was isolated in Hong Kong in July 1968, and like the previous pandemic, probably originated in Southern China. The H3N2 virus contained an avian H3 and PB1 gene, produced through reassortment (Wright *et al*, 2007; Neumann *et al*, 2009;

Taubenberger and Morens, 2010). The virus spread around the world, predominantly affecting the 10-14 year old age group, but although it caused a significant number of deaths, it was not as severe as the Asian influenza outbreak. This was probably due to pre-existing antibodies against the N2 protein in the population previously affected by the H2N2 pandemic (Wright *et al*, 2007; Taubenberger and Morens, 2010).

The re-emergence of H1N1 viruses in 1977 – Russian Influenza (H1N1)

First noticed in China in May 1977, this outbreak spread through China and Russia, reaching other countries the following winter. Confirmed as H1N1, the mortality was almost exclusively limited to persons younger than 25 years (Wright *et al*, 2007). This evidence of pre-existing immunity in the older members of the population, coupled with the lack of mutations normally acquired during replication in an animal host, has lead to the opinion that this pandemic was caused by a previous H1N1 strain, accidentally reintroduced by humans from a frozen source (Wright *et al*, 2007; Neumann *et al*, 2009). In addition, a characteristic of influenza virus infection in humans is that when a new pandemic strain emerges, it tends to displace the previously circulating subtype which disappears. The exception was the H1N1 Russian pandemic because since 1977, both H3N2 and H1N1 viruses have co-circulated in humans (Webster *et al*, 1992; Wright *et al*, 2007; Neumann *et al*, 2009).

The H5N1 Outbreak

Although it has not caused a pandemic, the H5N1 influenza virus was, and continues to be, a source of concern. In May 1997, the first documented infection of humans by an influenza A virus (H5N1) that was entirely of avian origin, occurred in Hong Kong. Prior to this it was believed that the difference in host receptor binding specificities would provide a host range barrier making the transmission highly unlikely (Wright *et al*, 2007). The outbreak started with the infection of a three year old boy, who later died. In total, eighteen people were infected, with six deaths. The source of the infection was domestic poultry but the virus did not appear to be

transmissible from human to human. Officials in Hong Kong ordered the culling of all poultry in the live bird markets, despite considerable economic loss, which prevented any further human cases (Wright *et al*, 2007; Neumann *et al*, 2009). Unfortunately the virus re-emerged in May 2001 and April 2002, again resulting in the depopulation of poultry stocks. An outbreak in July 2003, affecting poultry in Vietnam, Indonesia and Thailand, spread to Cambodia, Laos, South Korea, Malaysia, Japan, China and Mongolia. Since then the virus has spread to Russia, Ukraine, central and south east Europe, the Middle East, and Africa. This spread may have resulted from infected migratory birds or possibly the movement of infected poultry (Wright *et al*, 2007).

The dominant highly pathogenic H5N1 influenza virus strain, genotype Z, contains all eight RNA gene segments from avian viruses, indicating that genetic reassortment had arisen from multiple reassortment events among avian influenza viruses and not between human and avian strains (Li *et al*, 2004; Wright *et al*, 2007). Highly pathogenic H5N1 viruses are characterised as possessing the multibasic cleavage site in the HA protein (Wright *et al*, 2007) and are lethal to chickens and mice (Wright *et al*, 2007; Neumann *et al*, 2009). Most importantly, they continue to be transmitted to humans resulting in high levels of morbidity and mortality. As of April 21, 2011, 553 cases of human H5N1 influenza infection have been confirmed, resulting in 323 deaths (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2011_04_21/en/index.html). H5N1 viruses are characterised by their high mortality rate but inefficient spread among humans (Neumann *et al*, 2009). The obvious fear is that, through reassortment, this virus may acquire that ability causing a pandemic on the scale of the 1918 Spanish influenza.

Swine-Origin influenza virus (S-OIV) H1N1 - 2009 Pandemic

The first cases of infection with this virus were observed in the Mexican town of La Gloria, Veracruz, in February 2009. In April 2009, public health authorities alerted the Pan American Health organisation (PAHO), the regional office of the World Health Organisation (WHO), to a possible outbreak. The Centre for Disease Control

(CDC) subsequently identified two cases of S-OIV in Southern California, caused by a similar virus. By the end of April, the international spread of the virus, with human to human transmission reported in at least two countries, prompted the WHO to elevate the pandemic to phase 5 of 6 (Smith *et al*, 2009; Neumann *et al*, 2009). On 11th June 2009, the pandemic alert was raised to level 6 (Girard *et al*, 2010). As of 1st August 2010, more than 214 countries worldwide had reported laboratory confirmed cases of pandemic influenza H1N1 2009, including over 18449 deaths, although the majority of cases were mild (http://www.who.intt/csr/don/2010_08_06/en/index.html). The virus presented the highest risk to pregnant woman, younger children and young adults, while older adults displayed evidence of pre-existing immunity from previously circulating H1N1 viruses. Many of the deaths caused by the pandemic H1N1 virus occurred in patients with an underlying health condition (Peiris *et al*, 2009; Leung and Nicoll, 2010; Girard *et al*, 2010).

The H1N1 S-OIV was determined to have resulted from the reassortment of North American H3N2 and H1N2 swine viruses with Eurasian avian-like swine viruses. Prior to this, the American H3N2 and H1N2 swine viruses were avian/human/swine triple reassortments themselves, meaning the H1N1 S-OIV is a quadruple reassortant (Neumann *et al*, 2009; Girard *et al*, 2010; Taubenberger and Morens, 2010). The H1N1 S-OIV possesses a PB1 gene from human H3N2 virus origin, PB2 and PA genes of North American avian virus origin, NA (N1) and M genes of Eurasian avian-like swine virus origin and HA (H1), NP and NS genes of classical swine virus origin (Smith *et al*, 2009; Neumann *et al*, 2009; Girard *et al*, 2010). The influenza virus of classical swine origin, including the H1, is a direct descendent of the 1918 H1N1 influenza virus, that has circulated in pigs ever since. These viruses have remained antigenically stable in pigs, due to the short life span of the pig and this may provide an explanation as to why older humans possessed immunity to the pandemic H1N1 strain (Peiris *et al*, 2009).

Fortunately, the H1N1 S-OIV does not possess markers associated with high virulence, such as an HA with a multibasic cleavage site or a PB2 with a lysine at position 627 (Wright *et al*, 2007; Neumann *et al*, 2009; Peiris *et al*, 2009; Girard *et al*, 2010). The virus generally caused a mild, self-limiting upper respiratory tract

illness, however, the clinical spectrum ranged from asymptomatic to primary viral pneumonia with acute respiratory distress, multi-organ failure and death (Peiris *et al*, 2009; Girard *et al*, 2010). The severity of the pulmonary symptoms in some patients can be accounted for by the fact that S-OIV can bind to both α2,6-linked sialic acid in the upper respiratory tract, as well as α2,3-linked sialic acid receptors deep in the lungs (Childs *et al*, 2009; Girard *et al*, 2010). The S-OIV H1N1 continues to co-circulate with seasonal influenza strains and has now been incorporated into the trivalent seasonal vaccine for both hemispheres (Leung and Nicoll, 2010).

The next pandemic....

The Swine-Origin H1N1 2009 pandemic came as a complete surprise (Girard *et al*, 2010), for no-one had predicted the emergence of this subtype. The significance of this statement is profound as it underscores our inability to accurately predict when or where the next pandemic may occur, let alone what subtype it might be (Girard *et al*, 2010). Previous dogma had dictated that, as previous pandemics arose from a subtype that differed from the prevailing influenza in circulation, the most recent pandemic should have been anything other than an H1 or an H3 subtype (Peiris *et al*, 2009). It remains unclear as to whether H5N1, and other avian-adapted influenza viruses, have the ability to acquire the means necessary for efficient human to human transmission. It is unknown whether the S-OIV H1N1 will co-circulate with the current H3N2 and H1N1 strains or replace them, and whether current vaccination strategies will increase or decrease the likelihood of the next pandemic. However, it seems likely that reassortment will play a major role in the next pandemic; the last three pandemics have resulted from reassortment of pre-existing swine or human adapted viruses with imported genes from avian influenza viruses. Finally, it seems true of influenza that the more we learn, the less we know and are certain of, when it comes to the determinants of pandemics. What is clear is that to prevent and manage future pandemics, regardless of subtype, we need to develop pre-emptive and effective intervention strategies, which includes novel vaccines, combined with effective surveillance and communication (Taubenberger and Morens, 2010).

1.1.6. Avian Influenza

Influenza A viruses infecting poultry can be divided into two distinct groups, Highly Pathogenic Avian Influenza (HPAI) or Low Pathogenicity Avian Influenza (LPAI), on the basis of the severity of disease they cause. Influenza viruses that cause LPAI produce a localized infection resulting in mild disease consisting of respiratory disease, depression and decreased egg production in laying birds (Capua and Marangon, 2007; Wright *et al*, 2007). HPAI viruses, such as the H5N1 (Genotype Z), are very virulent and cause a systemic infection where flock mortality in susceptible species can be as high as 100% (Capua and Marangon, 2007; Wright *et al*, 2007). Other signs of HPAI infection include excessive lacrimation, cyanosis of unfeathered skin, and oedema of the head, diarrhoea and nervous disorders (Webster *et al*, 1992). As HPAI viruses possess a HA with a multibasic cleavage site, they are capable of replicating throughout the bird, resulting in a systemic infection that causes damage to vital organs and increased morbidity and mortality (Rott, 1992; Senne *et al*, 1996; Suarez and Schultz-Cherry, 2000; Alexander, 2003; Baigent and McCauley, 2003; Alexander, 2007; Capua and Marangon, 2007; Wright *et al*, 2007; Lee and Saif, 2009; Neumann *et al*, 2009). It is thought that that the conversion from a basic to a multi-basic cleavage site occurs through spontaneous duplication of purine triplets (Alexander, 2003; Perdue *et al*, 2003; Alexander, 2007), which appear only after the viruses have moved from their natural wild bird host to poultry (Capua and Marangon, 2007; Alexander, 2007). The current European Union legislation defines HPAI avian influenza as 'an infection of poultry caused by either any influenza A virus that has an intravenous pathogenicity index in 6-week-old chickens greater than 1.2 or any influenza A virus of H5 or H7 subtype' (CEC, 1992; Alexander, 2003; Capua and Marangon, 2003; Lee and Saif, 2009).

There have been several serious major outbreaks of HPAI avian influenza that have devastated the poultry industry and caused significant human health issues, including the risk of generating a new pandemic virus via the human-avian link. These outbreaks include the Italian outbreak of HPAI H7N1 in 1999-2000 which saw the culling of approximately 14 million birds; the Dutch outbreak in 2003 of HPAI H7N7 that resulted in the deaths of over 25 million birds; and the Canadian outbreak

of HPAI H7N3 in 2004, where approximately 16 million birds were killed (Alexander, 2007; Capua and Marangon, 2007). For years, vaccination against HPAI viruses was discouraged, as it was considered that it would interfere with the diagnosis of HPAI. However, the increase in outbreaks of HPAI combined with the spread of H9N2 and H5N1 has lead to the widespread introduction of avian influenza (AI) vaccines in combination with increased biosecurity (Alexander, 2007). AI vaccines, when administered properly, can help to prevent, manage or eradicate avian influenza and subsequently limit economic losses, improve the health and welfare of billions of poultry and positively impact on public health by reducing environmental contamination (Swayne and Kapczynski, 2008).

1.2. The Chicken Immune System

1.2.1. The immune system in chickens

It is evident that, while the immune systems of avian species differ from those of model mammalian species, there are also similarities. For example, as with mammalian species, dendritic cells and other immune cells such as macrophages are present, as are interferons type I-III that include IFN-α, IFN-β, IFN-γ and IFN-λ. There are also, however, many differences in terms of the genes, molecules, cells and organs that chickens possess, as well as the functional mechanisms that have evolved (Kaiser, 2010). The major difference is in the lymphatic system. In mammals, the lymph nodes contain B cells, T cells, macrophages and dendritic cells and are the primary sites for the immunologic interaction that is important for immune activation (Swayne and Kapczynski, 2008). Chickens, however, lack lymph nodes but they do possess small concentrations of non-encapsulated lymphoid tissue throughout the body (Swayne and Kapczynski, 2008; Kaiser, 2010). The avian-specific primary lymphoid organ is the bursa of Fabricius, which is the site of development for B cell differentiation and production (Swayne and Kapczynski, 2008; Kaiser, 2010). Other

lymphoid tissues include the caecal tonsils, Meckel's diverticulum, Peyer's patches and the Harderian gland (Swayne and Kapczynski, 2008).

In addition, chickens possess a different range of Toll-like receptors (TLRs) that show a pattern of gene duplication and gene loss (Table 2) when compared to mammals (Cormican *et al*, 2009; Kaiser, 2010). TLRs are type I integral membrane glycoproteins, expressed on various immune cells associated with the innate immune system, including macrophages, dendritic cells and B cells (Akira *et al*, 2006; Kaiser, 2007). They represent a set of immune pattern-recognition receptors (PRRs) capable of recognising pathogen-associated molecular patterns (PAMPs), thereby alerting the immune system as soon as an infection occurs and causing the recruitment and activation of cells of both the innate and adaptive immune response (Akira *et al*, 2006; Kaiser, 2010; Moser and Leo, 2010). Birds lack functional eosinophils and the avian functional equivalent of the neutrophil is the heterophil, a polymorphonuclear cell that actively phagocyte invading pathogens. Furthermore, in comparison to mammals, birds also have different cytokines, chemokines, defensins and integrins (Lynn *et al*, 2007; Kaiser, 2007; Kaiser, 2010). Finally, only three immunoglobulin classes have been shown to exist in chickens: IgA, IgM and IgY, where IgY is the functional equivalent to mammalian IgG (Warr *et al*, 1995; Karlsson *et al*, 2004).

1.2.2. The avian immune response

Despite the physiologic differences in structure and organisation between species, the functional aspects of the lymphoid cells and peripheral organs are similar. The avian immune system is a multilayered defence system, and as with mammals, it comprises the innate immune response and the adaptive immune response which is further divided into humoral and cell-mediated immune responses (Swayne and Kapczynski, 2008; Moser and Leo, 2010).

Table 2: - Comparison of the Toll-Like Receptor (TLR) repertoire between humans and chickens

TLR Pattern Recognition Receptor	Human	Chicken
TLRs recognising cell surface PAMPs	TLR1/6/10	TLR1LA and TLR1LB
	TLR2	TLR2A and TLR2B
	TLR4	Present
	TLR5	Present
	TLR11	Absent
		TLR15 (predicted)
TLRs recognising pathogen nucleic acid	TLR3	Present
	TLR7	Present
	TLR8	Pseudogene
	TLR9	Absent
		CpG recognised by TLR21

Innate immunity represents the first line of host defence against pathogenic organisms that have entered the body, providing almost immediate protection. However, it lacks memory and focuses on a limited set of microbial determinants, the PAMPs, which are shared by a large number of pathogens (Esser *et al*, 2003; Moser and Leo, 2010). Despite this, the innate immune response is of crucial importance, not only as an effective initial response, but also in determining the course of the adaptive immune response. Pathogens are targeted by the effector cells of the innate response, the dendritic cells (DC), heterophils and Natural Killer (NK) cells and, in the case of DCs, presents pathogen antigen to the adaptive immune response via the major histocompatability complex (MHC) class II pathway. In addition, the innate response also produces cytokines and chemokines that drive the inflammatory response and influence the adaptive response (Kaiser, 2010).

Adaptive immunity provides a second line of defence that is comparatively slower and is characterised by a very large set of effector molecules and cells, able to efficiently recognise and eradicate pathogens. After elimination, the immune response establishes 'memory', allowing it to mount a rapid and effective response upon re-infection with the same infectious agent (Kaiser, 2010; Moser and Leo, 2010). The adaptive immune response is further divided into humoral and cell-mediated immune responses which utilise antibodies and T cell receptors as recognition systems respectively (Swayne and Kapczynski, 2008; Moser and Leo, 2010). Intracellular pathogens are cleared by the cell-mediated immune response; extracellular pathogens are cleared via the antibodies of the humoral immune response. The end result of the adaptive immune response is clearance of the pathogen, infected cells and the establishment of immunological memory (Kaiser, 2010).

Cell-mediated Immunity

Subsets of T cells, within the cell-mediated immune response, include $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells that are matured in the thymus (Erf, 2004; Swayne and Kapczynski, 2008). There are two main subsets of $CD4^+$ T helper cells (Th1 and

Th2) that orchestrate and direct the immune response by secreting cytokines, playing a regulatory role in the adaptive immune response. Th1 corresponds to the cellular immune responses, releasing cytokines that activate macrophages, NK cells and $CD8^+$ cytotoxic T cells, whereas Th2 corresponds to the humoral response, producing cytokines that are associated with antibody production (Esser *et al*, 2003; Erf, 2004; Degen *et al*, 2005; Swayne and Kapczynski, 2008; Kaiser, 2010). In the course of an antibody response, antigen specific $CD4^+$ T helper cells interact with antigen MHC class II complexes, formed from peptides of endosomal origin, on the surface of antigen presenting cells such as DCs, macrophages or B cells. This interaction is essential for the induction of high affinity antibodies and immune memory through the induction of cytokines (Erf, 2004; Moser and Leo, 2010). The $CD8^+$ cytotoxic T cells, in comparison, perform targeted removal of host cells infected by intracellular pathogens though the delivery of proteases that induce cell death (Esser *et al*, 2003; Zepp, 2010; Moser and Leo, 2010). Cells that have been infected present peptides, processed intracellularly via the MHC class I pathway, which are displayed for surveillance by the immune system (York and Rock, 1996). Cells presenting viral peptides are targeted and destroyed by the effector T cells, the $CD8^+$ cytotoxic lymphocytes (CTLs) (Erf, 2004; Cinatl Jr *et al*, 2007; Moser and Leo, 2010). Both $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells can differentiate into memory cells, essential for long-term immunity (Esser *et al*, 2003; Erf, 2004; Ahmed and Gray, 1996).

Humoral Immunity

Upon an encounter with a specific antigen, in combination with a CD4+ T cell, B lymphocytes that express a given antibody are stimulated to divide and differentiate into plasma cells, which produce antigen-specific antibodies and memory B cells. Antibodies are bifunctional proteins that can both recognize and eliminate an antigen or pathogen. They are roughly Y-shaped molecules, made up of two heavy and light chains linked together, with constant regions that determine functional properties, as well as variable regions that contribute to the antigen binding sites (Moser and Leo,

2010). The initial binding of an antibody to its target activates and enhances effector mechanisms that lead to removal or destruction of the antigen. These effector mechanisms include activation of the classical complement pathway directly on the antigen; opsonisation of the antigen for more effective phagocytosis; antigen agglutination; and antigen neutralisation as binding would prevent it from interacting with cellular receptors, thus inhibiting its infectivity (Erf, 2004). Exposure to the same antigen, in combination with signals from CD4+ T helper cells, induces a rapid secondary response composed of IgY antibodies that rapidly neutralises the antigen (Moser and Leo, 2010). In poultry, abrogation of the bird's ability to produce a humoral response can be achieved by removing the bursa of Fabricius, *in ovo*, eliminating the ability of the chicken to produce B cells and subsequently protective antibodies (Swayne and Kapczynski, 2008).

1.2.3. Protective immunity

Protective immunity may be described as an achieved level of immune-related function with the production of antibodies or antigen-specific lymphocytes that allows a bird to resist disease following exposure to a pathogen. Protective immunity against the influenza virus in immunocompetent birds is predominantly due to neutralising IgY antibodies directed against the influenza HA proteins, blocking viral attachment, neutralising the infectivity of the virus and preventing infection (Swayne and Kapczynski, 2008). By evaluating the relative neutralisation titres of serum antibody, shedding of challenge virus and protection against lethal HPAI challenge virus, Nayak *et al* (2010) determined that the HA glycoprotein was the major contributor to the induction of neutralising IgY antibodies and protective immunity in chickens (Suarez and Schultz-Cherry, 2000; Swayne and Kapczynski, 2008; Nayak *et al*, 2010). NA was found to provide partial immunity but the M2 protein did not induce a detectable level of serum-neutralising antibodies or provide protection against lethal HPAI virus challenge (Nayak *et al*, 2010). Antibodies directed at the HA glycoprotein of the influenza virus are long-lived in the absence of antigenic drift (Cinatl Jr *et al*, 2007). The understanding of T cell immunity

against the influenza virus in chickens is limited, although it has been demonstrated that cross-reactive cellular immunity with CD8+ T cells, induced by the H9N2 influenza virus, protects chickens from the lethal H5N1 virus (Seo and Webster, 2001, Seo *et al*, 2002; Haghighi *et al*, 2009). Haghighi *et al* (2009) identified a T cell epitope of the HA antigen that induced T cell proliferation in chickens immunized with a fowlpox virus-based vaccine expressing the H5 protein. The peptide was presented by both MHC class I and II molecules, leading to activation of CD4+ and CD8+ T cell subsets. Finally, Singh *et al* (2010) determined that chicken CD8+ T lymphocytes respond to the influenza nucleocapsid (NP) and haemagglutinin (HA) protein, with the NP inducing a significantly greater response.

1.3. Vaccines

1.3.1. Early Beginnings

One of the most effective means to combat infectious diseases is though vaccination (Mäkelä, 2000). In 1798, Edward Jenner published his method for the prevention of smallpox, following his observation that inoculation with pus from a cowpox lesion, which caused a mild disease in humans, would provide immunity to smallpox (Jenner, 1798; Strassburg, 1982; Mäkelä, 2000; Borysiewicz, 2010). Regarded as the first vaccine, it should be noted that, by this time, human populations had been using a form of vaccination, known as variolation, to prevent smallpox for centuries (Mäkelä, 2000). With variolation, immunity was induced by inoculating the smallpox virus under the skin but the technique had variable outcome, could promote the spread of the disease and had a procedural mortality rate of 2-3% (Borysiewicz, 2010). When vaccination was first proposed, it was widely ridiculed, exemplified in James Gillray's satirical cartoon where the vaccinator is surrounded by his victims who have cows emerging from their bodies (Borysiewicz, 2010). However, mortality rates fell, as a direct result of vaccination, and the focus of smallpox control switched from national to global eradication. The last recorded case of naturally occurring

smallpox in the world was diagnosed on 31st October 1977 and on 8th May 1980, the World Health Organization declared the world free of smallpox (Strassburg, 1982; Borysiewicz, 2010).

1.3.2. Vaccines

Vaccination has been one of the most effective interventions to decrease mortality and morbidity due to infectious diseases. The best immune response is induced following natural infection with the pathogenic organism, although this may result in high mortality. Vaccines work on the principle that they induce protective immunity against the disease causing pathogen, but without causing the disease (Swayne and Kapczynski, 2008; Zepp, 2010). The goal of a vaccine is to induce long term immunological memory that, once reactivated, can respond quickly and efficaciously (Esser *et al*, 2003; Moser and Leo, 2010). An ideal vaccine is a non-virulent, attenuated or inactivated form of a pathogen, able to elicit a strong and protective immune response *in vivo* (Moser and Leo, 2010). The primary mechanism of protection is through the generation of neutralising antibodies, rather than the induction of cell-mediated immunity (Wilson-Welder *et al*, 2009). However, advances in biotechnology and the development of a greater understanding of the immunology and pathogenesis of disease, has allowed an increased level of sophistication of vaccine design (Mäkelä, 2000). This is necessary as, although vaccination is undoubtedly the most effective means of controlling infectious disease, the problem becomes more complex when dealing with zoonotic infections, such as avian influenza. The successful control of this virus continues to present a major challenge, although combating the influenza virus at the animal source would help to reduce human exposure to emerging strains by decreasing the global levels of virus in circulation (Marangon *et al*, 2008; Pastoret, 2009).

1.3.3. Vaccine Adjuvants

To improve the immunogenicity of vaccines, adjuvants (from the Latin word *adjuvare*, meaning 'to help') can be introduced into the vaccine formulations (Nicholls *et al*, 2010; Zepp, 2010). An adjuvant is an agent that increases the response to a vaccine by providing a reservoir for the slow release of antigen (Wilson-Welder *et al*, 2009). In addition, the presence of an adjuvant enhances the immune response by functioning as a delivery system, enhancing the uptake of the antigen by antigen presenting cells (APCs) (Wilson-Welder *et al*, 2009; Leroux-Roels, 2010). Only inactivated/killed or component vaccines such as subunit or DNA vaccines require the addition of adjuvants; live and recombinant vaccines induce effective humoral and cell-mediated immune responses so do not require the presence of adjuvants in the formulation (Brun *et al*, 2008; Nicholls *et al*, 2010). This is due to the fact that inactivated or highly purified vaccines lose part of their intrinsic immunostimulatory ability when produced, and thus are simply not considered threatening enough by the immune system (Moser and Leo, 2010; Leroux-Roels, 2010).

Aluminium compounds were originally identified as adjuvants more that eighty years ago, and since then aluminium salts have been widely used in vaccines to generate effective immune responses (Brewer, 2006; Leroux-Roels, 2010). Aluminium salts provide proinflammatory or immunostimulatory effects as well as prolonging the persistence of vaccine antigens by slowing down antigen degradation (Nicholls *et al*, 2010; Zepp, 2010). Oil emulsion vaccines slowly release antigen over time, resulting in higher immune responses than would be produced from antigen alone (Swayne and Kapczynski, 2008). Water-in-oil (w/o) emulsions, such as Freund's adjuvant and oil-in-water (o/w) emulsions, such as MF59, have both been utilised in avian influenza vaccine preparations (Leroux-Roels, 2010; Nicholls *et al*, 2010). In addition, modifying the oil phase can change the overall immune response to the vaccine. For example, non-metabolizable oils like mineral oil generally produce higher antibody responses when compared with biodegradable oils such as vegetable oil, although the non-metabolizable oils can cause increased damage to host tissue at the site of injection (Swayne and Kapczynski, 2008). Other adjuvants include

liposomes and virosomes, TLR ligands, the water soluble polymer polyphosphazene, saponins such as Quil A, and immune-stimulating complexes (ISCOM) that are composed of lipids, cholesterol, antigen and Quil A (Leroux-Roels, 2010; Nicholls *et al*, 2010). Finally, cytokines can enhance the immune response (Wilson-Welder *et al*, 2008). Chicken myelomonocytic growth factor (cMGF), for example, has a positive effect on the efficacy of Marek's Disease vaccines by activating macrophages, increasing survival time and lowering viraemia following challenge with a very virulent Marek's Disease Virus (vvMDV) strain (Djeraba *et al*, 2002; Asif *et al*, 2004; Gimeno, 2008). Other cytokines that have been shown to possess adjuvant activity include interleukin-18, IFN-α, IL-β and IFN-γ, all of which have the potential to be used in vaccine formulations to provide improved protection against disease (Asif *et al*, 2004; Hung *et al*, 2010; Nicholls *et al*, 2010).

1.3.4. Avian Influenza Vaccines

Avian influenza represents one of the greatest concerns to public health that has emerged from an animal reservoir. The emergence of H5N1 HPAI viruses in Asia, and the subsequent outbreaks of serious disease in humans and poultry, focused the attention of the world on the influenza virus. With the potential risk of human pandemic viruses being created via the avian-human link, infections caused by this virus have assumed a completely different profile in both the veterinary and medical scientific communities (Webster *et al*, 2006; Capua and Marangon, 2007). In June 2009, fears of a potential influenza pandemic were confirmed when a new influenza A H1N1 virus emerged and spread through the human population causing the first pandemic of the 21st century (Peiris *et al*, 2009; Smith *et al*, 2009; Neumann *et al*, 2009; Girard *et al*, 2010).

Vaccines are essential tools for the control of avian influenza because they increase resistance to infection, prevent illness and death, reduce virus replication and reduce virus transmission to other birds and mammals, including humans (Swayne and Kapczynski, 2008). The successful control of influenza would also help avoid severe economic losses that would result from mass depopulation policies in the event of an

outbreak (Capua and Marangon, 2003). Avian influenza vaccines, properly selected and correctly administered, will protect against clinical signs and mortality, reduce the levels and duration of virus excretion and increase the resistance of the host to infection. However, it is important that vaccination is never the sole method of disease control. It should be combined with other control measures such as good biosecurity and strict monitoring systems (Capua and Marangon, 2003; Marangon *et al*, 2008; Capua and Alexander, 2008; Swayne and Kapczynski, 2008).

In addition, the considerations for the development of veterinary vaccines differ from humans in some respects, one of the most important of which is cost, as a high cost would preclude the use of certain types of vaccines. Also, the ability to distinguish between infected and vaccinated animals (DIVA) is essential for the eventual eradication of HPAI or LPAI, without the mass culling of birds. The DIVA strategy is necessary due to the lack of clinical signs seen in birds infected with a field virus (Brun *et al*, 2008; Capua and Alexander, 2008). Most importantly, vaccines should produce a consistent immune response, be protective against circulating field strains, and be administered properly to a high percentage of the susceptible population to produce flock immunity (Swayne and Kapczynski, 2008).

There are six general types of avian influenza vaccine which include inactivated, subunit, DNA vaccines, live attenuated virus, recombinant vectors expressing foreign genes and most recently, virus like particles (VLPs). All have specific advantages and disadvantages but only inactivated and recombinant type AI vaccines have received licensure for commercial use (Swayne and Kapczynski, 2008, Brun *et al*, 2011).

Inactivated vaccines

Inactivated viral vaccines are essentially inert antigens that induce $CD4^+$ T cell and humoral responses (Dudek and Knipe, 2006). The overwhelming majority of AI vaccines produced and sold for use in poultry are oil emulsion, inactivated whole AI virus vaccines that are administered either subcutaneously or intramuscularly.

Conventional inactivated vaccines aimed at H5, H7 and H9 subtypes are now commercially available and have been licensed for use in a number of countries (Capua and Alexander, 2008). These vaccines have the advantage of being very safe as, although most of the proteins that induce the protective immune response are present, the organism cannot replicate and therefore establish a persistent infection or revert to a virulent form (Wilson-Welder *et al*, 2009). It is rare for such vaccines to contain residual infective virus that has resisted inactivation and there is no reversion to virulence by the vaccine (Graham and Crowe Jr, 2007). Inactivated vaccines are prepared by first propagating the virus in 9-11 day old embryonating chicken eggs, harvesting the infected allantoic fluid and chemically inactivating the viruses with formalin, β-propiolactone or binary ethylenimine (Capua and Alexander, 2008; Swayne and Kapczynski, 2008; Swayne, 2009). The inactivated vaccine can induce protection in multiple species of poultry but in order to ensure a high immunological protection, more than one administration may be required (Marangon *et al*, 2008). In general, inactivated vaccines primarily provide protection via humoral immunity and do not stimulate cell-mediated immunity. This is due to the fact that, for an effective CD8+ cytotoxic T lymphocyte response, antigens must be processed through the proteasome so that the peptides can be transported in the endoplasmic reticulum and associate with the MHC class I molecules (Graham and Crowe Jr, 2007; Cinatl Jr *et al*, 2007; Swayne and Kapczynski, 2008). To help increase the immunogenicity and produce a greater immune response, an adjuvant such as an oil emulsion can be added (Swayne and Kapczynski, 2008). Recent research has focused on developing safe and effacious oil-adjuvented H5N1 avian influenza vaccines (Kydyrbayev *et al*, 2010; Imamura *et al*, 2010).

Protein Subunit vaccines

Improvements in industrial fermentation, purification and production processes have allowed subcomponents of pathogens to be isolated and produced in large quantities (Zepp, 2010). Avian influenza protein subunit vaccines are based on *in vitro* expression of the HA gene in animal or plant cells, bacteria or yeast. Once expressed,

the HA protein is purified away from cell extracts, quantified, oil emulsified and injected parenterally. No live virus is involved so protein subunit vaccines are safe; however, production costs can be very high (Swayne and Kapczynski, 2008; Swayne, 2009). In addition, the immune response tends to be weaker so multiple doses, as well as adjuvantation, is required to induce sufficient protection (Cinatl Jr *et al*, 2007; Zepp, 2010). Baculovirus vectors have been used to express both H5 and H7 HA in cell culture supernatants, for the immunization of poultry, and induced 100% protection against lethal H5 and H7 strains (Crawford *et al*, 1999; Swayne, 2009).

DNA vaccines

DNA vaccination involves the introduction of DNA expression vectors encoding immunogenic proteins into cells, thereby inducing a $CD8^+$ cytotoxic T cell response (Ulmer *et al*, 1993). Using this method, plasmid DNA-based vaccines that express the AI HA gene can provide protective immunity towards influenza. Following vaccine application and uptake by host cells, the HA gene is transcribed into RNA and transported to the cytoplasm for protein translation. The endogenously expressed protein antigen is processed intracellularly via MHC class I proteins, stimulating cytotoxic T cells, or by MHC class II molecules for the stimulation of humoral immunity (Swayne and Kapczynski, 2008; Swayne 2009). DNA vaccines are safe as the production of plasmids does not require handling of infective agents and, because immunity is only directed towards the plasmid encoded antigen, it is easy to differentiate infected from vaccinated animals. However, although DNA vaccines do induce a humoral and cellular response, they have a relatively low efficacy and a large amount of DNA is required to produce a strong response (Brun *et al*, 2011). The development of DNA vaccines to deliver the haemagglutinin gene was first carried out in 1993 (Robinson *et al*, 1993; Fynan *et al*, 1993). Currently, however, no DNA vaccines have been licensed for use in poultry due to the high manufacturing costs and the requirement of multiple vaccinations to achieve immunity (Swayne and Kapczynski, 2008).

Live attenuated vaccines

A live attenuated vaccine is a live virus that has lost its virulence while maintaining its ability to replicate (Brun *et al*, 2008; Wilson-Welder *et al*, 2008). Live attenuated vaccines induce humoral and cellular immunity and provide superior and longer lasting protection compared with inactivated vaccines (Brun *et al*, 2008; Swayne and Kapczynski, 2008; Zepp, 2010). In addition, these vaccines have a relatively low manufacturing cost as they do not require adjuvants in the formulation (Brun *et al*, 2008; Wilson-Welder *et al*, 2009). However, in regards to avian influenza, there is a danger that live LPAI vaccines could reassort and mutate from LPAI to HPAI viruses (Graham and Crowe Jr, 2007; Swayne and Kapczynski, 2008; Zepp, 2010), although this could be avoided using *in ovo* vaccination as other influenza strains would not be present in the egg (Cai *et al*, 2011). Additionally, live LPAI vaccines may cause unacceptable respiratory disease or drops in egg production and can spread to neighbouring farms (Swayne and Kapczynski, 2008). One solution to address this was to use reverse genetics (Hoffmann *et al*, 2000; Hoffmann *et al*, 2002) to engineer an AI virus with reduced pathogenicity, either by mutating the haemagglutinin cleavage site (Cai *et al*, 2011), or by deleting the NSI gene which is associated with virulence and evasion of the host immune system (Palese and Shaw, 2007). The resulting vaccine displayed decreased replication and attenuation of infectivity, but retained immunogenicity, safely eliciting both humoral and cell-mediated immune responses (Palese and Shaw, 2007; Swayne and Kapczynski, 2008; Cauthen *et al*, 2007; Brahmakshatriya *et al*, 2010).

Recombinant vectors expressing avian influenza genes

Recombinant vaccines for avian influenza viruses have been produced by inserting the gene coding for the influenza virus HA protein into a live virus vector and using this recombinant virus to vaccinate against influenza. These live vaccines are ideal as they replicate, presenting the foreign antigen to the immune system in the context of an intracellular infection, with the expectation of stimulating humoral and cellular immunity. This intracellular expression and processing of the antigen allows for

presentation to the immune system in the context of the MHC class I system, giving the option to stimulate specific cytotoxic T cells (Capua and Alexander, 2008; Zepp, 2010). In addition, viral vectors can be mass applied to vaccinate with minimal labour, such as the robotic vaccination of fertile eggs or with rapid vaccinations used at one day of age (Swayne and Kapczynski, 2008). They allow easy distinction between vaccinated and infected birds as antibodies to the common influenza proteins, matrix and nucleoprotein, are not produced (Capua and Alexander, 2008). Disadvantages include a limited host range and vaccine failure if the birds to be vaccinated already possess immunity to the vector (Capua and Alexander, 2008).

There are several examples of viral vectors used for the *in vivo* expression of influenza haemagglutinin genes in poultry. A live attenuated fowlpox virus was originally developed which expressed the HA molecule from a highly virulent H5 strain of avian influenza (Taylor *et al*, 1988; Swayne, 2009). A recombinant fowlpox (recFP) vector expressing the H5 HA gene, designated TROVAC™-AIV H5 (TROVAC H5), was commercialized and used extensively in Mexico, El Salvador and Guatemala, with 1.6 billion doses used in the field (Bublot *et al*, 2006; Swayne and Kapczynski, 2008). Other examples of live attenuated viruses used to deliver an influenza HA gene include vaccinia virus (Chambers *et al*, 1988), adenovirus (Gao *et al*, 2006; Tang *et al*, 2009), Rous sarcoma virus (Hunt *et al*, 1988), infectious laryngotracheitis virus (Luschow *et al*, 2001; Pavlova *et al*, 2009), Venzuelan equine encephalitis virus (Schultz-Cherry *et al*, 2000) and Newcastle disease virus (Ge *et al*, 2007; Nayak *et al*, 2009).

Virus-Like particles

Virus-like particles (VLPs) are composed of recombinantly expressed viral proteins that spontaneously assemble into structures resembling infectious viruses. VLPs are highly immunogenic, however, because they lack viral nucleic acid, they are non-pathogenic and very safe (Brun *et al*, 2011). Gómez-Peurtas *et al* (2000) demonstrated that the matrix (M1) protein is the only viral component which is essential for VLP formation and that the viral RNPs are not required for virus

particle formation. By developing a recombinant baculovirus that expressed the HA, NA, M1 and M2 proteins of the influenza virus in Sf9 insect cells, Latham and Galarza (2001) demonstrated the successful formation of influenza VLPs. These closely resembled the influenza virus in size, morphology and fine structure of surface spikes, but lacked the influenza ribonucleoprotein complex (Latham and Galarza, 2001). A further study demonstrated that the influenza virus HA and NA, but not the M2 protein, are required for the formation of plasmid derived VLPs (Chen *et al*, 2007). In comparison with available influenza vaccines, the immunization of mice with influenza VLPs elicited better immune responses that inactivated whole virus vaccine or recombinant HA (Bright *et al*, 2007). Most recently, influenza VLPs have been created for the successful vaccination of ferrets against the 2009 pandemic H1N1 influenza strain (Pushko *et al*, 2010), and the vaccination of poultry against an H9N2 influenza virus (Lee *et al*, 2011).

1.3.5. A universal influenza vaccine

By evolving variants through genetic mutation, in response to the host immune response, influenza viruses can evade neutralisation. As a result of this antigenic drift, influenza vaccines must be prepared each year so that they closely match circulating strains. However, as the 2009 H1N1 pandemic demonstrated, completely new strains can unexpectedly emerge, against which the currently used strain-specific vaccine cannot protect (Nabel and Fauci, 2010). An ideal influenza vaccine, therefore, would be effective at producing a protective immune response against a range of virus subtypes, including emergent pandemic strains (Cinatl Jr *et al*, 2007; Du *et al*, 2010). To this end, research has focused on areas of the influenza virus that would induce heterosubtypic immunity, where protective immunity against a range of influenza subtypes can be induced by a single vaccine (Nabel and Fauci, 2010). For example, a human vaccine study has focused on the M2e peptide, the highly conserved ectodomain of the influenza M2 protein, rather than haemagglutinin, to provide protective immunity (Fiers *et al*, 2009). However, as previously stated, immunization of chickens with the M2 protein did not provide protection against

lethal HPAI virus challenge (Nayak *et al*, 2010). An alternative approach has concentrated on the creation of influenza vaccine constructs that contain the stem region of the haemagglutinin spike, HA2, but not the globular head, HA1. The rational is that HA2 is more conserved, and during normal infection or vaccination, it is masked from neutralising antibodies by the bulky, and immunogenic, globular head (Steel *et al*, 2010; Nabel and Fauci, 2010). What is not yet clear is whether these antibodies will be effective against wildtype strains, which still possess the globular head. However, with the continued identification of conserved regions in the HA glycoprotein, vaccines that can induce stronger immunity and improved cross-protective efficacy against divergent influenza virus strains may be created (Du *et al*, 2010).

1.4. Marek's Disease (MD)

1.4.1. Marek's Disease Virus (MDV)

Marek's Disease (MD), a lymphoproliferative disease of chickens, is caused by the Marek's Disease Virus (MDV), which was first identified in 1967 using electron microscopy and negative staining (Churchill and Biggs, 1967). Witter *et al* (1969) subsequently confirmed that MDV was the etiologic agent of Marek's Disease (Witter *et al*, 1969). The virus is highly cell-associated; cell-free virus is only found in the feather follicle epithelium (FFE) of infected birds, an important and efficient route of transmission for the disease (Calnek *et al*, 1970; Gimeno, 2008).

MDV, which belongs to the *Alphaherpesvirinae* family, includes three serotypes that were determined initially using indirect immunofluorescence tests (Bülow and Biggs, 1975). MDV serotype 1 (MDV-1) includes all the oncogenic strains and their attenuated forms; MDV serotype 2 (MDV-2) includes all the non-oncogenic strains isolated in chickens; serotype 3 includes all the non-oncogenic strains isolated in turkeys, Herpesvirus of Turkey (HVT) (Bülow and Biggs, 1975; Nair, 2005; Gimeno, 2008). All three serotypes are grouped in the taxonomic genus of

Mardivirus (Nair, 2005; Gimeno, 2008). MDV-1 is now classified as *Gallid herpesvirus 2* (GaHV-2), MDV-2 as *Gallid herpesvirus 3* (GaHV-3) and HVT as a *meleagrid herpesvirus* (Schumacher *et al*, 2000).

Initially, MDV was thought to belong to the same family as the Epstein-Barr virus, *Gammaherpesvirinae*, due to their similar biological properties. Both viruses grow slowly in cell culture and induce T cell lymphomas (Osterrieder *et al*, 2006). However, when the molecular structure and genomic organisation of MDV was analysed, it was found to have more similarities with Herpes Simplex Virus (HSV), an alphaherpesvirus (Fukuchi *et al*, 1984; Tulman *et al*, 2000). This was confirmed using gene sequence and mapping data from MDV and HVT, which indicated that the sequences bore a greater similarity to the alphaherpesvirus, Varicella Zoster Virus (VZV), than to EBV (Buckmaster *et al*, 1988).

1.4.2. Marek's Disease (MD)

Marek's Disease was first described by Joszef Marek in 1907 as a polyneuritis with low morbidity and negligible mortality (Marek, 1907; Gimeno, 2008). Also known as fowl paralysis, the disease was found to cause lymphoid tumours in visceral organs and pathological changes to the nervous system (Pappenheimer *et al*, 1929a; Pappenheimer *et al*, 1929b). Marek's disease is a great concern for the poultry industry as it is capable of causing devastating losses to commercial poultry stocks (Gimeno, 2008).

All chickens are susceptible to infection with MDV. The infection occurs by the inhalation of dust in the poultry house environment that has been contaminated with viruses shed from the FFE of infected birds. Lymphoid cells are the main targets of MDV, and after initial replication in the lungs, the virus replicates in the lymphoid tissue (bursa of Fabricius, thymus and spleen). B lymphocytes and macrophages undergo a lytic infection that results in the activation of T cells, which are targeted by the virus. Infected T cells carry the virus to the FFE. After the early cytolytic phase, infection switches to a latent phase in the infected T cells after 7-8 days.

These T cells become targets for neoplastic transformation resulting in the development of lymphomatous tumours in various visceral organs as well as nerves, the brain and the skin (Nair, 2005; Baigent *et al*, 2006; Gimeno, 2008).

Only MDV-1 viruses are pathogenic and capable of causing tumours. Highly virulent strains are capable of inducing a high frequency of lymphoproliferative lesions in the brain and a higher rate of mortality due to brain edema (Gimeno, 2008).

1.4.3. MDV vaccination

Over time, Marek's Disease has changed in severity and clinical manifestations, turning from an endemic, mild paralytic syndrome (mMDV), into a highly contagious, aggressive, neoplastic disease with a worldwide distribution. The ability of MDV strains to continuously evolve towards greater virulence is becoming a major concern for the poultry industry and veterinarians (Witter, 1997; Nair, 2005).

At the start of the twentieth century, poultry production was characterised by low population densities, low egg production and varied combinations of different ages and breeds. World War II accelerated the growth of the poultry industry and, as greater intensive poultry farming practices were introduced, the disease became more severe. With the new form of the disease, chickens developed MD at a younger age with a high incidence of visceral lymphoid tumours, nerve lesions and a mortality rate in excess of 30%. This much more severe form of the disease became known as acute or virulent MD (vMDV) and, by the 1960s, had become the predominant form in most countries that had a well-developed poultry industry (Witter, 1997; Gimeno, 2008).

Marek's Disease has been successfully controlled since 1969, when it was found that attenuated MDV protected chickens against challenge with a vMDV strain (Churchill *et al*, 1969). This first vaccine, an attenuated strain (HPRS-16) of MDV-1 (Churchill *et al*, 1969), was the first vaccine against a neoplastic disease, as well as being one of the most effective for a herpesvirus. The control of MD by vaccination was a major step in the development of the modern poultry industry (Gimeno, 2008).

Soon after, a second vaccine was developed (Okazaki *et al*, 1970), based on the HVT strain that had been isolated from the kidney cell culture of normal turkeys (Kawamura *et al*, 1969). This HVT vaccine brought about a widespread reduction in MDV-induced poultry losses (Nair, 2005), although this was not sustained as the use of MD vaccines was accompanied with an increase in virulence of MDV (Witter, 1997). A unique feature of HVT is that it can be obtained as a cell-free virus by the sonication of infected cell cultures, allowing the production of cell-free lyophilized HVT vaccines. MDV, unlike HVT, is highly cell-associated and must be produced in chicken embryo cells, stored in liquid nitrogen, and administered as a viable cell suspension (Baigent *et al*, 2006; Gimeno, 2008). Cell-associated vaccines are very labile, so although the efficacy of HVT vaccines is not as high, they are a good solution for some countries where the logistics of maintaining a cold chain is a factor (Gimeno, 2008).

The introduction of vaccines, combined with the major changes to the poultry industry, seemed to play a major role in this increased virulence (Nair, 2005). Prior to this, both the virus and the host co-existed in a balanced state. With the advent of highly intensive farming practices, the poultry house provided the virus with a large number of genetically susceptible hosts and an ideal environment to persist outside the host for long periods (Nair, 2005). This, coupled with the widespread use of vaccines, forced the virus to evolve rapidly and a new, more virulent MDV strain emerged. This strain was termed vvMDV, to differentiate it from the previous milder strains, mMDV and vMDV (Witter, 1983; Witter, 1997; Gimeno, 2008).

To respond to these new, more virulent strains, a bivalent vaccine was created, consisting of HVT and the apathogenic, MDV-2 strain, SB-1 (Schat *et al*, 1982; Gimeno, 2008). Combinations of strains provide better protection than either vaccine alone, an effect known as protective synergism (Baigent *et al*, 2006; Gimeno, 2008). Although this was initially successful, fresh outbreaks of the disease occurred due to even more virulent strains, designated vv+MDV (Witter, 1997; Nair, 2005). Subsequently, a third vaccine was introduced. Known as the CVI988 Rispens strain, it had been developed from a natural isolate of MDV-1 and had been used widely in Europe since 1972 (Rispens *et al*, 1972a; Rispens *et al*, 1972b; Nair, 2005; Gimeno,

2008). The CVI988 strain, which was found to be serologically identical to the (HPRS-16) strain (Bülow, 1977), had a low oncogenic potential upon isolation but was further attenuated by passaging the virus in duck embryo fibroblast (DEF) cell culture 26-35 times (Gimeno, 2008). As before, the introduction of this vaccine saw a reduction in MD-induced mortality caused by the vv+MDV strain (Nair, 2005).

The step-wise evolution of MDV is shown in Figure 6, demonstrating the increasing virulence of emerging MDV strains in response to the introduction of different generations of vaccines (Nair, 2005). Currently the CVI988 vaccine provides the best protection when compared to other vaccines (Witter *et al*, 1995; Gimeno, 2008). It has, however, been suggested that there may be a limit to the ability of the chicken to develop protective immunity in response to vaccination and this threshold has already been achieved with CVI988 (Witter and Kreager, 2004; Gimeno, 2008).

Outbreaks of MDV-induced morbidity and mortality in vaccinated birds, by these increasingly virulent strains, may be in part due to inherent weakness in the vaccination strategies. For example, the traditional vaccination of 1 day old chicks does not allow sufficient time for the formation of an adequate immune response (7-14 days of age). Early immunity is essential as the chicks generally face challenge from MDV within a few days of being introduced into the brooding pens. Instead, it was found that *in ovo* vaccination with CVI988 at 18 days of embryonation induced early post-hatch protection against vvMDV (Zhang and Sharma, 2001; Baigent *et al*, 2006; Gimeno, 2008). Currently, over 80% of US broilers are immunised *in ovo* with MD vaccine. Compared with field vaccination, *in ovo* vaccination provides uniform and fast delivery, reduced labour costs and decreased stress to the birds and the production of an early immune response (Cai *et al*, 2011). In addition, vaccination with suboptimal vaccine doses, or the immunosuppression caused by other pathogens, means that MDV strains can replicate in partially immune birds. Under such conditions, the virus will not be prevented from adapting and evolving into more virulent strains (Nair, 2005).

1.4.4. The future of MDV vaccines

Current MDV vaccines greatly reduce the level of latency of the wild-type virus and the development of tumours. This is due to the vaccine-stimulated host defences acting on the replication of MDV, reducing the viral load. Subsequently, the probability of neoplastic transformation of lymphoid cells is decreased and the immunosuppressive effects of MDV are diminished, allowing the host to mount a more effective immune response (Payne *et al*, 1978; Gimeno, 2008). Vaccinated birds can, however, be superinfected by virulent strains, and although the quantity of virulent MDV shed from the FFE is reduced, a minimum level of replication in the FFE is enough to spread the disease very efficiently to other chickens. This failure of vaccines to induce sterile immunity in the host and prevent the infection, replication and shedding of virulent virus strains means that the evolution of the virus is not halted. Fortunately, through the continued development of MDV vaccines, MD has been successfully controlled (Nair, 2005; Gimeno, 2008).

There is concern, however, that the CVI988 strain is one of the last in the line of effective vaccines against MDV and that, as the evolution of MDV produces greater virulence that the CVI988 vaccine cannot protect against, the effect on the poultry industry will be catastrophic (Nair, 2005; Spatz *et al*, 2007). Consequently, vaccination techniques are monitored, improved and revaccination is repeated if necessary. A real-time polymerase chain reaction (PCR) assay for the quantification of CVI988 vaccine virus in the feather tips has been developed commercially to confirm the successful vaccination of chicks (Baigent *et al*, 2005; Baigent *et al*, 2006). Any disease outbreaks of MD that occur are managed using polyvalent vaccines (combined serotypes 1, 2 and 3). In addition, research has focused on gaining a better understanding of the biology of MDV and its genome, potentially paving the way for a new generation of molecularly defined vaccines (Nair, 2005). To this end, the MDV genome was cloned into a Bacterial Artificial Chromosome, so that the functions of various genes could rapidly and efficiently be ascertained.

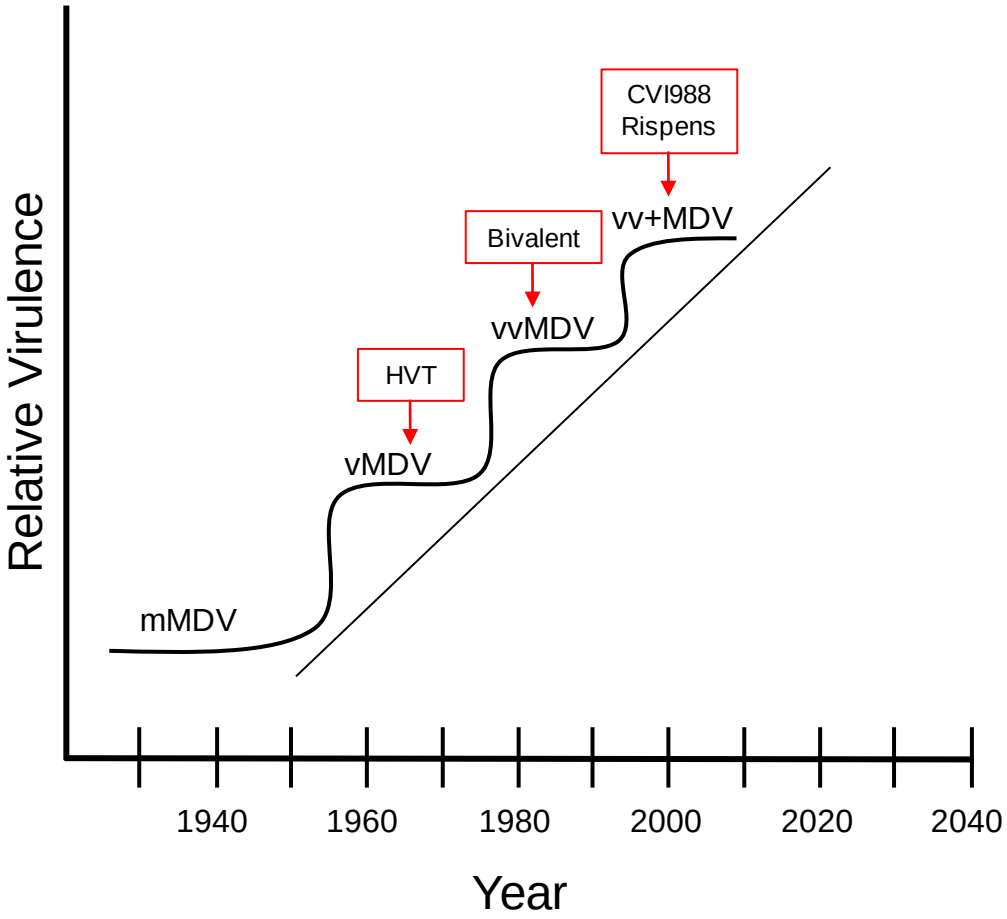


Figure 6. Step-wise evolution of virulence of MDV. Adapted with permission (Nair, 2005).

1.5. Bacterial Artificial Chromosomes (BACs)

Bacterial Artificial Chromosomes (BACs) are DNA constructs derived from the naturally occurring F' (Fertility) plasmid found in the bacterium *Escherichia coli* (*E. coli*) (Kim *et al*, 1992; Shizuya *et al*, 1992). F' plasmids, carrying inserted bacterial DNA, are capable of maintaining fragments as large as 1 Mb, which led to speculation that they would suitable for cloning viral genomes (Shizuya *et al*, 1992).

The first herpesvirus BAC mutant was created for murine cytomegalovirus in 1997 and since then the technology has proved to be an invaluable tool for studying herpesvirus pathogenesis (Messerle *et al*, 1997; Warden *et al*, 2011). Knowing the function of individual, or families of, genes is important in understanding their role in pathogenesis, in determining their potential as a therapeutic target or for the purpose of vaccine design (Adler *et al*, 2003). The BAC approach was conceived because the herpesvirus genomes, ranging in size from 125-240 kb, are too large to be incorporated into an individual plasmid or cosmid construct which can incorporate up to 10 kb or 30 kb of DNA, respectively (Warden *et al*, 2011). A technique for the construction of recombinant herpesviruses from cloned overlapping cosmid clones was developed, allowing the study of herpesvirus pathogenesis through the introduction of mutations (Zijl *et al*, 1988; Brune *et al*, 2000; Reddy *et al*, 2002). This technique, however, relies on several recombination events to create the mutant virus and this was difficult to control (Messerle *et al*, 1997). In addition, cosmid technology does not easily allow the construction of revertants that can prove the altered property is a function of the deliberately introduced mutation (Brune *et al*, 2000). Yeast artificial chromosomes (YACs) can also incorporate large DNA sequences, but BACs are more suitable for virus studies as they are more stable and less prone to undesired genomic rearrangements (Monaco and Larin, 1994; Brune *et al*, 2000; Warden *et al*, 2011). It was found that the F' plasmid could propagate mammalian genomic DNA inserts with significantly greater stability (Kim *et al*, 1992). Using the BAC system, large DNA fragments from varied genomic sources can be cloned into *E. coli*, where the DNA is stable, easy to manipulate and represents a single foreign DNA source (Shizuya *et al*, 1992). In addition, purified BAC DNA containing the CMV genome was infectious and this allowed the

generation of replication-competent viruses following transfection into an appropriate cell line (Messerle *et al*, 1997).

1.5.1. BAC Vectors

To create the viral BACs, the F' plasmid or BAC vector cassette sequence was inserted into the viral genome using site-directed mutagenesis (Warden *et al*, 2011). A typical BAC vector is about 10 kb in length and contains an origin of replication (*oriS*) and BAC replication (*repE*) gene which are both responsible for the unidirectional replication of the F' plasmid. The genes *parA* and *parB* both control the rate of replication so that the number of copies in a bacterial cell is limited to one or two (Shizuya *et al*, 1992; Warden *et al*, 2011). In addition, an antibiotic resistance gene such as chloroamphenicol and a selectable marker (SacB) must be present so that bacterial colonies containing the BAC can be selected. The SacB gene encodes a protein called levansucrase, which catalyzes the polymerization of fructosyl groups in sucrose to form levan, a substance toxic to *E. coli* (Lepesant *et al*, 1972; Bramucci and Nagarajan, 1996; Wagner *et al*, 2002). The BAC vector must be flanked by 500-1000 base pairs that are homologous to the target sequence of the viral genome where the BAC vector will be inserted via homologous recombination (Warden *et al*, 2011). For this to be successful, a long non-essential region of the virus must be identified to avoid severe growth defects in the virus (Brune *et al*, 2000; Warden *et al*, 2011). Finally, the BAC vector containing viral DNA is transformed into an *E. coli* strain (DH10B or DH5-α) via electroporation, made easier due to the tendency of the herpesvirus genome to circularise during replication (Warden *et al*, 2011). It is necessary to use a bacterial strain in which the *recABCD* recombination system is disrupted, as the repetitive sequences in herpesviruses genomes are targets for recombination. As the DH10B and DH5-α strains are *rec*A-negative, this helps to increase the stability of cloned sequences. The *rec*A enzyme, however, is essential for allelic exchange by homologous recombination to occur so is transiently expressed by the shuttle plasmid used in BAC mutagenesis (see below) (Brune *et al*,

2000; Wagner *et al*, 2002; Adler *et al*, 2003). Once created, viral BACs in *E. coli* can be stored at -80^{o}C (Warden *et al*, 2011).

1.5.2. BAC Mutagenesis

The large size of herpesvirus BAC genomes prohibits the use of conventional enzymatic cleavage and ligation techniques to introduce or delete DNA (Wagner *et al*, 2002). BAC mutagenesis by homologous recombination is achieved by constructing a shuttle plasmid that contains the mutation to be introduced, flanked by sequences homologous to the integration site. The alignment of similar sequences allows a crossover between the aligned DNA strands resulting in an exchange of material, subsequently introducing the mutation into the herpesvirus BAC. Once constructed, the shuttle plasmid (encoding the *rec*A gene, as well as the SacB gene and a temperature sensitive origin of replication, RepTS), is subsequently transformed into the same *E. coli* host (DH5-α) as the BAC and allelic exchange takes place through a two-step process of co-integrate formation and resolution (Wagner *et al*, 2002; Brune *et al*, 2000).

1.5.3. Marek's Disease Virus BACs

Viral BACs have been created for many human and animal herpesviruses, listed in Table 3 (Warden *et al*, 2011). With regards to MDV, several BAC clones have now been developed for different strains. The development of a MDV BAC was critical to the understanding of this pathogen. A MDV BAC clone of the attenuated MDV-1 strain, 584Ap80C, was created by inserting the F' plasmid into the US2 region of the virus by homologous recombination. The growth of the BAC mutant, assessed by plaque formation and growth curves, was found to be indistinguishable from that of the parental strain, 584Ap80C (Schumacher *et al*, 2000).

This MDV BAC clone was used to successfully investigate the essential role of various genes in virus growth, including the UL46-UL49 genes which encode the

Table 3: - BAC-based human and animal herpesvirus

BAC-based human herpesvirus	BAC-based animal herpesvirus
Herpes Simplex Virus-1 (HSV-1)	Bovine Herpesvirus Type I (BHV-1)
Herpes Simplex Virus-2 (HSV-2)	Equine Herpesvirus Type I (BHV-1)
Varicella Zoster Virus (VZV)	Feline Herpesvirus Type I (BHV-1)
Kaposi's Sarcoma-Associated Herpesvirus (KSHV)	Guinea Pig Cytomegalovirus (GPCMV)
Cytomegalovirus (CMV)	Herpesvirus Saimiri (HVS)
Human Herpesvirus 6 (HHV-6)	Koi Herpesvirus (KHV)
Epstein-Barr Virus (EBV)	Marek's Disease Virus (MDV)
	Murine Cytomegalovirus (mCMV)
	Murine Gammaherpesvirus 68 (MHV-68)
	Pseudorabies Virus (PrV)
	Rhesus Cytomegalovirus (rhCMV)
	Rhesus Rhadinovirus (RRV)
	Turkey Herpesvirus (HVT)

MDV-1 homologues of HSV-1 major tegument proteins (Dorange *et al*, 2002), and the UL10 and UL49.5 genes which encode the viral membrane glycoprotein M (gM) and envelope/tegument protein, respectively (Tisher *et al*, 2002). Recently, research to delete both copies of the *Meq* gene from a MDV BAC has produced a highly efficacious vaccine that was non-oncogenic *in vivo* and which provided protection greater than that of the CVI988 Rispens strain *(*Silva *et al*, 2010). The *Meq* protein is a 339-aa-long protein encoded within the MDV *EcoRI* Q fragment (MEQ) of MDV-1 strains that is consistently expressed in MDV-induced tumour cells and has been proposed as a major virulence determinant (Jones *et al*, 1992; Lupiani *et al*, 2004; Spatz *et al*, 2007; Lee *et al*, 2008).

In an attempt to understand why the CVI988 Rispens vaccine strain provided the best protection when compared to other vaccines, and to meet the challenges raised by the continuing increase in MDV virulence, the MDV CVI988 genome was cloned into a BAC (Petherbridge *et al*, 2003). As with the BAC clone of the MDV-1 strain, 584Ap80C, the F' plasmid was inserted into the US2 region of the CVI988 Rispens virus by homologous recombination. The viruses rescued from the BAC clones displayed plaque morphology and growth kinetics indistinguishable from those of the parental virus, despite the presence of the BAC vector sequence (Petherbridge *et al*, 2003; Baigent *et al*, 2006). The BAC clones were stable during *in vitro* and *in vivo* passages in chicken embryo fibroblast (CEF) cells and they provided complete protection against the virulent RB1B MDV strain, demonstrating that MDV BAC clones could be developed as commercial vaccines (Petherbridge *et al*, 2003).

To date, real time PCR to distinguish the CVI988 Rispens from virulent challenge viruses has not been possible because sequences differences between them are very limited (Spatz *et al*, 2007; Baigent *et al*, 2010). This puts limitations on investigating the effect of the CVI988 vaccine on challenge virus replication. The development of the MDV CVI988 BAC, where the BAC vector sequence replaced the US2 gene has, however, allowed it to be used as a marker virus to develop and validate real time quantitative PCR assays to quantify and distinguish between CVI988 and virulent MDV. The ultimate goal is to develop a Q-PCR system that can distinguish commercial CVI988 from virulent strains (Baigent *et al*, 2010).

1.5.4. BACs: Vaccine Vectors

In addition to the study of herpesvirus genes and their role in virus growth and pathogenesis, BACs may also be used to develop novel vaccines. The large size of the herpesvirus genome, which can be manipulated easily using BAC technology, combined with the presence of virulence genes that are not essential for productive viral replication *in vitro* or *in vivo*, make herpesvirus BACs ideal candidates as potential vaccine vectors (Brun *et al*, 2008).

There are several examples of animal herpesvirus vector vaccines, which have utilised the BAC technology for the generation of viruses expressing heterologous antigens. A novel live attenuated vaccine, protecting against varicella-zoster virus (VZV) and mumps virus (MuV), was created by replacing the ORF 13 gene of the VZV Oka vaccine strain with the MuV haemagglutinin-neuraminidase (HN) gene using the BAC system. The inserted MuV HN gene was expressed using a CMV immediate early (IE) promoter. The recombinant virus displayed growth characteristics similar to the parental strain and vaccination of guinea pigs induced both VZV and HN-specific antibodies, producing a strong neutralising activity against VZV and MuV (Somboonthum *et al*, 2007). A BAC clone of the murine cytomegalovirus (mCMV) strain was used as a vaccine vector to express the self fertility antigen, murine zona pellucid 3, and rendered female mice infertile with a single inoculation (Redwood *et al*, 2005). The most commonly used BAC vaccine vector is the equine herpesvirus type I (EHV-1) BAC which, due to its ability to enter cells of various host origins (Trapp *et al*, 2005), has been developed as a delivery vector in various species, including non-equine hosts. It has been used to deliver the West Nile Virus (WNV) prM and E proteins, both viral envelope proteins and important determinants of virulence. The prM and E proteins were expressed under the transcriptional control of the CMV IE promoter. Horses immunized with the recombinant virus produced WNV E-protein specific antibodies (Rosas *et al*, 2007b). The equine herpesvirus BAC has also been used as a vector to deliver bovine viral diarrhoea virus (BVDV) structural proteins, inducing BVDV-specific neutralising antibodies and causing the reduction of levels of viraemia and nasal viral shedding in cattle after BVDV challenge infection (Rosas *et al*, 2007a). Finally, and

importantly for this study, recombinant EHV-1 BACs expressing influenza genes have been created. An EHV-1 BAC expressing the haemagglutinin, H3, under the control of the CMV IE promoter induced a robust immune response and caused a reduction of clinical signs and viral shedding in dogs (Rosas *et al*, 2008) and horses (Van de Walle *et al*, 2010).

As stated previously, the identification of a non-essential region, where the foreign gene may be inserted, is crucial to avoid severe growth defects in the virus (Brune *et al*, 2000; Warden *et al*, 2011). When introducing the MuV haemagglutinin-neuraminidase (HN) gene into the VZV BAC, the ORF 13 gene which codes for the thymidylate synthetase protein was replaced, as this was reported to be non-essential for virus replication *in vitro* (Cohen and Seidel, 1993; Somboonthum *et al*, 2007). The EHV-1 modified-live virus vaccine strain RacH, commonly used to vaccinate horses against EHV-1, was incorporated into a BAC by inserting the F' plasmid, combined with the gene encoding the green fluorescent protein (GFP), into the Orf 71 open reading frame. Despite the deletion of Orf 71, which encodes a major immunogenic glycoprotein (gp2), the recombinant virus was able to replicate to high titres and exhibited growth kinetics comparable to the attenuated strain RacH (Rosas *et al*, 2007a; Rosas *et al*, 2007b). The H3, WNV proteins and BVDV genes were all inserted into the Orf 71 open reading frame, replacing the GFP gene (Rosas *et al*, 2007a; Rosas *et al*, 2007b; Rosas *et al*, 2008). Finally, a second strain of EHV-1 (NY03) was produced where an H3 influenza gene was introduced into the Orf 1 open reading frame, as deletion of the Orf 1 gene attenuated EHV-1, causing shorter primary pyrexia and significantly reduced nasal shedding (Van de Walle *et al*, 2010; Hussey *et al*, 2011).

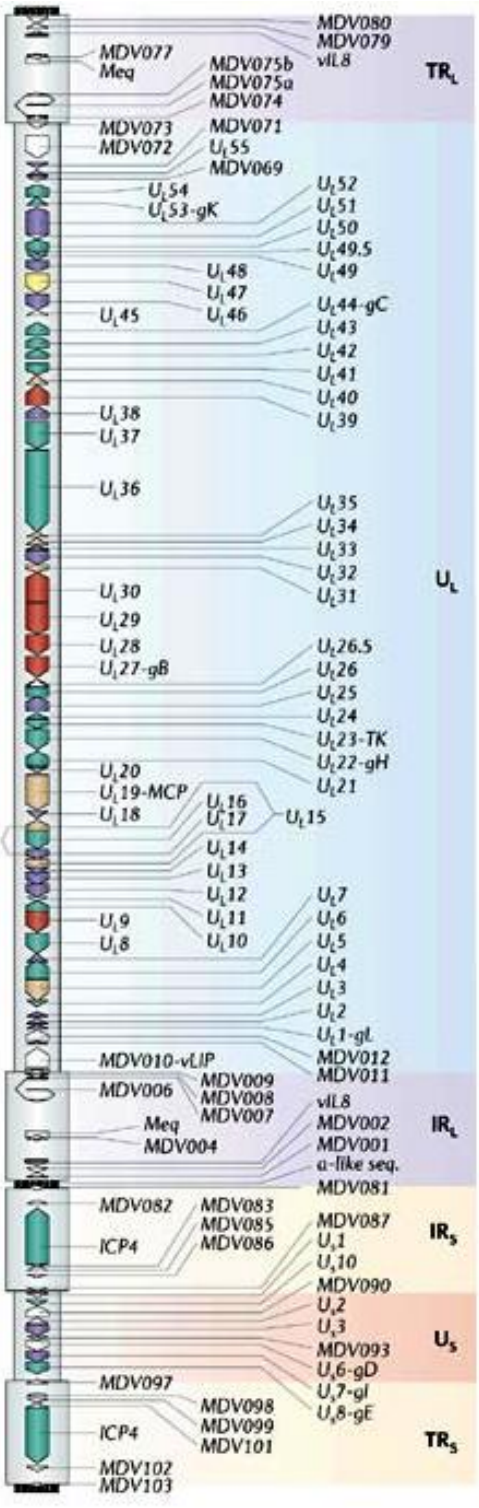
The use of MDV strains as vectors expressing foreign antigenic determinants could provide an efficient, safe, polyvalent vaccine against poultry diseases (Sakaguchi *et al*, 1993). MDV has several advantages as a recombinant vector because it has a natural host range limited to avian species and induces lifetime protection against MD (Sakaguchi *et al*, 1994). The first step in this process was to identify a non-essential gene in the MDV viral genome so that the influenza haemagglutinin gene could be inserted, creating the recombinant MDV-HA construct.

1.6. MDV CVI988 BAC: Non-essential genes

1.6.1. Non-essential genes

The MDV genome encodes more than 100 genes (Figure 7), the functions of which have been deduced on the basis of homology to genes from other well studied alphaherpesviruses such as HSV-1 (Chattoo *et al*, 2006). However, it is recognised that although some HSV-1 genes may be regarded as non-essential for virus propagation, the term non-essential only applies to one cell culture system. Homologous genes and their products, although non-essential in one cell type, may have critical functions in another (Parcells *et al*, 1994).

To gain an accurate understanding of the functions of MDV genes and their role in virus replication, deletion studies have been performed. As discussed previously, the deletion of both copies of the *Meq* gene from the MDV genome did not affect *in vitro* or *in vivo* viral replication but did cause attenuation of the virus (Lupiani *et al*, 2004). Similar studies have determined that the UL10 gene, which encodes the gM protein, and the UL49.5 gene that encodes an envelope/tegument protein, are both essential for MDV growth (Tischer *et al*, 2002). In a study to determine which genes were essential for the horizontal spread of the MDV virus from chicken to chicken, it was determined that the UL44 gene (Glycoprotein C) and UL13 (Protein Kinase) were both essential, however the US2 gene was dispensable (Jarosinski and Osterrieder, 2010). This corroborated previous work by Cantello *et al* (1993) who stably inserted the *lac*Z gene into the MDV US2 open reading frame, demonstrating that it was not essential for MDV growth in cell culture. And, as stated earlier, the CVI988 Rispens virus BAC clone was created by inserting the BAC vector sequence into the US2 region by homologous recombination, emphasising the fact that the US2 open reading frame was non-essential for MDV growth (Petherbridge *et al*, 2003).



TR_L – Terminal Repeat (long)
TR_S – Terminal Repeat (short)
IR_L – Internal Repeat (long)
IR_S – Internal Repeat (short)
U_L – Unique long region
U_S – Unique short region

Figure 7. Organisation of the MDV genome. Adapted with permission (Osterrieder *et al*, 2006).

The primary aim of this study was to incorporate an influenza haemagglutinin gene into the MDV CVI988 genome. The following genes were determined, using previously published research, to be non-essential for MDV replication and growth *in vitro* and *in vivo*.

1.6.2. UL41 Open Reading Frame

The UL41 open reading frame encodes the virion host shutoff (VHS) protein (Kwong and Frenkel, 1987; Kwong *et al*, 1988, Kwong and Frenkel, 1989; Gimeno and Silva, 2008). Common to alphaherpesviruses, the VHS protein is an endoribonuclease protein that degrades pre-existing and newly transcribed mRNAs in newly infected cells, reducing host protein synthesis (Read and Frenkel, 1983; Taddeo and Roizman, 2006; Gimeno and Silva, 2008). This process of reducing the host macromolecular metabolism precludes the host response to infection and diverts cell resources to the synthesis of viral proteins (Esclatine *et al*, 2004).

Read and Frenkel (1983) determined that the expression of the VHS protein in HSV-1 was not essential for virus replication in HEp-2 or Vero cells. In Marek's Disease Virus, using random transposon mutagenesis, the MDV Orf 54 (UL41) was not found to be essential for MDV replication *in vitro*. Following the mutation of this gene, the mutant showed evidence of virus replication in CEF cells, indicating that the VHS protein was dispensable (Chattoo *et al*, 2006). Gimeno and Silva (2008) confirmed that the MDV UL41 gene was non-essential by creating a UL41 deletion mutant that replicated as well as the parental strain *in vitro.* The pattern and degree of neurovirulence and tumour lesions produced by the deletion mutant *in vivo* was the same as the pattern of lesions induced by the parental virus. Deletion of the MDV UL41 gene did not decrease the replication of the virus in the lymphoid organs or in the FFE. The only observable difference between the parental MDV and the UL41 deletion mutant was that the early *in vivo* cytolytic infection was of a longer duration (Gimeno and Silva, 2008).

1.6.3. US10 Open Reading Frame

The US10 gene, located in the short unique region (Us) of the MDV genome, is a minor virion protein (Sakaguchi *et al*, 1993) that binds the stem cell lymphocyte antigen 6 complex (Spatz *et al*, 2007). The MDV US10 gene is homologous to the US10 open reading frame of HSV-1 (Sakaguchi *et al*, 1992) which encodes a polypeptide of 313 amino acids predicted to have a molecular mass of 33 kDa (McGeoch *et al*, 1985; Parcells *et al*, 1994; Yamada *et al*, 1997).

Jones *et al* (1991) determined that the gene product of a US10 homologue in human cytomegalovirus was not essential for virus replication and growth in tissue culture. Parcells *et al* (1994) replaced a 4.8 kb region of MDV that included US1, US10 and US2, as well three MDV-specific genes (Sorf1, Sorf2, Sorf3), with the *lac*Z gene, demonstrating that these genes were all non-essential for MDV growth in CEF cells. Sakaguchi *et al* (1994) determined that the US10 gene of MDV-1 was an effective site for the insertion of foreign genes from which to construct a polyvalent live vaccine for poultry. They incorporated the *E. coli lac*Z gene into the US10 open reading frame of the MDV-1 genome by homologous recombination. When tested *in vitro*, the recombinant virus replicated as well as the parental strain, indicating that the US10 gene was non-essential for viral growth in culture. *In vivo*, the deletion of this gene did not affect vaccine-induced immunity. Chickens immunized with the recombinant virus were protected when challenged with virulent MDV-1, producing a high level of antibodies against β-galactosidase as well as against MDV-1 antigens (Sakaguchi *et al*, 1994).

1.6.4. UL50 Open Reading Frame

The UL50 gene product is the ubiquitous enzyme dUTPase (deoxyuridine triphosphatase), which is required during deoxythymidine triphosphate (dTTP) synthesis and for the prevention of uracil-incorporation into DNA (Shlomai and Kornberg, 1978; Fuchs *et al*, 2000). This is significant as the presence of uracil in

DNA might result in premutagenic U:G mispairing unless removed and repaired (Shlomai and Kornberg, 1978; Krokan *et al*, 2002).

UL50 homologues have been studied in several other alphaherpesviruses including HSV-1, BHV-1, VZV and ILTV. In HSV-1, it is dispensable for normal virus replication *in vitro* (Fisher and Preston, 1986; Pyles *et al*, 1992), whereas *in vivo*, HSV-1 dUTPase deficient mutants were attenuated for neurovirulence and neuroinvasiveness and displayed reduced reactivation frequency (Pyles *et al*, 1992). In BHV-1, a dUTPase negative mutant was fully viable and therefore the dUTPase was not required for virus growth in cell culture (Liang *et al*, 1993), a finding that was later corroborated using random-insertion mutagenesis with a Tn5 transposition system to identify non-essential genes (Robinson *et al*, 2008). In VZV, deletion of the Orf 8 gene, encoding the viral dUTPase, had no effect on growth and syncytia formation *in vitro* (Ross *et al*, 1997). Fuchs *et al* (2000) determined that an ILTV UL50 deletion mutant propagated like wild-type ILTV in cell culture, demonstrating that the gene was non-essential for virus replication. A GFP-expressing UL50-deletion mutant of ILTV, however, showed reduced cell-to-cell spread *in vitro*, and was attenuated *in vivo*. To date, the impact of deleting the UL50 gene from the MDV has not been studied.

1.6.5. Construction of a live recombinant MDV vaccine expressing HA

Conventional MD vaccines stimulate both the B and T cell dependent immune systems, eliciting antibody and cell-mediated immune responses (Baaten *et al*, 2004). Kermani-Arab *et al* (1975) demonstrated that passive immunization with purified IgY, that possessed anti-MDV antibody activity, delayed the development of viraemia and lesions. Chickens vaccinated with a recombinant fowlpox vaccine expressing MDV gB elicited neutralising antibodies and were protected against virulent MDV (Nazerian *et al*, 1992). In addition, as MDV is strictly cell-associated in chickens, with the exception of the FFE, the cell-mediated immune response is important for protective immunity to MD (Markowski-Grimsrud and Schat, 2002).

CD8+ T cells, induced by MD vaccination, are essential for anti-virus activity (Morimura *et al*, 1998).

A recombinant MDV CVI988 virus has been developed that expresses the infectious bursal disease virus (IBDV) host protective antigen, VP2, providing protection against both pathogens (Tsukamoto *et al*, 1999). However, to date, MDV has not been used for the expression of influenza haemagglutinin. A live recombinant MDV CVI988 vaccine, expressing the HA protein, would provide humoral and cellular immunity against MDV as well as the chosen strain of haemagglutinin.

1.6.6. The 2A Polyprotein – expression of multiple HAs

The 2A polyprotein has been derived from the picornavirus family, which includes poliovirus and Foot and Mouth Disease Virus (FMDV) (Ryan *et al*, 1991). FMDV encodes all of its proteins within a single open reading frame, producing a long polyprotein which is co- and post-translationally processed into smaller products. Rather than use proteases, FMDV employs the 18 aa long, 2A polypeptide that mediates a co-translational cleavage at its own COOH-terminus by manipulating the ribosome into 'skipping' the synthesis of a specific peptide bond, producing a discontinuity in the peptide backbone. When the ribosome reaches the 2A sequence, it skips the synthesis of the glycyl-prolyl peptide bond at the COOH-terminus of 2A. Following the release of the protein, and without disruption, translation of downstream genes is re-initiated by the ribosome (Felipe and Ryan, 2004; Felipe *et al*, 2006; Felipe *et al*, 2010).

2A and 2A-like oligopeptide sequences are widely used in biotechnology to co-express heterogeneous proteins within the same cell (Amrani et al, 2004; Felipe *et al*, 2010). Multiple genes that encode proteins can be linked using the 2A sequence to form a single open reading frame (ORF). The translation of this single ORF results in the production of each protein, expressed as a discrete product, from a single transformation step (Felipe *et al*, 2006; Felipe *et al*, 2010). By linking HA genes with 2A, and incorporating the entire open reading frame into MDV, this system may be

used to induce heterosubtypic immunity to influenza, through the simultaneous and stable co-expression of multiple HA proteins within the same cell.

1.6.7. Project Aims

The primary objective in this study was to incorporate a haemagglutinin gene into the MDV viral genome with the intention of creating a novel virus vector that would induce protective immunity against avian influenza (AI) and Marek's Disease. By manipulating the MDV CVI988 BAC genome, HA genes and the GFP gene were inserted, replacing the identified non-essential genes. Subsequently, using *in vitro* analysis, CEF cells transfected with the MDV-HA and MDV-GFP constructs expressed detectable levels of HA and GFP proteins, determined by immunohistochemistry. In collaboration with the Istituto Zooprofilattico Sperimentale delle Venezie in Italy, the viral constructs will be tested *in vivo* to determine if they induce a protective level of antibody response in chickens to the H10 protein, while insuring adequate protection to MDV challenge.

Chapter 2: Materials and Methods

2.1. Molecular Techniques

2.1.1. Polymerase Chain Reaction

Polymerase Chain Reactions (PCR) were carried out using the reaction mixtures and thermocycler conditions listed in Appendix: PCR programs. High fidelity amplification of DNA was carried out using Phusion High Fidelity DNA Polymerase (Finnzyme, Finland). Reactions were set up using the supplied buffers and were made up to a final volume of 50 µl in a 0.2 ml thin-walled PCR tube (Applied Biosystems, UK). Taq DNA Polymerase (Invitrogen, UK) was used for colony PCR screening. When carrying out colony PCR, the 50 µl reaction mix was aliquoted into 10 µl quantities, and inoculated with the specific bacterial colony to be tested. Reactions were carried out in a PCR Thermal cycler (Applied Biosystems, UK). 10 mM dNTPs were supplied by Sigma, UK. Primers were supplied by MWG Biotech, Germany, and were made up to a concentration of 100 pmol/µl with stated volumes of dH_2O. All primers are listed in Appendix: PCR programs.

2.1.2. Cloning of PCR products

Once appropriately sized PCR products were successfully amplified and purified, they were cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, UK) according to the manufacturer's instructions. A 1 µl sample of the PCR product was mixed with 0.5 µl TOPO vector, 0.5 µl salt solution and 1 µl H_2O and incubated at room temperature (RT) for 30 minutes. Competent *Escherichia coli* (*E. coli*) were subsequently transformed with the ligation reaction (Section 2.2.2).

2.1.3. Purification of PCR products using Purification kit

PCR products were purified using the QIAquick PCR purification kit (Qiagen, UK). Five volumes of supplied Buffer PB were added to one volume of PCR sample and mixed by pipetting. The total sample was added to the supplied spin column and centrifuged at 18,000 x g for 1 minute to bind the DNA to the column membrane.

Flow through was discarded. 750 µl of supplied buffer PE were added to the column to wash the sample, which was centrifuged twice to ensure complete removal of the wash buffer. The column was placed in a labelled, fresh, 1.5 ml microcentrifuge tube and 30 µl EB buffer (10 mM Tris, pH 8.5) were added. After 10 minutes incubation, the DNA was eluted by centrifugation at 18,000 x g.

2.1.4. Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to analysis DNA. Samples up to 50 µl in volume were mixed with 5-10 µl Orange G dye (Section 2.5.2) and loaded into 0.7-1% (w/v) agarose gels containing 0.5 µg/ml ethidium bromide (EtBr) in Tris Acetic acid EDTA (TAE) (Section 2.5.2). Electrophoresis was carried out at 80 V/cm^2 in horizontal gel tanks containing TAE. Samples were compared with DNA ladders to estimate the size of DNA products (1 kb DNA ladder, Invitrogen, UK; 1 kb Plus DNA Ladder, Invitrogen, UK). Gels were visualised on a UV transilluminator and photographed.

Digested Bacterial Artificial Chromosome (BAC) DNA (2 µg) was analysed using agarose gel electrophoresis. Samples were loaded into a 0.8% (w/v) agarose gel and run slowly overnight at 40 V/cm^2. Agarose gels were post-stained with 0.5 µg/ml EtBr in TAE buffer and incubated on an orbital shaker for 1 hour. Following two 30 minute washes in TAE, the gel was visualised on a UV transilluminator and photographed.

2.1.5. Concentration of DNA by Ethanol Precipitation

DNA was concentrated by adding 0.1 volume of 3 M Sodium Acetate (NaAc) pH 5.2, and 3 volumes of ice-cold 100% ethanol. The sample was mixed by vortexing and incubated at -80^{o}C overnight or on dry ice in ethanol for 30 minutes. The DNA precipitate was pelleted by centrifugation at 18,000 x g in a bench top microcentrifuge for 30 minutes. The pellet was rinsed gently with 70% ethanol and

pelleted again by centrifugation at 18,000 x g for 10 minutes. The pellet was air-dried for 2-3 minutes and resuspended in an appropriate volume of EB buffer.

2.1.6. Restriction Enzyme Digestion of DNA

Restriction enzyme digestion was carried out using a broad range of restriction endonucleases, for the purposes of cloning and plasmid identification. Unless otherwise stated all restriction enzymes were supplied by New England Biolabs (NEB, UK). DNA was digested according to the manufacturer's instructions, using the recommended buffers and conditions. DNA digests were carried out with up to 5 µg of DNA per reaction, in a total volume of 20-100 µl, with no more than 1 µl of enzyme per 10 µl total volume to reduce the chances of star activity. Digests were incubated at the appropriate temperature for between 1-4 hours depending on the enzyme activity.

2.1.7. DNA Blunting

Digested plasmid DNA, with 5' overhangs, was prepared for blunt cloning using a Quick Blunting Kit (NEB, UK). The enzyme mix was used to convert the 5' overhangs to 5' phosphorylated, blunt-ended DNA. Digested plasmid DNA was incubated at room temperature with the Blunt Enzyme mix and dNTPs (0.1 mM final concentration), in the recommended buffer, for 30 minutes. The enzyme was heat inactivated at 70^{o}C for 10 minutes.

2.1.8. DNA De-phosphorylation

To remove 5' phosphate groups and prevent self ligation, plasmids digested with a single enzyme were incubated with Antarctic Phosphatase (AP) (New England Biolabs, UK), in the recommended buffer, for 1 hour. AP was inactivated by heating to 65^{o}C for 15 minutes.

2.1.9. DNA extraction from Agarose Gels

Prior to ligation, all plasmids and PCR products digested with restriction enzymes were gel purified. Gel electrophoresis was used to isolate the DNA fragments. Following separation, the area containing the DNA of interest was excised from the gel using a clean scalpel blade. DNA was recovered from the agarose gel using a Qiagen Gel Extraction Kit (Qiagen, UK), according to the manufacturer's protocol. The recovered gel slice was weighed and solubilised in the supplied Buffer QG, at a ratio of 3 µl per 1 mg. The sample was incubated at 50^{o}C, vortexed until the gel slice had completely dissolved, and then added to the supplied spin column. The column was centrifuged at 18,000 x g for 1 minute to bind the DNA and the flow through was discarded. Residual agarose was removed by washing the column with 500 µl Buffer QG. Again, the flow though was discarded following centrifugation at 18,000 x g. A second wash was carried out by adding 750 µl Buffer PE to the column and centrifuging as above. The flow through was discarded and the column was centrifuged again to remove residual wash buffer. The column was placed in a labelled, fresh, 1.5 ml microcentrifuge tube and 30 µl EB buffer were added. After 30 minutes incubation, the DNA was eluted by centrifugation at 18,000 x g.

2.1.10. DNA ligation

The Ligafast Rapid DNA Ligation system (Promega, UK) was used for all ligations. A restriction enzyme digested vector and insert were combined at a molar ratio of 2:1. T4 DNA ligase (1 µl) was added, along with 10x Ligase Buffer (1 µl). The sample was made up to a final volume of 10 µl with H_2O and incubated overnight, on thawing ice. 50 µl of competent *E. coli* were subsequently transformed with the ligation reaction (Section 2.2.2).

2.1.11. Quantification of Nucleic acid

The concentration of DNA and RNA was determined using the NanoDrop Nd-1000 UV/Vis 1 µl Spectrophotometer (Thermo Fisher Scientific, UK) at an absorbance reading of OD_{260nm} and OD_{280nm}.

2.1.12. Sequencing of Plasmid DNA

To sequence selected DNA, 1 µl plasmid sample (300-500 ng/µl) was mixed with 1 µl specific sequencing primers (3.2 pmol/µl), 1.5 µl 5x Big Dye Buffer and 1 µl Big Dye (Applied Biosystems, UK). Sequencing reactions were performed on a PCR thermal cycler using the recommended reaction conditions (96^{o}C, 10 sec; 50^{o}C, 5 sec; 60^{o}C, 2 minutes for 30 cycles). Sequencing was carried out by Genepool at the School of Biological Sciences Sequencing Service (SBSSS) (Ashworth Laboratory, Kings Buildings, University of Edinburgh).

2.1.13. Sequence Analysis and Primer Design

DNA sequences were analysed using BioEdit software and NCBI nucleotide-nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Vector NTI (Invitrogen, UK) was used for sequence analysis, plasmid construction, primer design and restriction digest mapping.

2.1.14. Extraction of RNA

RNA was extracted from cultured Chicken Embryo Fibroblast (CEF) cells that had been transfected with various Bacterial Artificial Chromosomes (BACs). Approximately 4 x 10^6 cells were used as starting material. Cells were scraped off and resuspended in 1 ml TRI reagent (Applied Biosystems, UK) and incubated for 5 minutes at room temperature. RNA was extracted by adding 200 µl chloroform per 1 ml TRI reagent solution. Sample was vortexed, incubated for 10 minutes at room temperature and centrifuged at 18,000 x g for 15 minutes at 4^{o}C to separate the

aqueous and organic phases. The RNA was isolated from the colourless, upper aqueous layer.

RNA was isolated using the RNeasy Mini Kit (Qiagen, UK). The aqueous layer, containing the extracted RNA, was mixed with 350 µl 70% ethanol by pipetting and added directly to the RNeasy mini column. All tubes and samples were kept on ice at 4^{o}C. Columns were centrifuged at 18,000 x g for 2 minutes, in a bench top centrifuge at 4^{o}C, to bind the RNA and the flow through was discarded. 350 µl of supplied buffer RW1 were added to the column, the column was centrifuged as above and the flow through was discarded. RNA samples were treated using the RNase-Free DNase set (Qiagen, UK). 10 µl DNase stock were added to 70 µl buffer RDD for every sample and mixed by gentle inversion. The total 80 µl mix was added to the column and incubated at room temperature for 15 minutes. Following DNase treatment, 350 µl of buffer RW1 were added to the column, which was centrifuged at 18,000 x g for 2 minutes. The column was transferred to a fresh 2 ml collection tube and washed twice using 500 µl RPE buffer, centrifuging and discarding the flow through as above. The column was transferred to a fresh 1.5 ml microcentrifuge tube. The RNA was eluted by adding 30 µl RNase-free H_2O and centrifuging as above.

2.1.15. Reverse transcription of RNA

Reverse transcription was performed using Superscript III reverse transcriptase (Invitrogen, UK). Extracted, DNase-treated RNA was mixed with 3 µl random primers (50 ng/µl) or 2 µl gene-specific primers (1 pmol/µl), 1 µl dNTP (10 mM) mix and made up to 12 µl with sterile, distilled H_2O. The sample was incubated at 65^{o}C for 5 minutes, rapidly chilled on ice for 1 minute and centrifuged briefly. 4 µl 5x First strand buffer, 1 µl 0.1 M Dithiothreitol (DTT), 1 µl RNase OUT and 1 µl Superscript III reverse transcriptase were added, mixed by pipetting and incubated at 25^{o}C for 5 minutes, followed by 50^{o}C for 60 minutes. If gene-specific primers were used, the reaction temperature was increased to 55^{o}C. The enzyme was inactivated by heating the samples to 70^{o}C for 15 minutes. cDNA samples were frozen at -20^{o}C.

2.2. Bacterial Techniques

2.2.1. Bacterial Culture

Laboratory strains of *E. coli* were grown in Luria (LB) medium (Section 2.5.4) or on LB plates with 1.5% (w/v) agar. The agar was melted and sterilised by autoclaving. Agar that contained additional sucrose was autoclaved using the sugar cycle. Following autoclaving, and once it had cooled to 50^{o}C, LB agar was supplemented with the appropriate antibiotic and poured into 10 cm diameter petri dishes. The antibiotics ampicillin (100 µg/ml), kanamycin (50 µg/ml), chloramphenicol (20 µg/ml) and gentamycin (15 µg/ml) were used (Sigma, UK). Bacteria were plated onto LB agar by streaking with a wire loop or spreading with a glass spreader and were incubated overnight, inverted, at 30^{o}C or 37^{o}C. Single colonies were used to inoculate sterile, liquid LB medium, which was also supplemented with the appropriate antibiotic. Liquid cultures were incubated overnight in an orbital shaker, shaking at 225 rpm, at 30^{o}C or 37^{o}C.

2.2.2. Transformation of TOP10 / NEB chemically competent *E. coli*

One-Shot TOP10 chemically competent *E. coli* (Invitrogen, UK) were used for routine cloning. Two strains of competent cells were purchased from NEB, UK. 10-beta competent *E. coli* (High Efficiency) were used for routine cloning. The dam^{-} /dcm^{-} competent *E. coli* strain was used for cloning plasmids that required digestion with methylation sensitive restriction enzymes.

Cells were transformed using aseptic bacteriological technique. Single aliquots (50 µl) were thawed on ice. 10 µl ligation reaction or 1 µl plasmid DNA (20-100 ng/µl) were added and the cells were incubated, on ice, for 30 minutes. The cells were then incubated at 42^{o}C for exactly 30 seconds (heat pulsed), and placed on ice for 2 minutes. 900 µl prewarmed SOC medium (Invitrogen, UK) were added and the samples were shaken in an orbital shaker at 225 rpm for 1 hour at 30^{o}C or 37^{o}C.

Subsequently, the cells were pelleted, resuspended in 100 µl and serially titrated out to 10^{-2} in 90 µl SOC medium. These titrations were plated onto labelled, prewarmed LB plates containing the appropriate antibiotic. Plates were inverted and incubated overnight at 30°C or 37°C.

2.2.3. Transformation of XL10-Gold Ultracompetent *E. coli*

XL10-Gold Ultracompetent *E. coli* (Stratagene, UK) cells were used for cloning large plasmids. Single aliquots (100 µl) were thawed on ice and gently added to pre-chilled 14 ml Falcon tubes. 4 µl of β-mercaptoethanol were added and the cells were incubated on ice for 10 minutes, swirling gently every two minutes. 10 µl ligation mixture were added and the cells were incubated on ice for 30 minutes. Following incubation, the cells were heat pulsed at 42°C for exactly 30 seconds and then placed on ice for 2 minutes. 900 µl of prewarmed SOC medium were added and the samples were shaken in an orbital shaker at 225 rpm for 1 hour at 37°C. Subsequently, the cells were pelleted, resuspended in 100 µl and serially titrated out to 10^{-2} in 90 µl SOC medium. These titrations were plated onto labelled, prewarmed LB plates containing the appropriate antibiotic. Plates were inverted and incubated overnight at 30°C or 37°C.

2.2.4. Preparation of Bacterial Stocks for Long Term Storage

Bacteria containing plasmids of interest were stored in glycerol at -80°C. A single bacterial colony was used to inoculate 3 ml of LB medium containing the appropriate antibiotic. Cultures were incubated overnight at 225 rpm in an orbital shaker at 37°C until the culture had an absorbance reading of 0.6 OD (Optical Density)$_{600}$. 850 µl of culture were mixed with 150 µl sterile glycerol in a 1.8 ml cryovial (Thermo Fisher Scientific, UK) and incubated on ice for 2 hours before storage at -80°C.

2.2.5. Plasmid DNA isolation from Bacteria (Small scale)

Plasmid DNA was extracted from transfected cultures using a Qiagen MiniPrep Kit (Qiagen, UK). A single bacterial colony, isolated from LB agar plates, was used to inoculate 7 ml of LB medium containing the appropriate antibiotic. Cultures were incubated overnight at 225 rpm in an orbital shaker at 30^{o}C or 37^{o}C.

After 16 hours, 3 ml of the bacterial overnight culture were pelleted by centrifugation at 6000 x g for 3 minutes at room temperature (15–25 °C). The bacterial pellet was resuspended in 250 µl Buffer P1 and transferred to a 1.5 µl microcentrifuge tube. 250 µl Buffer P2 was added to lyse the cells. This was mixed thoroughly by inverting the tube several times. The lysis reaction was stopped after 3 minutes by the addition of 350 µl Buffer N3, followed by gentle inversion. The sample was then centrifuged for 10 minutes at 18,000 x g in a bench top microcentrifuge to pellet the precipitate.

The supernatant was applied to the QIAprep spin column by pipetting. The column was subsequently centrifuged at 18,000 x g for 1 minute to bind the plasmid DNA to the silica gel membrane. The flow through was discarded and the sample was washed by adding 500 µl Buffer PB, followed by 750 µl Buffer PE. After each wash the column was centrifuged at 18,000 x g for 1 minute and the flow through was discarded. Following the addition of Buffer PE, the column was centrifuged twice to completely remove residual wash buffer. The QIAprep column was transferred to a clean, 1.5 ml microcentrifuge tube. To elute DNA, 50 µl Buffer EB were applied to the column and allowed to stand for 1 minute. The plasmid DNA was eluted by centrifugation at 18,000 x g for 1 minute and stored at -20^{o}C.

2.2.6. Plasmid DNA isolation from Bacteria (Large scale)

Large-scale isolation of plasmid DNA was carried out using the Qiagen Endofree Plasmid Maxi Kit (Qiagen, UK) according to the manufacturer's instructions. A single colony was isolated and inoculated into 3 ml LB medium supplemented with the appropriate selective antibiotic. This starter culture was incubated at 37^{o}C for 8 hours in an orbital shaker at 225 rpm. 200 µl of the starter culture were used to

inoculate 100 ml LB medium supplemented with the appropriate antibiotic. A large, sterile, 500 ml Erlenmeyer flask was used for suitable aeration. The large culture was incubated overnight at 225 rpm in an orbital shaker at 37^{o}C.

After 16 hours, using the Beckman J2-21 centrifuge prechilled to 4^{o}C, bacteria were pelleted by centrifugation at 6000 x g for 15 minutes at 4^{o}C. The bacterial pellet was resuspended in 10 ml Buffer P1, before being lysed by the addition of 10 ml Buffer P2 with vigorous inversion. The lysis reaction was stopped after 5 minutes by the addition of 10 ml Buffer P3, followed by vigorous inversion. The lysate was poured into the barrel of the QIAfilter Cartridge immediately and incubated at room temperature for 10 minutes, during which time the precipitate floated to the top of the solution. Using the supplied plunger, this lysate was gently filtered into a fresh 250 ml polypropylene centrifuge tube (Beckman Coulter, UK). 2.5 ml Buffer ER was then added to the filtered lysate, mixed by inverting the tube approximately 10 times, and incubated on ice for 30 minutes.

A QIAGEN-tip 500 was equilibrated by applying 10 ml of Buffer QBT, which was allowed to enter the silica resin by gravity flow. The filtered lysate was applied to the column and allowed to drain through the QIAGEN-tip by gravity flow. The flow through was discarded and the column was washed twice with 30 ml Buffer QC. Plasmid DNA was eluted using 15 ml of Buffer QF and collected in a sterile, 30 ml glass corex tube. DNA was precipitated by adding 10.5 ml room temperature isopropanol to the eluate, mixed by inversion and centrifuged immediately at 15,000 x g for 30 minutes at 4 °C. Following centrifugation, the supernatant was carefully decanted and the pelleted DNA was washed with 5 ml of endotoxin-free room temperature 70% ethanol. The sample was centrifuged again at 15,000 x g for 10 minutes, the supernatant was removed and the pellet was allowed to air dry. The pellet was redissolved in 500 µl Buffer TE (10 mM Tris·Cl, 1 mM EDTA, pH8.0) and stored at -20^{o}C.

2.2.7. BAC DNA isolation from Bacteria (Large scale)

Large-scale isolation of Bacterial Artificial Chromosome (BAC) DNA was carried out using the Qiagen Large-Construct Kit (Qiagen, UK) according to the manufacturer's instructions. A single colony was isolated and inoculated into 3 ml LB medium supplemented with the appropriate selective antibiotic. This starter culture was incubated at 37^{o}C for 8 hours in an orbital shaker at 225 rpm. 1 ml of the starter culture was used to inoculate 500 ml LB medium supplemented with the appropriate antibiotic. A large, sterile, 2000 ml Erlenmeyer flask was used for suitable aeration. The large culture was shaken overnight at 225 rpm in an orbital shaker at 37^{o}C.

After 16 hours, using the Beckman J2-21 centrifuge prechilled to 4^{o}C, bacteria were pelleted in 250 ml polypropylene centrifuge tubes by centrifugation at 6000 x g for 15 minutes at 4^{o}C. The bacterial pellet was resuspended in 20 ml Buffer P1, before being lysed by the addition of 20 ml Buffer P2 with gentle inversion. The lysis reaction was stopped after 5 minutes by the addition of 20 ml Buffer P3, followed by gentle inversion. The lysate was incubated on ice for 10 minutes before being mixed again by gentle inversion and centrifuged at 20,000 x g for 30 minutes at 4 °C. The supernatant was filtered into a 250 ml polypropylene centrifuge tube through folded filter paper prewetted with distilled water. BAC DNA was precipitated by adding 36 ml room temperature isopropanol to the cleared lysate. This was mixed gently and centrifuged immediately at 15,000 x g for 30 minutes at 4 °C. Subsequently, the supernatant was carefully decanted, the pellet was washed with 5 ml room-temperature 70% ethanol and centrifuged again at 15,000 x g for 15 minutes at 4^{o}C. The pellet was allowed to air dry for 2–3 minutes and dissolved in 9.5 ml Buffer EX by very gentle shaking. 200 µl ATP-Dependent Exonuclease and 300 µl ATP solution (100 mM, pH 7.5) were added to the dissolved DNA, mixed gently and incubated at 37^{o}C for 60 minutes.

A QIAGEN-tip 500 was equilibrated by applying 10 ml of Buffer QBT, which was allowed to enter the silica resin by gravity flow. 10 ml Buffer QS were added to the BAC DNA sample and the whole sample was applied to the QIAGEN-tip and

allowed to enter the resin by gravity flow. The flow through was discarded and the column was washed twice with 30 ml Buffer QC. BAC DNA was eluted using 15 ml of Buffer QF, prewarmed to 65 °C, and collected in a sterile, 30 ml glass corex tube. DNA was precipitated by adding 10.5 ml isopropanol to the eluate, mixing by inversion and centrifuged immediately at 15,000 x g for 30 minutes at 4 °C. Following centrifugation, the supernatant was carefully decanted and the pelleted DNA was washed with 5 ml of room-temperature 70% ethanol. The sample was centrifuged again at 15,000 x g for 10 minutes, the supernatant was removed and the pellet was allowed to air-dry. The pellet was redissolved in 250 µl TE Buffer at 55^{o}C for 1 hour and stored at 4^{o}C.

2.2.8. Preparation of Electrocompetent DH5-α cells

The genome of the Marek's Disease Virus (MDV) vaccine strain (MDV CVI988 Rispens) has been incorporated into a BAC by replacing the non-essential US2 gene with the 7.2 kb BAC vector. The MDV CVI988 BAC was transformed into DH5-α bacteria and stored as a glycerol stock which was acquired from Prof Nair (Institute for Animal Health, Compton, UK).

The glycerol stock of DH5-α/CVI988 was streaked onto a selective LB agar plate containing the appropriate antibiotic and was incubated overnight at 37^{o}C. A single bacterial colony, isolated from LB agar plates, was used to inoculate 5 ml of LB medium containing 20 µg/ml chloramphenicol and 0.1% L-arabinose (Sigma, UK). The culture was incubated overnight at 225 rpm in an orbital shaker at 37^{o}C.

The following day, 2.5 ml of the overnight culture were used to inoculate 20 ml LB medium containing 20 µg/ml chloramphenicol and 0.1% L-arabinose. The cultures were incubated for 3.5 hours at 225 rpm in an orbital shaker at 37^{o}C. Bacterial cells were pelleted at 1300 x g at 4^{o}C for 30 minutes. The supernatant was removed and the pellet, kept on ice throughout, was resuspended in 5 ml ice cold 10% glycerol in H_2O (1:9 v/v). Bacterial cells were pelleted at 1300 x g at 4^{o}C for 30 minutes. The supernatant was removed and the pellet, kept on ice throughout, was resuspended in

1 ml ice cold 10% glycerol in H_2O (1:9 v/v) and transferred to a prechilled 1.5 ml microcentrifuge tube. Bacterial cells were pelleted at 9000 x g at 4^oC for 2 minutes. The supernatant was removed and the pellet of competent DH5-α cells, kept on ice throughout, was resuspended in 400 µl ice cold 10% glycerol in H_2O (1:9 v/v).

2.2.9. BAC Mutagenesis

A recombination plasmid (shuttle plasmid) must be constructed first, where the mutation to be introduced is flanked by sequences (2-3 kb) homologous to the integration site. This alignment of similar sequences allows a crossover between the aligned DNA strands resulting in an exchange of material. The shuttle plasmids must be handled at 30^oC, due to the presence of a temperature sensitive origin of replication (repTS). It is, therefore, constructed first in the high copy vector, Zero Blunt II TOPO (Invitrogen, UK). The insert (Flank – Gene – Flank) was transferred to the shuttle plasmid (pST76k) in the final cloning step. The shuttle plasmid, pST76k, is kanamycin resistant and contains the recA gene and the SacB gene, which allows for selection in the presence of 5% sucrose.

Electrocompetent DH5-α cells (200 µl) were transferred to a 0.2 cm electroporation cuvette containing 1 µl of the shuttle plasmid DNA (5-20 ng/µl). Cells were transfected by electroporation with the Micro Pulsar (BioRad, UK) using the preset EC-1 program. Following electroporation, 1.5 ml SOC broth (37^oC) were added and the culture was transferred to a universal. The culture was incubated at 30^oC at 225 rpm in an orbital shaker for 90 minutes. Subsequently, the bacterial cells were pelleted, diluted to 10^{-2} in SOC broth and plated onto selective LB agar containing 20 µl/ml chloramphenicol and 50 µl/ml kanamycin. Plates were inverted and incubated for 48 hours at 30^oC. To identify co-integrates, 5 colonies were picked and spread onto selective LB agar plates containing 20 µl/ml chloramphenicol and 50 µl/ml kanamycin. Plates were inverted and incubated overnight at 43^oC to eliminate bacteria containing both uncombined plasmids but not the co-integrate. This selection is possible due to the presence of the repTS, which ensures that the shuttle plasmid cannot replicate at this temperature and therefore provide resistance to

kanamycin. To allow the temperature-resistant co-integrates to resolve, 5 colonies were picked and spread onto selective LB agar plates containing 20 µl/ml chloramphenicol. Plates were inverted and incubated for 48 hours at 30^{o}C. To identify clones with resolved BAC plasmids, 5 colonies were picked and spread onto selective LB agar plates containing 20 µl/ml chloramphenicol and 5% sucrose. Plates were inverted and incubated for 48 hours at 30^{o}C. To confirm resolution, 20 colonies were picked and inoculated onto a grid-labelled LB agar plate containing 50 µl/ml kanamycin and, in parallel, a grid-labelled LB agar plate containing 20 µl/ml chloramphenicol. Both plates were incubated at 37^{o}C overnight. 80-100% of the clones were expected to be kanamycin sensitive. For those that were, colony PCR incorporating specific primers (Appendix: PCR Programs) was used to characterise clones where the non-essential gene had been deleted or replaced by the gene of interest.

2.3. Tissue Culture and Virus growth

2.3.1. Growth of Established Cell lines

Vero cells (monkey kidney epithelial cells) were cultured in Dulbecco's Modified Essential Medium (DMEM) (Gibco, UK), supplemented with 10% (v/v) Foetal Calf Serum (FCS) (Harlan, UK) and 1% (v/v) Penicillin and Streptomycin (Invitrogen, UK). Cells were maintained in 175 cm^2 tissue culture flasks (Thermo Fisher Scientific, UK) and grown in a 37^{o}C incubator with 5% CO_2. Cell monolayers were split every three-four days. Confluent cells were rinsed with approximately 100 µl versene solution (0.2 g/L EDTA-4Na, Gibco, UK) per cm^2 of the cells. The versene was removed and the cells were incubated with approximately 30 µl trypsin/EDTA (Gibco, UK) per cm^2 of the cells until the cells had detached. The cells were resuspended in 10 ml DMEM medium and centrifuged for 5 minutes at 500 x g in a bench top centrifuge. The supernatant was discarded, the pellet was resuspended in 10 ml DMEM and live cells were counted using a haemocytometer (50 µl cell

suspension and 50 µl Trypan Blue Solution, Sigma, UK). Viable cell numbers were calculated using the following equation:

$$\text{Cells/ml} = \left(\frac{\text{cell count}}{\text{middle 25 squares (haemocytometer)}} \right) \text{ x dilution in trypan blue x } 10^4$$

Cells were used to seed six-well plates at a cell density of 4.0 x 10^5 cells per well and twelve-well plates containing sterile 10 mm glass coverslips (Scientific Laboratory Supplies, UK) at a cell density of 2.0 x 10^5 cells per well. In addition, 175 cm^2 flasks were seeded with approximately 5 x 10^6 cells in 40 ml DMEM media.

2.3.2. Preparation of Primary Chicken Embryo Fibroblast cells

Primary Chicken Embryo Fibroblast (CEF) cells were prepared from ten-day-old incubated eggs from the Roslin Institute. Embryos were euthanised by removing the head, and the wings and legs were excised. The remaining bodies were cut in half and placed in an Erlenmeyer flask containing 20 ml warm PBS (Section 2.5.1). The supernatant was removed and replaced with 20 ml trypsin/EDTA (Gibco, UK), which was incubated for 5 minutes at 37^{o}C and subsequently discarded. 20 ml of fresh trypsin were added and incubated for 10 minutes at 37^{o}C, shaken vigorously after 5 minutes. This was subsequently decanted into a universal tube containing 1 ml Foetal Calf Serum (FCS) (Harlan, UK) to deactivate the trypsin. This was repeated three more times. The four universals were centrifuged (8 minutes, 200 x g), the supernatant was removed and the cells were resuspended in 5% CEF media (See below). Cells were pooled and counted using a haemocytometer and 175 cm^2 flasks were seeded with approximately 1.6 x 10^7 cells in 40 ml 5% CEF Media. CEF cells were grown overnight in a 38.5^{o}C incubator with 5% CO_2. After twenty-four hours, once the cells were confluent, the media was changed to 2% CEF Media (See below) and again the flasks were incubated at 38.5^{o}C with 5% CO_2 overnight.

2.3.3. Growth of Primary Chicken Embryo Fibroblast cells

CEF cells were cultured in 2% or 5% CEF Media (M199, Gibco, UK) supplemented with 10% Tryptose Phosphate Broth (Invitrogen, UK), 7.5% $NaHCO_3$ (Sigma, UK), 2% or 5% Foetal Calf Serum (FCS) (Harlan, UK) and 1% Penicillin and Streptomycin (Invitrogen, UK). Cells were maintained in 75 cm^2 or 175 cm^2 tissue culture flasks, as well as grown in six-well dishes (Thermo Fisher Scientific, UK). CEFs were grown in a 38.5^oC incubator with 5% CO_2. Confluent cell monolayers were passed only once. As in Section 2.3.1, CEF cells were removed with trypsin/EDTA and counted using the haemocytometer. Cells were used to seed six-well plates at a cell density per well of 5.0 x 10^5 cells; 175 cm^2 flasks were seeded with approximately 1 x 10^7 cells in 40 ml 5% CEF media and 75 cm^2 flasks were seeded with 5 x 10^6 cells in 20 ml 5% CEF media.

2.3.4. Preparation of cells for long term storage

Confluent cell monolayers were removed and counted as described previously (Section 2.3.1). The cells were pelleted by centrifugation at 200 x g for 5 minutes and resuspended in 1 ml of freezing solution (90% v/v FCS, 10% v/v dimethyl sulphoxide). Vero cells were resuspended at a concentration of 5 x 10^6 cells per ml; CEF cells were resuspended at a concentration of 1 x 10^7 cells per ml. Cells were aliquoted into cryovials and frozen overnight at -80^oC, wrapped in cotton wool. Samples were transferred to liquid nitrogen the following day for long-term storage.

2.3.5. Growing cells from frozen stocks

All media were prewarmed to 37^oC. Cryovials were removed from liquid nitrogen and kept on dry ice until ready to thaw. Cells were thawed rapidly in a 37^oC water bath and transferred to 10 ml of the appropriate medium immediately. Cells were pelleted by centrifugation at 200 x g for 5 minutes, resuspended in 10 ml of fresh medium and transferred to a 75 cm^2 flask. Two cryovials of CEF cells were used to

seed one 175 cm^2 flask. Vero cells were grown in a 37°C incubator with 5% CO_2; CEF cells were grown in a 38.5°C incubator with 5% CO_2.

2.3.6. Transfection of Vero cells with plasmid DNA using Lipofectamine

The day prior to transfection, Twelve-well and six-well plates were seeded with 2.0 x 10^5 and 4.0 x 10^5 Vero cells per well, respectively. Seeded plates were incubated overnight at 37°C with 5% CO_2. Previously, the concentration of each plasmid was determined using the NanoDrop Nd-1000 UV/Vis 1 µl Spectrophotometer (Section 2.1.11). For each well of a six-well plate, 1 µg of plasmid DNA was made up to 100 µl in volume with serum-free Opti-mem media (Gibco, UK). For each well of a twelve-well plate, 0.5 µg of plasmid DNA was made up to 100 µl in volume with Opti-mem. Separately, 5 µl of lipofectamine were mixed with 95 µl of Opti-mem media. Both the DNA and lipofectamine samples were mixed and incubated for 45 minutes at room temperature, allowing complexes to form. During this incubation, media was removed from the Vero cells, which were subsequently rinsed with 2 ml sterile PBS. 800 µl of Opti-mem were added to the wells of the six-well dishes; 400 µl Opti-mem were added to the wells of the twelve-well dishes. The DNA/Lipofectamine solution was diluted in 600 µl of Opti-mem media and added carefully onto the cell sheet. For each well of the six-well dishes, 800 µl of plasmid DNA/lipofectamine mix were added; for each well of the twelve-well dishes, 400 µl of plasmid DNA/lipofectamine mix were added. Plates were incubated at 37°C with 5% CO_2. Media was changed from serum free Opti-mem media to DMEM containing 10% FCS after 6 hours.

2.3.7. Transfection of CEF cells with plasmid/BAC DNA using Lipofectamine

The day prior to transfection, six-well plates were seeded with 5.0 x 10^5 CEF cells per well. Seeded plates were incubated overnight at 38.5°C with 5% CO_2. For each well, 1 µg of BAC DNA or 0.5 µg of plasmid DNA were made up to 100 µl in

volume with serum-free Opti-mem media. Separately, 5 µl of lipofectamine were mixed with 95 µl of Opti-mem media. Both the DNA and lipofectamine samples were mixed and incubated for 45 minutes at room temperature, allowing complexes to form. During this incubation, media was removed from the CEF cells, which were subsequently rinsed with 2 ml sterile PBS. 800 µl of Opti-mem were added to the wells of the six-well dishes. The DNA:Lipofectamine solution was diluted in 600 µl of Opti-mem and the total 800 µl of plasmid/BAC DNA:lipofectamine mix was added to each well. Plates were incubated at 38.5^{o}C with 5% CO_2. Media was changed from serum free Opti-mem media to prewarmed 5% CEF Media after 5 hours.

2.3.8. 'Reverse' transfection of CEF cells with BAC DNA using Lipofectamine

The transfection of CEFs with MDV DNA is improved if DNA is introduced at the seeding stage, rather than once the CEF monolayer is formed (Morgan *et al*, 1990). This method was referred to as a 'Reverse' transfection.

For each well of a six-well dish, 1 µg of BAC DNA was made up to 100 µl in volume with serum-free Opti-mem media. Separately, 5 µl of lipofectamine were mixed with 95 µl of Opti-mem media. The DNA and lipofectamine samples were mixed and incubated for 45 minutes at room temperature. During this incubation, CEF cells were removed from the T175 cm^2 flask using trypsin/EDTA, resuspended in 10 ml of Opti-mem and counted, as previously described in Section 2.3.1. For each well, the DNA:Lipofectamine solution was diluted in 600 µl Opti-mem and the total 800 µl of plasmid/BAC DNA:lipofectamine was mixed with 5.0 x 10^5 CEF cells. The DNA:lipofectamine:CEF cell culture was made up to 3 ml with Opti-mem and added to one well. Seeded plates were incubated at 38.5^{o}C with 5% CO_2. Media was changed from serum-free Opti-mem media to prewarmed 5% CEF media after 5 hours. Media was changed to prewarmed 2% CEF media once cells were confluent.

2.3.9. Growth and storage of MDV Cell-Associated Viral stocks

Six-well dishes containing CEF cells transfected with MDV CVI988 BAC and mutant BACs, displayed cytopathic effect (CPE) in the form of plaques after 5-7 days. The entire contents of three wells of a six-well dish were removed using trypsin/EDTA as described previously in Section 2.3.1. The infected cells were resuspended in 10 ml of 5% CEF medium and centrifuged for 5 minutes at 200 x g. The supernatant was discarded and the pellet was resuspended in 10 ml prewarmed 5% CEF media.

One cryovial of CEF cells was removed from liquid nitrogen and kept on dry ice until ready to thaw. The cells were thawed rapidly in a 37^{o}C water bath, transferred to a universal containing 10 ml of prewarmed 5% CEF media and pelleted by centrifugation at 200 x g for 5 minutes. Pelleted cells were resuspended in 10 ml of fresh 5% CEF media and transferred to a labelled T75 cm^2 flask. 10 ml infected cells were transferred to the flask which was incubated at 38.5^{o}C with 5% CO_2.

Flasks were checked daily until extensive cytopathic effect was seen, approximately after 2-3 days. The infected cell monolayers were removed using trypsin/EDTA as described previously. The cells were resuspended in 10 ml 5% CEF media and pelleted by centrifugation at 200 x g for 5 minutes. Four cryovials of CEF cells were removed from liquid nitrogen and kept on dry ice until ready to thaw. Two cryovials of CEF cells were thawed rapidly in a 37^{o}C water bath and transferred to a universal containing 10 ml of prewarmed 5% CEF media. This was repeated and cells in both universals were pelleted by centrifugation at 200 x g for 5 minutes. Pelleted cells were resuspended in 10 ml of fresh 5% CEF media and transferred to a labelled T175 cm^2 flask. 5 ml of infected cells were transferred to each T175 cm^2 flask as well as 25 ml fresh, prewarmed 5% CEF media. Both T175 cm^2 flasks were incubated in a 38.5^{o}C incubator with 5% CO_2. Once extensive cytopathic effect was visible, the infected cell monolayers were removed using trypsin/EDTA as described previously and pelleted by centrifugation at 200 x g for 5 minutes.

Pelleted cells were resuspended in 2 ml of freezing solution (90% v/v FCS, 10% v/v dimethyl sulphoxide) and aliquoted into cryovials (200 µl/cryovial). All cryovials

were frozen overnight at -80^{o}C, wrapped in cotton wool. Samples were transferred to liquid nitrogen the following day for long term storage.

2.3.10. Plaque Assay

Plaque assays were used to determine the infectivity titre of the frozen stocks of cell associated MDV CVI988 and MDV mutant viruses. CEF cells were used to seed six-well plates at a density of 5.0 x 10^5 cells per well, and these were incubated at 38.5^{o}C with 5% CO_2 until confluent. For each construct, one 200 µl cryovial of infected CEF cells was removed from liquid nitrogen and kept on dry ice until ready to thaw. Infected CEF cells were thawed rapidly in a 37^{o}C water bath and, in duplicate, 100 µl was serially diluted six times in 900 µl of prewarmed 2% CEF media. The diluted cells, 10^{-1} to 10^{-6}, were pipetted onto a labelled well of the seeded 6 well dish. Plates were incubated at 38.5^{o}C with 5% CO_2 overnight. Media was changed to prewarmed 2% CEF media after 24 hours.

2.3.11. Immunohistochemistry of transfected Vero cells

Vero cells, transfected with GFP or DsRed plasmid constructs, did not require antibody staining. Media was pipetted from the well and the coverslip was gently rinsed twice with PBS. Transfected cells were fixed onto coverslips for 20 minutes using 4% (w/v) paraformaldehyde (BDH laboratories, UK) in PBS. Following fixing, coverslips were washed twice for 5 minutes using PBS and mounted onto microscope slides using Mowiol Mounting medium (EMD Biosciences, Germany). These were subsequently stored, wrapped in foil, at 4^{o}C until viewed using a confocal microscope.

2.3.12. Immunohistochemistry of CEF Plaques with antibody specific for MDV glycoprotein B (gB)

MDV-induced plaques can be visualised using the MDV-specific antibody, HB3 (Millipore, UK), to detect the virally expressed glycoprotein B (gB). Media from

cells displaying CPE in the form of plaques were removed and the cells were gently rinsed twice with PBS. Transfected cells were fixed using ice-cold acetone:methanol (1:1 v/v) for 2 minutes at room temperature. Following fixing, cells were washed twice for 5 minutes using PBS.

The cells were incubated for 1 hour in CAS Block (Invitrogen, UK). Blocking solution was pipetted off and the cells were incubated for 1 hour with HB3 (α-gB) antibody, diluted 1/100 in CAS block. Cells were washed three times for 5 minutes with PBS. The secondary antibody, horse radish peroxidase-conjugated rabbit anti-mouse IgG (Dako P0260), was diluted 1/200 in CAS Block and applied to the cells for 1 hour. Cells were washed three times for 5 minutes with PBS. The developing solution was prepared: 513 µl of 3-amino-9-ethylcarbazole (AEC) substrate buffer (0.1 M sodium acetate, pH 4.8), 27 µl of 3-amino-9-ethylcarbazole (AEC) stock (4 mg/ml^{-1}) and 9 µl Hydrogen Peroxide (H_2O_2), per well. The developing solution was added to the well and the plates were incubated at 38.5^oC until the red colour developed. After 1 hour, the developing solution was removed by washing the cells with dH_2O.

2.3.13. Immunohistochemistry of CEF Plaques with α-myc/HIS antibody

Using BAC mutagenesis to replace MDV genes with an influenza gene tagged with the Myc/His tag meant that plaques could be visualised using either an α-myc antibody (Clone 4A6, Millipore, UK) or an α-His antibody (Penta-His, Qiagen, UK). Media from cells displaying CPE in the form of plaques were removed and the cells were gently rinsed twice with PBS. Transfected cells were fixed for 20 minutes using 4% (w/v) paraformaldehyde in PBS and subsequently were washed twice for 5 minutes using PBS. Cells were incubated for 20 minutes with 0.3% Triton X-100 (t-Octylphenoxypolyethoxyethanol, Sigma, UK) in PBS to permeablise the cell membranes before being washed three times with PBS for 5 minutes.

Cells were subsequently incubated for 30 minutes in CAS Block. This was removed by pipetting and the cells were incubated for 2 hours with either α-myc or α-His antibody, diluted 1/50 (v/v) in CAS block. Cells were washed three times for 5 minutes with PBS. The secondary antibody, goat anti-mouse Alexa Fluor 488 conjugate (Invitrogen, UK), was diluted 1/500 in CAS block and applied to the cells for 1 hour. Cells were washed twice for 10 minutes with PBS. PBS was left on the cells, the plates were sealed and wrapped in foil at 4^{o}C until viewed and photographed.

Cells transfected with GFP constructs did not require antibody staining but were prepared, fixed and permeablised as described above.

2.4. Western Blots

2.4.1. Protein Extraction

Media from cells displaying CPE in the form of plaques was removed and the cells were gently rinsed twice with ice cold PBS. 250 µl ice cold lysis buffer (Section 2.5.3) containing 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (Pefabloc) (Fluka, UK) were added to the cells and incubated on a rocking platform at 4^{o}C for 1 hour. Lysed cells were transferred to a non-stick, 1.5 ml microcentrifuge tube (Ambion, UK) and centrifuged at 18,000 x g at 4^{o}C for 5 minutes to remove cell debris. The remaining supernatant, containing protein only, was divided into two aliquots and frozen at -80^{o}C. Immediately prior to protein quantification, a 1/100 dilution (v/v) of each protein sample in lysis buffer was prepared.

2.4.2. BCA protein quantification

Total protein was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, UK) which was used to determine protein concentration with reference to standards of the common protein, bovine serum albumin (BSA). A series of dilutions

of known concentrations are prepared from the protein and assayed alongside the unknown samples so that the concentration of each may be determined using a standard curve. Using the enhanced test tube protocol, the albumin standard (BSA) (2 mg/ml) was used to prepare a set of diluted standards (Table 2.1).

The BCA working reagent (WR) was prepared (2 ml per sample) by mixing 50 parts of BCA reagent A with 1 part BCA reagent B (50:1, Reagent A:B). 0.1 ml of each standard and unknown sample was mixed with 2 ml working reagent and incubated at 60^{o}C for 30 minutes. A spectrophotometer was set to 562 nm and, using a cuvette filled with H_2O, the instrument was zeroed. Once the samples had cooled to room temperature, the absorbance measurements of all tubes were made within 10 minutes of each other.

Table 2.1 Preparation of diluted albumin (BSA) standards

Vial	Volume of diluent	Volume and source of BSA	Final BSA concentration
A	700 µl	100 µl of stock	250 µg/ml
B	400 µl	400 µl of vial A Dilution	125 µg/ml
C	450 µl	300 µl of vial B Dilution	50 µg/ml
D	400 µl	400 µl of vial C Dilution	25 µg/ml
E	400 µl	100 µl of vial D Dilution	5 µg/ml
F	400 µl	0	0

The average 562 nm absorbance measurement of the blank standard was subtracted from the 562 nm absorbance measurement of all other individual standards and extracted protein samples. A standard curve was prepared by plotting the average blank-corrected 562 nm measurement for each BSA standard versus its concentration

in µg/ml. This standard curve was used to determine the protein concentration of each unknown, extracted protein sample.

2.4.3. Calculation of protein weight

The theoretical molecular weight of the proteins of interest was calculated using the Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of Bioinformatics (SIB).

2.4.4. Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE).

The gel plates were assembled with spacers and poured, first with a 10% acrylamide resolving gel (Section 2.5.3), followed by a stacking gel (Section 2.5.3). All gels were poured immediately after the addition of ammonium persulphate (AP) and N,N,N',N'-tetramethylethylenediamine (TEMED). Resolving gels were poured, overlayed with butan-2-ol and allowed to set for 30 minutes. The butan-2-ol was removed and the top surface of the gel was rinsed with stacking gel solution. The stacking gel was poured, the 10 well comb was added and the gel was allowed to set for 30 minutes. Once polymerisation of the gel was complete, the gels were assembled in a vertical gel tank with two gels per tank. The reservoir was filled with SDS running buffer (Section 2.5.3). The combs were removed and the wells were rinsed out with SDS running buffer to remove any unpolymerised acrylamide.

20 µg of each protein sample were mixed with 16 µl of SDS-PAGE boiling buffer (Section 2.5.3) by vortexing and heated at 95^{o}C for 5 minutes. Samples were loaded into the wells of the gel and run at 175 V/cm^{2} until the bromophenol blue dye front had reached the bottom of the gel. For comparative size estimation of proteins, the ColorPlus Prestained Protein Marker (NEB, UK) was used.

2.4.5. Tank blotting transfer of proteins to nitrocellulose membranes

Nitrocellulose membrane (Amersham Hybond-ECL, UK) was cut to the same size as the polyacrylamide gel (approximately 4.5 cm x 7.5 cm) and nine pieces of chromatography paper (Whatman, UK) were cut to a slightly larger size. Both the chromatography paper and membrane were soaked in tank blotting transfer buffer (Section 2.5.3) for 10 minutes prior to use. The tank blotting buffer contains a higher proportion of methanol and, as the buffer capacity is greater, it can be performed over extended periods (2-8 hours). This ensures more efficient transfer of proteins.

Three sheets of the chromatography paper were laid on an absorbent filter pad in a blotting cassette. The nitrocellulose membrane was laid on top of this, followed by the resolving gel. The remaining six sheets of chromatography paper were placed on the gel and a pipette was used to roll out any trapped air bubbles. Finally, a second absorbant filter pad was laid on top and the blocking cassette was transferred to the tank containing tank blotting transfer buffer. The voltage used was 6 mV/cm^2 of filter paper. Following blotting, the nitrocellulose membrane was carefully removed and washed twice in dH_2O and stained with Ponceau S solution (Section 2.5.3) for 1 minute. After a further two washes with dH_2O, staining revealed defined bands confirming even transfer of protein. Subsequently, the blot was incubated for 1 hour in freshly prepared 5% reconstituted dried milk in TBS (Section 2.5.3) to block non-specific binding sites.

2.4.6. Immunological Detection of Protein Blots

Primary antibodies used were specific for the myc (Millipore, UK), HIS (Qiagen, UK) and MDV gB (Millipore, UK).

The primary α-myc and α-HIS and α-gB antibodies were diluted 1:500 in TBS with 5% reconstituted dried milk. Membranes were incubated for 1.5 hours and subsequently washed twice with 0.05% Tween-20/TBS for 15 minutes. Membranes

were then incubated for 1 hour with the secondary biotinylated anti-mouse IgG (rabbit) antibody (Dako, UK), which was diluted 1:2,000 in TBS with 5% reconstituted dried milk. Membranes were washed twice with 0.05 % Tween-20/TBS for 15 minutes and rinsed with 5 changes of dH_2O. The tertiary stage used a Streptavidin Alkaline Phosphatase (AP) conjugate, diluted 1:500 in TBS with 5% reconstituted dried milk, which formed a biotin-avidin peroxidise complex with the secondary biotinylated antibody (Roche, UK). Following this, membranes were washed twice with 0.05% Tween-20/TBS for 15 minutes, twice with TBS for 15 minutes and rinsed with dH_2O.

Bound antibody was detected by incubating the membrane with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution (1 SIGMAFAST BCIP/NBT tablet dissolved in 10 ml dH_2O) until clear bands were visible. All antibody incubations and washes were performed on an orbital platform at room temperature.

2.5. Recipes

2.5.1. Commonly used Solutions

PBS

10 mM Sodium Phosphate
140 mM Sodium Chloride
pH 7.4

2.5.2. Nucleic Acid Electrophoresis

Tris-Acetate EDTA (TAE)

40 mM TRIS
20 mM Acetic Acid
1 mM EDTA
pH 8.0

Loading Buffer

15% (w/v) Ficoll

0.25% (w/v) Orange G

2.5.3. Protein Electrophoresis

TBS

10 mM Tris-HCL

150 mM Sodium Chloride

pH 7.5

Lysis buffer

50 mM Tris

250 mM Sodium Chloride

50 mM Sodium Fluoride

5 mM EDTA

1.25 mM 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (Pefabloc)

0.1% (v/v) Nonyl phenoxypolyethoxylethanol (Np40)

pH 7.4

SDS-PAGE boiling buffer

125 mM Tris (pH 6.7)

5% (w/v) Sodium Dodecyl Sulphate

25% (v/v) Glycerol

12.5% (v/v) β-mercaptoethanol

0.25% (w/v) Bromophenol Blue

Stacking gel

4% (w/v) Acrylamide/bis-Acrylamide (37.5:1)
125 mM Tris
0.1% (w/v) Ammonium Persulphate
0.1% (w/v) Sodium Dodecyl Sulphate
0.1% (v/v) N, N, N', N'- Tetramethylethylenediamine (TEMED)
pH 6.8

Resolving gel

10% (w/v) Acrylamide/bis-Acrylamide (37.5:1)
375 mM Tris
0.1% (w/v) Ammonium Persulphate
0.1% (w/v) Sodium Dodecyl Sulphate
0.1% (v/v) N, N, N', N'- Tetramethylethylenediamine (TEMED)
pH 8.8

SDS running buffer

100 mM Tris
38 mM Glycine
0.1% (w/v) Sodium Dodecyl Sulphate
pH 8.8

Tank blotting transfer buffer

25 mM Tris
150 mM Glycine
20% (v/v) Methanol
pH 8.3

Ponceau S

0.5% (w/v) Ponceau S
1% (v/v) Acetic Acid

2.5.4. Bacterial Media

Luria-Bertani (LB) broth

1% (w/v) Tryptone
0.5% (w/v) Yeast extract
1% (w/v) Sodium Chloride
pH 7.0

Chapter 3: The Construction and Characterisation of recombinant CVI988 Bacterial Artificial Chromosomes (BACs) expressing Haemagglutinin and GFP within the UL41 Open Reading Frame

3.1. MDV-HA BAC Construct

3.1.1. Aims and Approach

The ongoing threat of an influenza virus pandemic has highlighted the need to continually develop novel and improved vaccines as a successful control strategy. Vaccination of commercial poultry flocks against influenza could decrease the global levels of virus in circulation, thus managing the pandemic potential and help to preserve the poultry industry (Capua and Marangon, 2003). The aim of this research was to identify a viral vector that could deliver influenza antigens to poultry, eliciting both humoral and cellular immune responses. The viral vector chosen was the widely used vaccine for Marek's Disease (MD), a common but important herpesvirus-induced, lymphoproliferative disease of poultry (Gimeno, 2008). If influenza genes could be incorporated into the live attenuated Marek's Disease Virus (MDV) CVI988 vaccine genome, then dual vaccination would be possible, allowing simultaneous vaccination against both pathogens.

Vaccine-induced protection against avian influenza (AI) is the result of the humoral immune response targeted against the haemagglutinin (HA) protein and, to a lesser extent, the neuraminidase (NA) protein, both found on the surface of the influenza virus (Swayne and Kapczynski, 2008). The primary objective of this study, therefore, was to incorporate a HA gene into the MDV viral genome. The approach taken was to first establish the location of a non-essential gene in the MDV genome, using previously published research, which could be deleted with no effect, or at most slight attenuation, on the ability of the parental virus to replicate. The manipulation of the MDV CVI988 viral genome is possible due to the creation of an MDV CVI988 Bacterial Artificial Chromosome (BAC), which allows genes of interest to be inserted or deleted using mutagenesis (Petherbridge *et al*, 2003). This method has the additional advantage of avoiding unmapped and intragenic promoters, the disruption of which may have impacted on essential genes in the region. Once identified, the non-essential gene can be replaced by the influenza HA gene, creating MDV-HA BAC constructs. Subsequently, using *in vitro* analysis, Chicken Embryo Fibroblast (CEF) cells, transfected with the MDV-HA constructs, should express

detectable levels of both MDV and HA proteins, determined by immunohistochemistry and western blot techniques. HA genes may be inserted into the MDV genome in the 'forward' orientation (F), where genes are transcribed from left to right, or the 'reverse' orientation (R), where genes are transcribed from right to left. Left is closest to the UL1 gene in the MDV genome or the origin of replication in a plasmid.

To replace the non-essential gene with the influenza HA gene, a recombination plasmid based on the shuttle plasmid, pST76k, will be constructed, where the gene to be introduced (HA) is flanked by sequences (2-3 kb) homologous to the sequences flanking the gene to be replaced. This alignment of similar sequences will allow a crossover between the DNA strands resulting in an exchange of material (Figure 3.1). pST76k is kanamycin resistant and contains the RecA gene and the SacB gene, which allows for selection in the presence of 5% sucrose. The shuttle plasmid, pST76k, has a low copy number and should be propagated at 30^{o}C. Therefore, the insert (Flanking Sequence – Gene – Flanking Sequence) will first be constructed in the high copy vector, Zero Blunt II TOPO (Invitrogen, UK). This insert will be transferred to the shuttle plasmid (pST76k) in the final cloning step. The following sections describe the construction of this shuttle plasmid and the creation of the MDV-HA BAC construct.

3.1.2. Identification of the non-essential genes in MDV

Several genes were identified as non-essential in the MDV genome, based on previously published research. The UL41 open reading frame was the first to be targeted as a potential site for the insertion of the HA gene (Read and Frenkel, 1983; Chattoo *et al*, 2006; Gimeno and Silva, 2008). Gimeno and Silva (2008) determined that the MDV UL41 gene functioned as a viral host shut-off (VHS) protein. A UL41 deletion mutant replicated as well as the parental strain *in vitro*. *In vivo*, the pattern and degree of neurovirulence and tumour lesions produced by the deletion mutant was the same as the pattern of lesions induced by the parental virus. The only observable difference between the parental MDV and the UL41 deletion mutant was

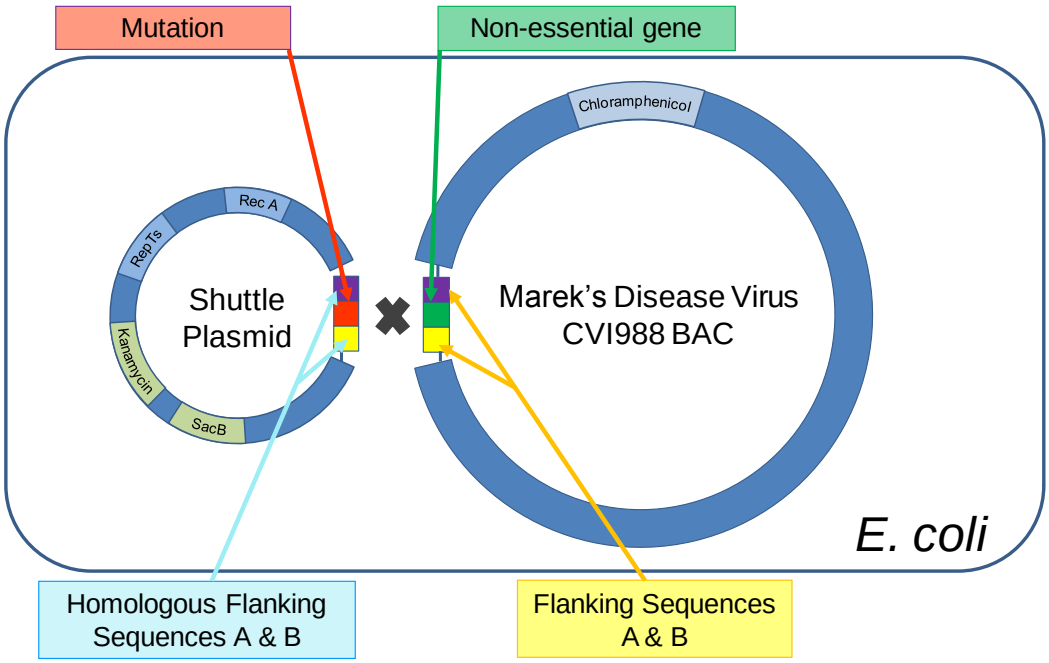


Figure 3.1 A diagrammatic representation of the shuttle plasmid and the MDV CVI988 BAC. The alignment of similar flanking sequences allows a crossover between the aligned DNA strands resulting in an exchange of material.

that the early *in vivo* cytolytic infection was of a longer duration. Subsequently, as it was concluded that the UL41 was non-essential, this open reading frame was targeted as the site for the insertion of the influenza HA gene.

3.1.3. MDV UL41 Flanking sequences

The MDV CVI988 BAC was isolated as described in Section 2.2.7. Primers were designed to amplify approximately 3 kb of the DNA sequence on either side of the UL41 gene (Appendix A.1.3.1). The unique restriction enzyme sites, *Kpn*I, *Pac*I and *Age*I, were incorporated into the primers to facilitate downstream cloning. The flanking sequences (FSA and FSB) were successfully amplified as described in Section 2.1.1 and Appendix A.1.4.1. These flanking sequences were gel purified according to the protocol in Section 2.1.9 and cloned into Zero Blunt II TOPO, as described in Section 2.1.2. TOP10 chemically competent *E. coli* cells were transformed (Section 2.2.2) with the ligated plasmids UL41pTOPFSA and UL41pTOPFSB (Figure 3.2), and plated onto Luria Bertani (LB) agar containing kanamycin (50 µg/ml). Plasmid DNA was isolated from small cultures (Section 2.2.5) and shown by restriction enzyme digest analysis (Section 2.1.6) to contain an insert of the correct size, in the correct orientation.

3.1.4. UL41pTOPFSAB

The plasmids, UL41pTOPFSA and UL41pTOPFSB, were digested with *Kpn*I and *Age*I, in preparation for cloning. The digested fragments were gel purified according to the protocol in Section 2.1.9. The purified FSB fragment was ligated into UL41pTOPFSA, as described in Section 2.1.10. Competent *E. coli* cells were transformed with the ligation mix (Section 2.2.2) and plasmid DNA was isolated (Section 2.2.5). The successfully ligated plasmid, UL41pTOPFSAB (Figure 3.2), was confirmed using restriction enzyme digestion with *Kpn*I and *Age*I, *Pac*I and *Age*I, and *Kpn*I and *Pac*I (Section 2.1.6).

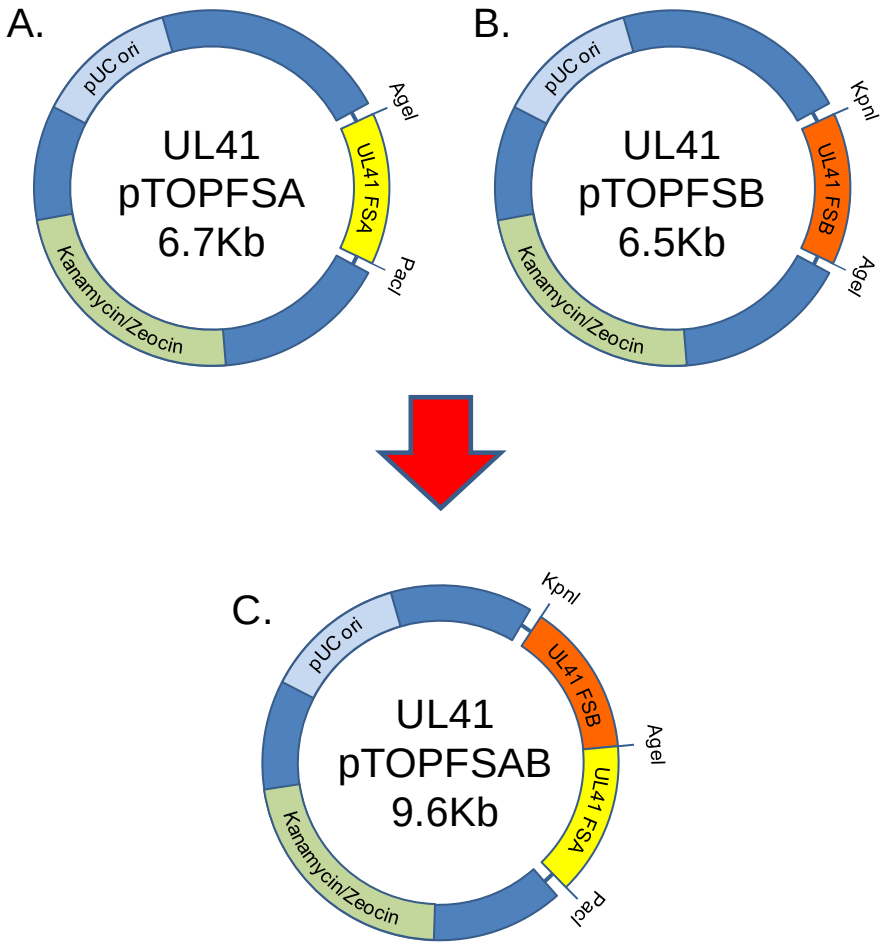


Figure 3.2 A) A diagrammatic representation of the created plasmid UL41pTOPFSA. B) A diagrammatic representation of the created plasmid UL41pTOPFSB. C) The 3.1 kb UL41FSB DNA fragment was ligated into UL41pTOPFSA to create UL41pTOPFSAB.

3.1.5. Digestion and phosphatase treatment of UL41pTOPFSAB

In preparation for cloning, the plasmid UL41pTOPFSAB was digested with *Age*I (Promega, UK) (Section 2.1.6). The digested plasmid was gel purified (Section 2.1.9) and concentrated using ethanol precipitation (Section 2.1.5). The digested plasmid DNA was treated with Antarctic Phosphatase (AP) to remove 5' phosphate groups and prevent self ligation upon ligation with the insert (Section 2.1.8).

3.1.6. H10 gene

In order to help facilitate future *in vivo* studies, it was decided that the HA gene from a low pathogenic (LP) strain of influenza virus should initially be incorporated into the MDV CVI988 genome. Any avian influenza virus with an intravenous pathogenicity index (IVPI) greater than 1.2 is classified as highly pathogenic (HP) (Lee and Saif, 2009). By locating an influenza strain with an IVPI with a value of 1.0, it was reasoned that any ill-health could be used as readout, and the location of facilities with suitable containment levels for viral challenge studies would be easier.

Dr Ilaria Capua, Director of the Virology Department and of the International Reference Laboratories at the Istituto Zooprofilattico Sperimentale delle Venezie in Italy, kindly supplied the genomic RNA of the strain: A/teal/Romania/4385/06 (H10N1). Dr Capua advised that the H10 influenza viruses had the closest available match to an IVPI of 1.0.

Using the gene-specific primer H10N1RT (Appendix A.1.3.1), 9 µl of the genomic RNA (0.7 ng/µl) were converted to cDNA by reverse transcription (Section 2.1.15).

Primers (H10N1For and H10Rev) that incorporated the unique restriction sites, *Xho*I and *Xba*I, were designed to amplify the H10 cDNA (Appendix A.1.3.1). The H10 DNA was successfully amplified as described in Section 2.1.1 and Appendix A.1.4.1. The amplified H10 DNA was purified using a QIAquick PCR purification kit (Section 2.1.3). In preparation for cloning, the purified H10 DNA was digested with *Xho*I *and Xba*I (Section 2.1.6) and gel purified according to the protocol in Section 2.1.9.

3.1.7. pCMVH10

To ensure a high level of expression, the H10 DNA was cloned downstream of the cytomegalovirus (CMV) immediate early (IE) promoter. This promoter was acquired from the plasmid, pEGFP-Nl, which contains the CMV promoter upstream of a Green Fluorescent Protein (GFP) gene (Figure 3.3). The GFP gene, flanked by the restriction sites, *Xho*I *and Xba*I, was removed by restriction enzyme digestion (Section 2.1.6). However, to digest this plasmid with the methylation sensitive *Xba*I enzyme, Dam^{-} competent cells were first transformed with pEGFP-Nl (Section 2.2.2). The unmethylated pEGFP-N1 plasmid DNA was isolated (Section 2.2.5) and, following digestion with *Xho*I *and Xba*I, the vector backbone was gel purified in preparation for cloning (Section 2.1.9).

The digested and purified H10 DNA fragment was ligated downstream of the CMV promoter in the vector backbone (Section 2.1.10). Following transformation of competent *E. coli* cells with the ligation mix (Section 2.2.2), and plasmid DNA isolation (Section 2.2.5), the successfully created plasmid, pCMVH10 (Figure 3.3), was confirmed using *Xho*I and *Ahd*I.

3.1.8. pTOPCMVH10

Primers (pCMVHA_For and pCMVHA_Rev; Appendix A.1.3.1) which both incorporated the unique restriction site, *Age*I, were designed to amplify the CMV-H10 DNA fragment so that this insert could be ligated between the flanking sequences in UL41pTOPFSAB (Figure 3.2). Using pCMVH10 as a template (20 ng), the CMV-H10 DNA was amplified (Section 2.1.1 and Appendix A.1.4.1), isolated by gel purification (Section 2.1.9) and cloned into Blunt II TOPO (Section 2.1.2) to create pTOPCMVH10 (Figure 3.3). Following the transformation of competent *E. coli* cells with the ligation mix (Section 2.2.2), and plasmid DNA isolation (Section 2.2.5), the isolated plasmid, pTOPCMVH10, containing the CMVH10 insert in the forward orientation, was identified by digestion with *Xho*I. Following confirmation, pTOPCMVH10 plasmid DNA was prepared according to the protocol in Section 2.2.6.

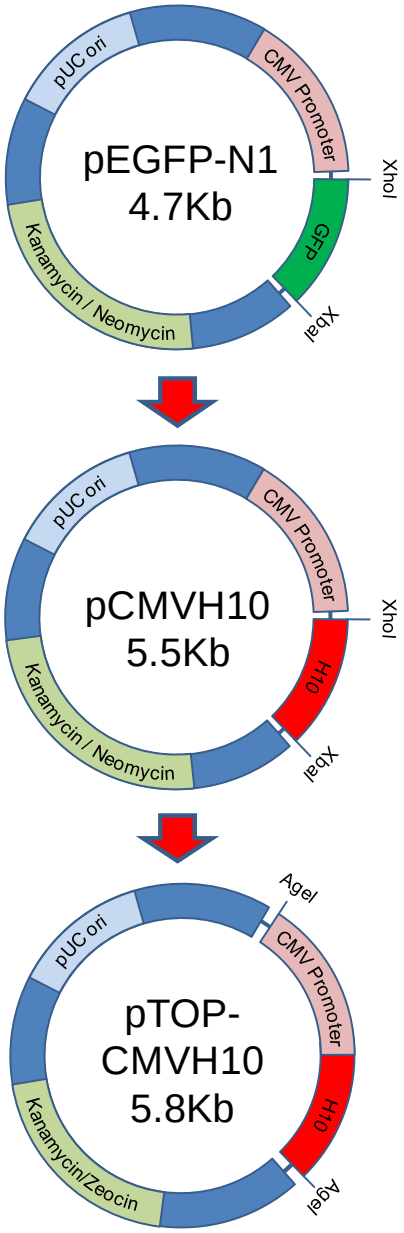


Figure 3.3 A) A diagrammatic representation the plasmid pEGFP-Nl. B) The H10 gene was cloned downstream of the CMV promoter to create pCMVH10. C) Using PCR, the CMVH10 DNA fragment was re-amplified with primers that both incorporated the *Age*I restriction enzyme site and cloned into Blunt II TOPO (Invitrogen, UK) to create pTOPCMVH10.

Primers were designed to sequence the CMV promoter and H10 gene (Appendix A.1.3.1). Sequence reactions were carried out using Big Dye (Section 2.1.12) and sequencing was performed by GenePool (University of Edinburgh). Sequence results indicated no errors in the CMV promoter or the downstream H10 gene with complete identity with the published sequences.

3.1.9. UL41pTOPFSABH10 (F)

The plasmid, pTOPCMVH10, was digested with *Age*I in preparation for cloning. The digested CMVH10 DNA fragment was gel purified according to the protocol in Section 2.1.9. As described in Section 2.1.10, the purified CMVH10 insert was ligated into the *Age*I digested and phosphatased UL41pTOPFSAB (Section 3.1.5) to create UL41pTOPFSABH10(F) (Figure 3.4). XL-Gold ultracompetent *E. coli* cells were transformed with the ligation reaction (Section 2.2.3) and plated onto LB agar plates containing 50 µg/ml kanamycin. For the purpose of colony PCR and the subsequent propagation of colonies identified that contained the correct plasmid, 20 colonies were inoculated onto a grid numbered (1-20) LB agar plate (50 µg/ml kanamycin) using a sterile pipette tip each time. The plate was incubated at 37^{o}C overnight. To identify bacteria that contained the plasmid, UL41pTOPFSABH10(F), colony PCR was carried out with the H10-specific primers, pCMVHAID_For and pCMVHAID_Rev, which detected the H10 insert in the forward orientation (Section 2.1.1 and Appendices A.1.3.1 and A.1.4.1). Three bacterial colonies tested positive for the plasmid, UL41pTOPFSABH10(F).

These three colonies were cultured overnight in LB agar containing kanamycin (50 µg/ml). Plasmid DNA was isolated from small cultures, as described in Section 2.2.5. The plasmids were digested with the restriction enzymes, *Nde*I, *Age*I and *Apa*I (Section 2.1.6), but only one sample contained a plasmid of the correct size and with the expected digest pattern. Following confirmation, UL41pTOPFSABH10(F) plasmid DNA was prepared according to the protocol in Section 2.2.6.

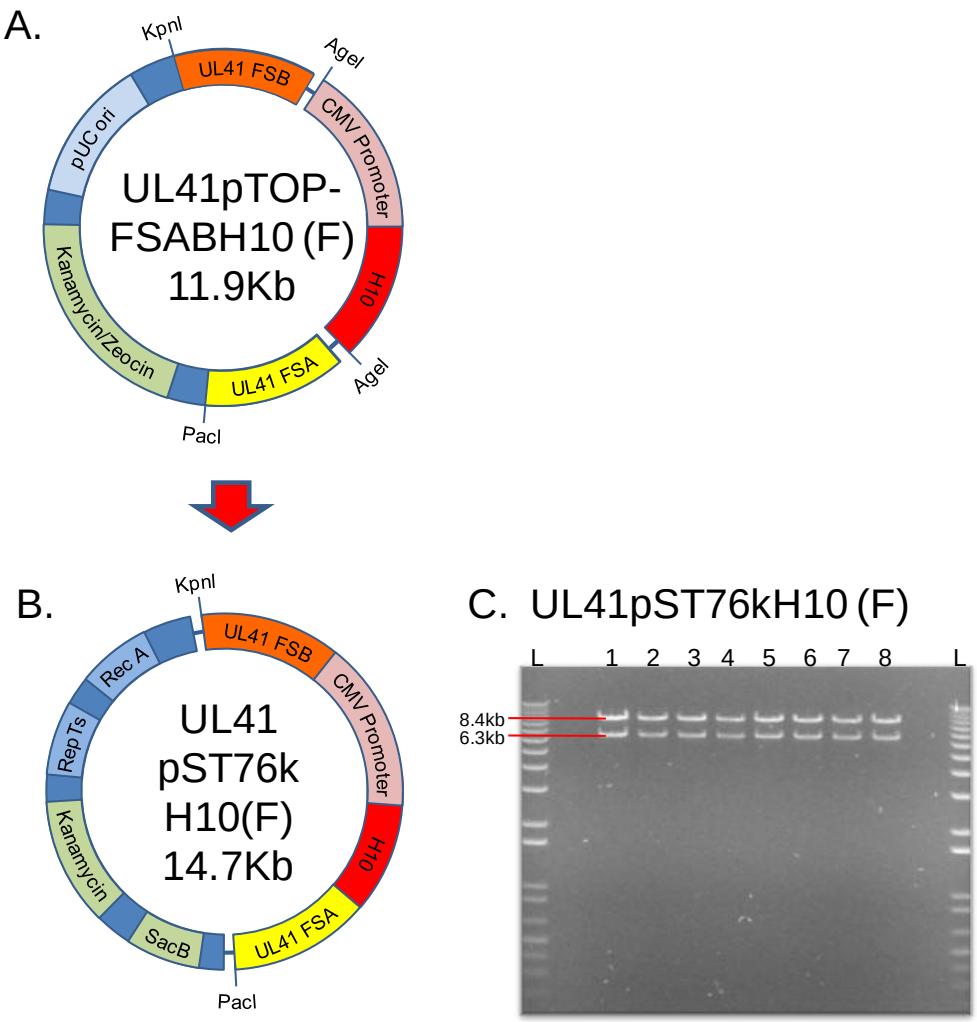


Figure 3.4 A) A diagrammatic representation of the created plasmid UL41pTOPFSABH10 (F). The CMV-H10 DNA fragment was cloned into the plasmid UL41pTOPFSAB, between the flanking sequences FSA and FSB. B) In the final cloning step, the FSABCMVH10 DNA fragment was transferred to the shuttle plasmid, pST76k, to create the plasmid UL41pST76kH10(F). C) A restriction enzyme digestion of eight isolated UL41pST76kH10(F) plasmids (No. 1-8), using the enzymes *Kpn*I and *Pac*I.

3.1.10. pST76k

The shuttle plasmid, pST76k, was kindly provided by Mr Ian Bennet as a glycerol stock which was streaked onto a LB agar plate containing 50 µg/ml kanamycin. The plate was incubated at 30^{o}C for 48 hours to allow the growth of single colonies. To ensure that the shuttle plasmid was sucrose sensitive, one kanamycin (50 µg/ml) LB agar plate and one kanamycin (50 µg/ml) with 5% sucrose LB agar plate was grid labelled with 6 squares. For each colony, a sterile pipette tip was used to inoculate a grid square with a single colony in both the kanamycin and kanamycin/sucrose agar plate. A colony that grew well on the kanamycin plate but that displayed complete sucrose sensitivity was isolated as described in Section 2.2.6.

In preparation for cloning, the plasmid pST76k was digested with the restriction enzymes, *Kpn*I *and Pac*I, and gel purified (Section 2.1.9). Following purification, the DNA concentration was determined, as described in Section 2.1.11.

3.1.11. UL41pST76kH10 (F)

The plasmid, UL41pTOPFSABH10(F), was digested with *Kpn*I and *Pac*I, in preparation for cloning. The digested FSABH10 fragment was gel purified (Section 2.1.9) and ligated into the digested and purified pST76k plasmid (Section 2.1.10) to create UL41pST76kH10(F) (Figure 3.4). Following transformation at 30^{o}C (Section 2.2.2), plasmid DNA was isolated from small cultures (Section 2.2.5). To confirm that the 6.3 kb plasmid, pST76k, contained the 8.4 kb FSABH10 DNA insert, eight plasmid DNA preparations were digested with *Kpn*I and *Pac*I. All colonies displayed the correct digest pattern (Figure 3.4).

3.1.12. BAC Mutagenesis using UL41pST76kH10(F)

BAC mutagenesis was performed, as described in Section 2.2.9. DH5-α bacteria, containing the MDV CVI988 BAC, were made competent according to the protocol in Section 2.2.8 and transformed with 10 ng of the shuttle plasmid,

UL41pST76kH10(F), by electroporation. Kanamycin-sensitive colonies containing UL41(Rep) BACs, where the UL41 gene had been replaced with H10, were identified using colony PCR (Section 2.1.1 and Appendix A.1.4.1). The primers, pCMVHAID_For and pCMVHAID_Rev (Appendix A.1.3.1), were used to identify the 2.3 kb CMVH10 DNA fragment. UL41pTOPFSABH10(F) plasmid DNA was included as a positive control; CVI988 BAC DNA acted as a negative control. Both the positive and negative control produced expected results. 6 colonies out of the 20 colonies tested were positive for the 2.3 kb H10 DNA sequence, indicating that the H10 gene had replaced the UL41 gene. A UL41(Rep) H10(F) BAC was selected and isolated, as described in Section 2.2.7.

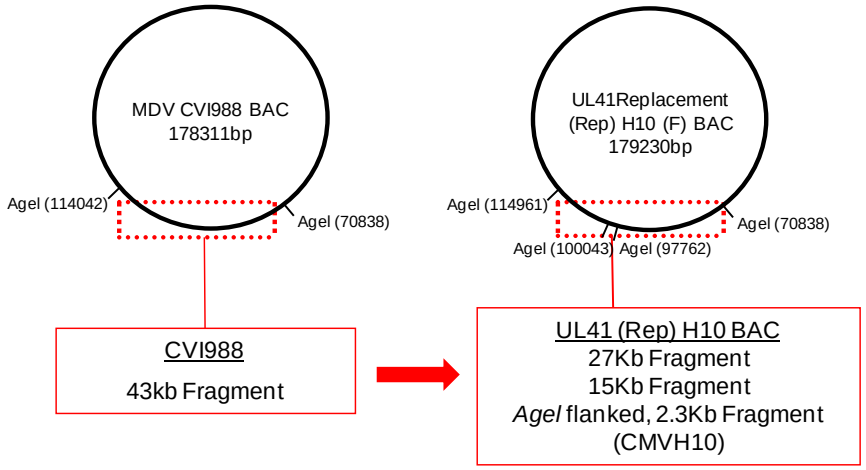
The CVI988 and UL41(Rep) H10(F) BAC were analysed by restriction enzyme digestion, using *Age*I (Section 2.1.6 and Section 2.1.4). The introduction of the *Age*I-flanked CMVH10 DNA fragment into the MDV CVI988 BAC genome altered the predicted restriction enzyme digestion pattern. The results clearly indicated the successful insertion of the 2.3 kb CMV-H10 DNA fragment into the MDV CVI988 genome (Figure 3.5).

3.1.13. *In vitro* Characterisation of UL41(Rep) H10(F) BAC

CEF cells, transfected with MDV CVI988 BAC DNA, develop focal cytopathic areas (CPE) as the highly cell-associated MDV virus spreads through the cell sheet by cell to cell contact (Churchill and Biggs, 1967) (Figure 3.6). These foci, also referred to as plaques, consist of rounded, highly refractile cells and appear after 7-10 days. They can be difficult to see by eye so to aid visualisation, plaques were stained red (Figure 3.6) using antibody specific for the MDV gB glycoprotein (HB3), combined with the specific developing solution, 3-amino-9-ethylcarbazole (AEC) (Section 2.3.12).

To determine if the UL41(Rep) H10(F) BAC DNA also induced the formation of plaques, CEF cells were transfected separately with CVI988 BAC DNA and UL41(Rep) H10(F) BAC DNA (Section 2.3.7). After 7 days, plaques were visible in CEF cells that had been transfected with CVI988 BAC DNA, with approximately 15

A.



B.

MDV CVI988 BAC	UL41 (Rep) H10 BAC
43204	26924
21601	21601
17062	17062
16098	16098
	14918
13923	13923
10282	10282
9560	9560
9560	9560
8830	8830
7690	7690
5482	5482
4305	4305
3599	3599
	2281 (CMVH10)
1776	1776
1776	1776
1633	1633
1414	1414
516	516

C.

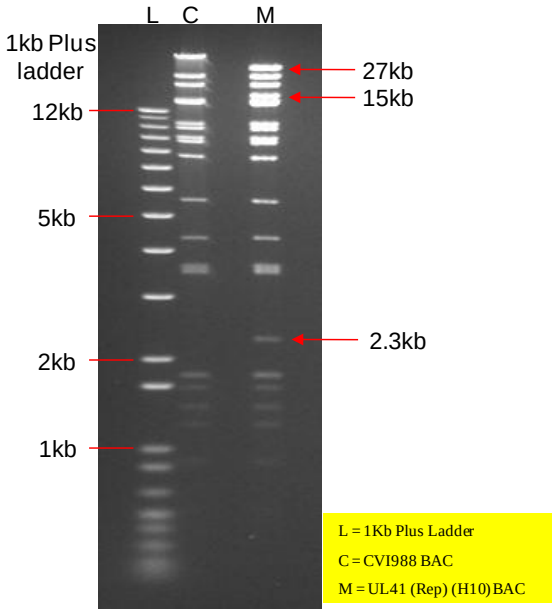
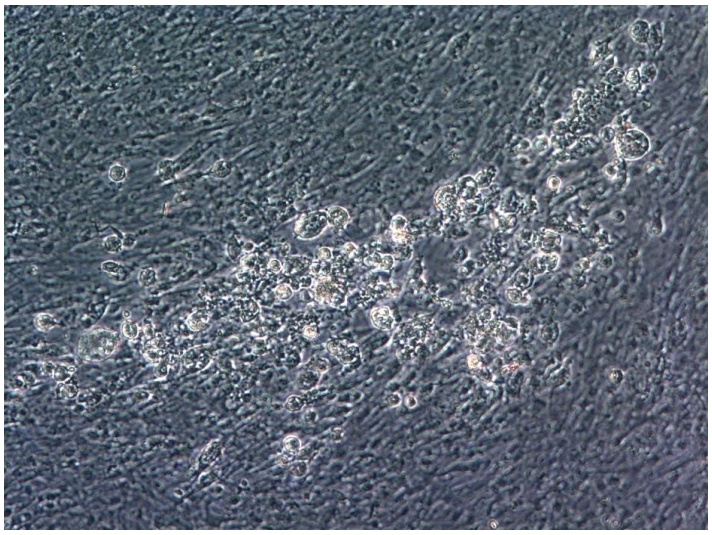


Figure 3.5 A) Predicted *Age*I sites in the area affected by mutagenesis (for clarity, other *Age*I sites are not shown). The changes caused by the introduction of the *Age*I flanked CMVH10 DNA fragment are highlighted. B) Table displaying the predicted digest fragments of the CVI988 and UL41 (Rep) H10 BAC following *Age*I enzyme digestion. C) A restriction enzyme digestion of the CVI988 and the UL41 (Rep) H10 BAC using the enzyme, *Age*I, clearly demonstrating the successful introduction of the 2.3 kb CMVH10 insert into the MDV CVI988 genome.

A.



B.

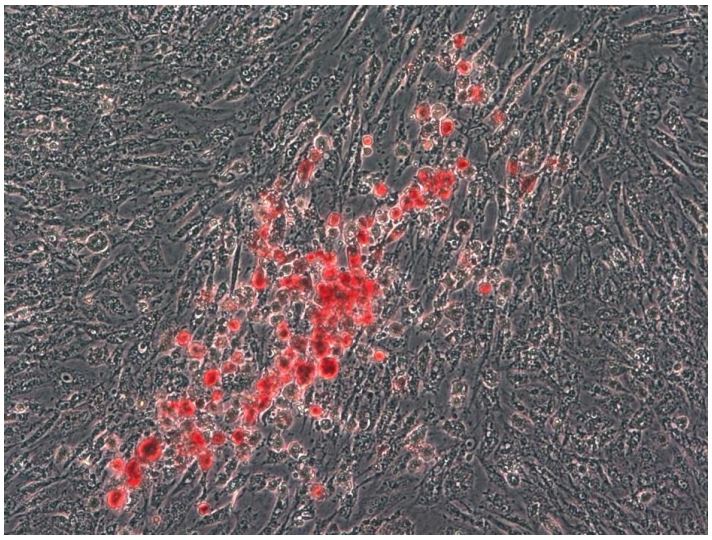


Figure 3.6 A) A photograph displaying a single plaque caused by the transfection of the MDV CVI988 BAC into Chicken Embryo Fibroblast (CEF) cells. B) Visualisation of a plaque using immunohistochemistry. The plaque was immunostained with 1^{o} HB3 antibody, specific for the MDV gB glycoprotein, 2^{o} Horseradish peroxidise-conjugated goat anti-mouse IgG and 3-amino-9-ethylcarbazole (AEC) developing solution.

15 plaques in each of the three wells. No plaques were visible in CEF cells that had been transfected with UL41(Rep) H10(F) BAC DNA. The BAC transfections were repeated, with similar results. Three wells of CEF cells were transfected with each BAC (Section 2.3.7). After 7 days, CVI988 BAC DNA transfection resulted in the formation of plaques (an average of 15 plaques per well) but UL41(Rep) H10(F) BAC DNA did not.

To ascertain if the BAC transfected CEF cells were expressing MDV-encoded mRNA or H10 mRNA, total RNA was extracted from unstained, transfected cells and isolated using an RNeasy Mini Kit (Section 2.1.14). 3 µg of the extracted, DNase-treated RNA was converted to complementary DNA (cDNA) by reverse transcription using random primers (Section 2.1.15).

3.1.14. CVI988/UL41(Rep) H10 BAC cDNA PCR

The cDNA created in Section 3.1.13 was tested by PCR using primers for H10, as well as two sets of MDV gene-specific primers (MDV UL18, vIL-8), kindly provided by Dr B. M. Dutia (Section 2.1.1 and Appendix A.1.3.1). Following PCR, the subsequent reactions were analysed using agarose gel electrophoresis on a 1% gel (Section 2.1.4). Results indicated that, as expected, the CVI988 BAC-transfected cells expressed MDV mRNA but not H10 mRNA. cDNA made from CEF cells transfected with the UL41(Rep) H10(F) BAC tested positive for H10 but only very weakly positive for the MDV genes (Figure 3.7). This indicated that the CEF cells were being successfully transfected with the UL41(Rep) H10(F) BAC and that the CMV promoter was driving H10 mRNA expression. However, when compared to the parental strain, MDV CVI988, the apparently weaker expression of the MDV genes (although the PCR was not quantitative), indicated that either the introduction of the H10 gene, or the deletion of UL41 gene, was having a disruptive effect on the ability of the MDV BAC to form plaques upon transfection.

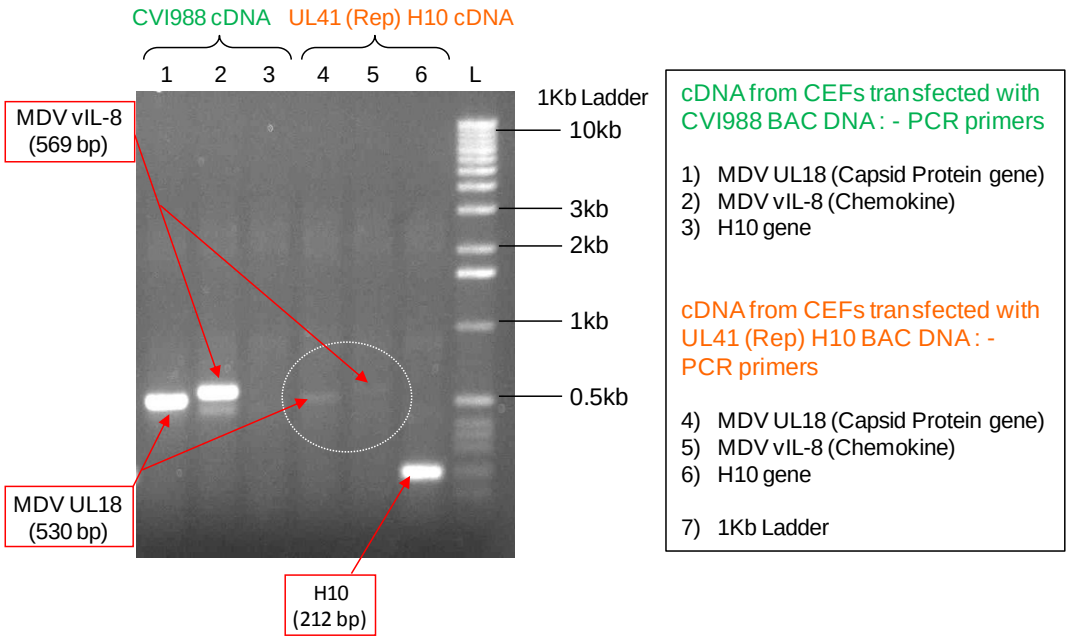


Figure 3.7 cDNA extracted from CEF cells transfected with CVI988 BAC DNA and UL41(Rep) H10 BAC DNA was tested using PCR with primers specific for the UL18, vIL-8 and the H10 gene.

3.1.15. UL41 Flanking sequences

Although the CMVH10 DNA insert had been fully sequenced (Section 3.1.8), the flanking sequences were not. It was speculated that if errors were present in the flanking sequences, they could be incorporated into the UL41(Rep) H10(F) BAC and subsequently disrupt an essential gene in the region. The MDV genes adjacent to the UL41 gene that were homologous to the flanking sequences were UL39 (Ribonucleotide reductase, large subunit), UL40 (Ribonucleotide reductase, small subunit), UL42 (DNA polymerase processivity factor), UL43 (Probable membrane protein) and UL44 (Virion Glycoprotein C) (Spatz *et al*, 2007). Sequencing primers were designed accordingly (Appendix A.1.3.1). Sequencing reactions were carried out (Section 2.1.12), using UL41pTOPFSABH10 DNA (Section 3.1.9) as a template. Sequencing results highlighted two issues. The first was that there was a point mutation in the FSB sequence, changing an amino acid of the UL40 gene from a histidine to a tyrosine, although the potential effect of this change is unknown. However, the UL40 open reading frame codes for the ribonucleotide reductase (small subunit) protein. Ribonucleotide reductase is a key enzyme in DNA synthesis reducing all four ribonucleotides to the corresponding deoxyribonucleotides (Reichard, 1988). Disruption of this gene, caused by the point mutation, may explain why the UL41(Rep) H10(F) BAC did not form CPE when transfected into CEF cells. The second issue was that there was an error in the design of the MareksFSB_Rev primer (Appendix A.1.3.1). Its position lay 17 base pairs upstream of the UL40 stop codon and therefore this would have been deleted in the subsequent UL41(Rep) H10 BAC. Without the stop codon, the signal to terminate transcription was lost and this may have affected downstream open reading frames. To resolve both of these issues, the FSB sequence was re-amplified and replaced in the plasmid, UL41pTOPFSAB. Consequently, the UL41(Rep) H10(F) BAC was recreated without these errors.

3.2. UL41(Rep) H10 BAC (Corrected)

3.2.1. Re-amplification of MDV Flanking Sequence B

Using MDV CVI988 BAC DNA (20 ng) as a template, the flanking sequence B (FSB) was successfully re-amplified as described in Section 2.1.1 and Appendix A.1.3.1. The amplified sequence was gel purified (Section 2.1.9) and cloned into Zero Blunt II TOPO (Section 2.1.2). Competent *E. coli* cells were transformed with the ligation mixture (Section 2.2.2) and plasmid DNA was isolated from small cultures (Section 2.2.5). The plasmid, UL41pTOPFSB (Figure 3.2), was shown by restriction enzyme digest analysis (*Kpn*I and *Age*I) to contain a product of correct size.

3.2.2. UL41pTOPFSAB (#2)

The plasmid, UL41pTOPFSB, was digested with *Kpn*I and *Age*I, and the FSB DNA fragment was gel purified in preparation for cloning (Section 2.1.9). The vector backbone, UL41pTOPFSA, was digested and purified previously (Section 3.1.4). The purified FSB fragment was ligated into UL41pTOPFSA (Section 2.1.10) and following transformation (Section 2.2.2), the plasmid DNA was isolated (Section 2.2.5). The successfully ligated plasmid, UL41pTOPFSAB (#2), was confirmed using restriction enzyme digestion with *Apa*I. UL41pTOPFSAB (#2) plasmid DNA was prepared according to the protocol in Section 2.2.6.

This plasmid, UL41pTOPFSAB (#2), was sequenced using pTOPFSABHA forward sequencing primers only (Appendix A.1.3.1). Sequencing results indicated that no mutations were present and that the UL40 gene was completely intact.

The plasmid, UL41pTOPFSAB (#2) was digested with *Age*I (Promega, UK) in preparation for cloning. The digested plasmid was gel purified (Section 2.1.9) and concentrated using ethanol precipitation (Section 2.1.5). The digested plasmid DNA was treated with AP (Section 2.1.8).

3.2.3. UL41pTOPFSABH10 (#2)

The plasmid pTOPCMVH10 was previously digested with *Age*I and purified in preparation for cloning (Section 3.1.9). This purified CMVH10 fragment was ligated into the *Age*I digested and AP treated UL41pTOPFSAB (#2) (Section 3.2.2). Competent *E. coli* cells were transformed with the ligation mixture according to the protocol in Section 2.2.3 and plated onto LB agar plates (50 µg/ml kanamycin). All plates and subsequent cultures were incubated at 30^{o}C to prevent recombination events occurring.

Following transformation, 21 colonies were inoculated onto a grid numbered (1-21) LB agar plate (50 µg/ml kanamycin) using a sterile pipette tip. To identify bacteria that contained the plasmid, UL41pTOPFSABH10 (#2), colony PCR was carried out (Section 2.1.1 and Appendix A.1.4.1). The primers, H10N1For and H10Rev (Appendix A.1.3.1) were used to identify the H10 DNA fragment, regardless of orientation. Uncorrected UL41pTOPFSABH10 plasmid DNA (20 ng) acted as a positive control (Section 3.1.9). Five bacterial colonies tested positive for the plasmid, UL41pTOPFSABH10 (#2).

As the insert was flanked by the same restriction site, *Age*I, it could be ligated into UL41pTOPFSAB in either the forward or reverse orientation. To determine the orientation of the CMVH10 insert, these five positive colonies were tested using orientation-specific primers (Section 2.1.1 and Appendix A.1.4.1). The primers pCMVHAID_For and pCMVHAID_Rev were used to detect the CMVH10 insert in the forwards orientation; the primers pCMVHAID_For and CMVHA[REV]ID_Rev were used to detect the CMVH10 insert in the reverse orientation (Appendices A.1.3.1 and A.1.4.1). All five colonies were tested using both sets of primers to ensure no colony was positive for both orientations. Uncorrected UL41pTOPFSABH10 plasmid DNA (20 ng) acted as a positive control (Section 3.1.9). In this plasmid, the H10 insert lies in the forward orientation, therefore, it was only positive for the forward orientation primers. Of the five H10 positive colonies, one plasmid was identified where the CMVH10 DNA fragment had been inserted in

the forward orientation, and four plasmids where it was found to be in the reverse orientation (Figure 3.8).

Two bacterial colonies containing the plasmids, UL41pTOPFSABH10(F) and UL41pTOPFSABH10(R) (Figure 3.8) were cultured overnight in LB agar containing kanamycin (50 µg/ml). Plasmid DNA was isolated according to the protocol in Section 2.2.5. The plasmids were analysed using restriction enzyme digestion with *Kpn*I and *Pac*I, *Apa*I, *Xho*I, *Eco*RI and *Age*I (Section 2.1.6). In both cases, the plasmids digested as expected, displaying bands of the appropriate sizes according to the orientation of the CMVH10 insert. The exception was the *Age*I enzyme digest which failed to digest the DNA in either plasmid, indicating that the *Age*I sites that flanked the CMVH10 insert may have been destroyed upon ligation.

To confirm the successful ligation of CMVH10 with the plasmid UL41pTOPFSAB, the two plasmids were sequenced over the ligation sites only. Both of the plasmids, UL41pTOPFSABH10 (F and R), were sequenced using the primers pTOPFSABHA _For5 and pTOPFSABHA_Rev9 (Appendices A.1.3.1 and A.1.4.1). Sequencing results indicated that in both plasmids, the CMVH10 DNA fragment had been correctly inserted between the flanking sequences. Sequencing also confirmed that, in both cases, the *Age*I sites had been destroyed but neither the MDV genes, nor the CMVH10 DNA, were affected by this.

3.2.4. pST76kH10 (Forward and Reverse)

The plasmids, UL41pTOPFSABH10(F) and UL41pTOPFSABH10(R), were digested with *Kpn*I and *Pac*I in preparation for cloning with the shuttle plasmid, pST76k. The digested FSABH10 fragments were gel purified (Section 2.1.9) and ligated into the digested and purified pST76k plasmid (Section 3.1.10) to create the plasmids UL41pST76kH10(F) and UL41pST76kH10(R) (Figure 3.8). Competent *E. coli* cells were transformed with each ligation reaction (Section 2.2.2) and plasmid DNA was isolated (Section 2.2.5). The plasmids were digested with the restriction enzymes, *Kpn*I and *Pac*I, to confirm that the ligation was successful. All plasmids gave the correct digest pattern.

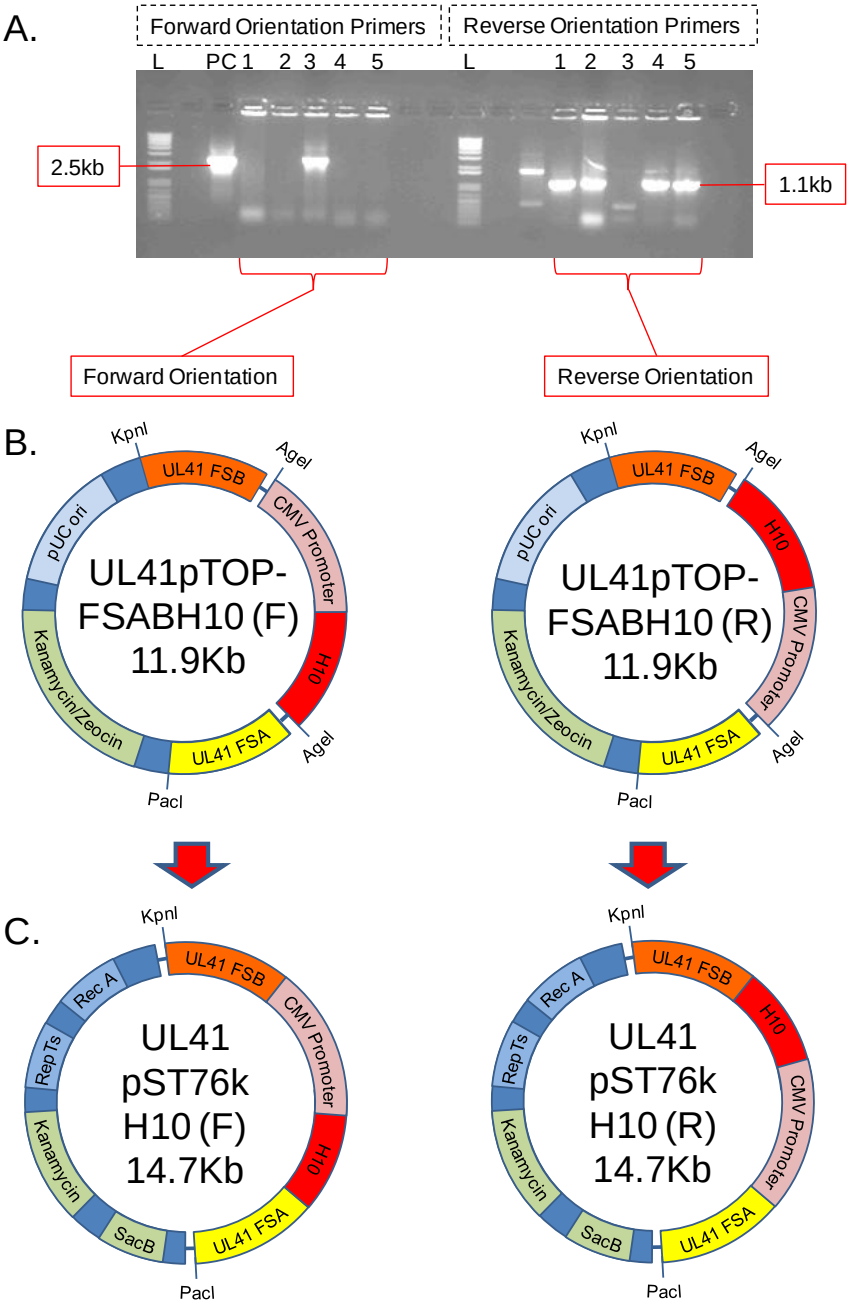


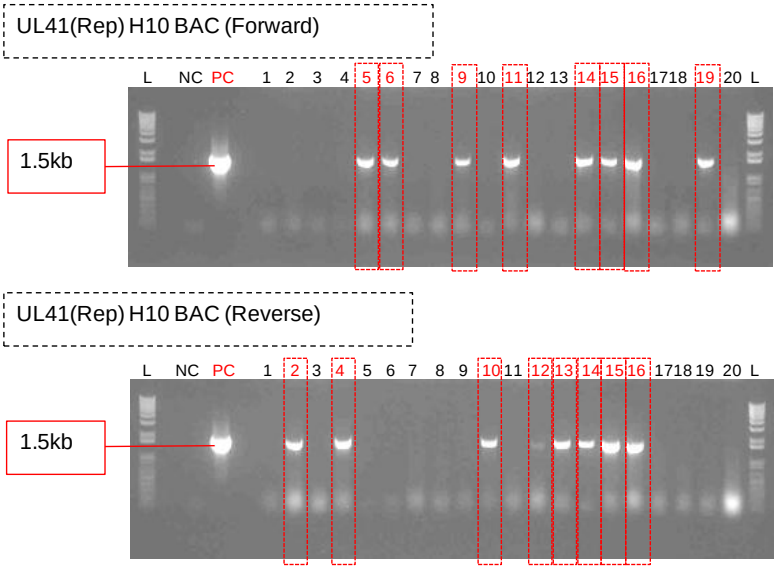
Figure 3.8 A) Agarose gel electrophoresis showing positive results from an orientation specific PCR designed to detect the CMVH10 DNA insert in the plasmid pTOPFSABH10 (Forward and Reverse). B) A diagrammatic representation of the plasmids UL41pTOPFSABH10 (Forward and Reverse). The CMV-H10 DNA fragment was cloned into the plasmid UL41pTOPFSAB, between the flanking sequences FSA and FSB. C) In the final cloning step, the FSABCMVH10 DNA fragments were transferred to the shuttle plasmid, pST76k, to create the plasmids UL41pST76kH10 (Forward and Reverse).

3.2.5. BAC Mutagenesis using the plasmids UL41pST76kH10(F) and UL41pST76kH10(R)

BAC mutagenesis was performed successfully for both plasmids (Section 2.2.9). DH5-α bacteria containing the MDV CVI988 BAC were made competent (Section 2.2.8) and, using electroporation, were transformed with 10 ng of the shuttle plasmids, UL41pST76kH10(F) and UL41pST76kH10(R). Kanamycin-sensitive colonies containing UL41(Rep) H10 BACs, where the UL41 gene had been replaced with the CMVH10 DNA fragment, were identified using colony PCR (Section 2.1.1 and Appendix A.1.4.1). The primers, H10N1For and H10Rev (Appendix A.1.3.1) were used to identify the 1.5 kb H10 gene, regardless of orientation. UL41pTOPFSABH10 plasmid DNA (20 ng) was included as a positive control; CVI988 BAC DNA (20 ng) acted as a negative control. Both the positive and negative control produced expected results. BAC mutagenesis that had utilised the UL41pST76kH10(F) shuttle plasmid yielded eight colonies that were positive for the H10 DNA insert. BAC mutagenesis carried out using the UL41pST76kH10(R) shuttle plasmid, also produced eight colonies positive for the H10 DNA insert (Figure 3.9). A UL41(Rep) H10(F) BAC and a UL41(Rep) H10(R) BAC colony was selected and isolated (Section 2.2.7).

The CVI988 BAC, and one of each of the UL41(Rep) H10 BACs (Forward and Reverse), were analysed by restriction enzyme digestion using *Xho*I (Section 2.1.6 and Section 2.1.4). *Age*I was used previously (Section 3.1.12) but the *Age*I sites flanking the CMV-H10 were destroyed so the results of that digest would have been ambiguous. The results indicated that the H10 gene had been successfully inserted into the MDV CVI988 genome, in the forward and reverse orientation, producing the UL41(Rep) H10(F) BAC and UL41(Rep) H10(R) BAC (Figure 3.9).

A.



B.

MDV CVI988 BAC	UL41(Rep) H10 BAC (For)	UL41(Rep) H10 BAC (Rev)
17000	17000	17000
15860	15860	15860
12087	12087	12087
9596	9596	9596
9286	9286	9286
9161	-	-
9145	9145	9145
9143	9143	9143
9102	9102	9102
8245	8245	8245
-	-	7526
-	6614	-
5276	5276	5276
5102	5102	5102
4638	4638	4638
4562	4562	4562
4060	4060	4060
3995	3995	3995
3990	3990	3990
3798	3798	3798
-	3641	-
2843	2843	2843
2825	2825	2825
-	-	2703
2436	2436	2436
2301	2301	2301

C.

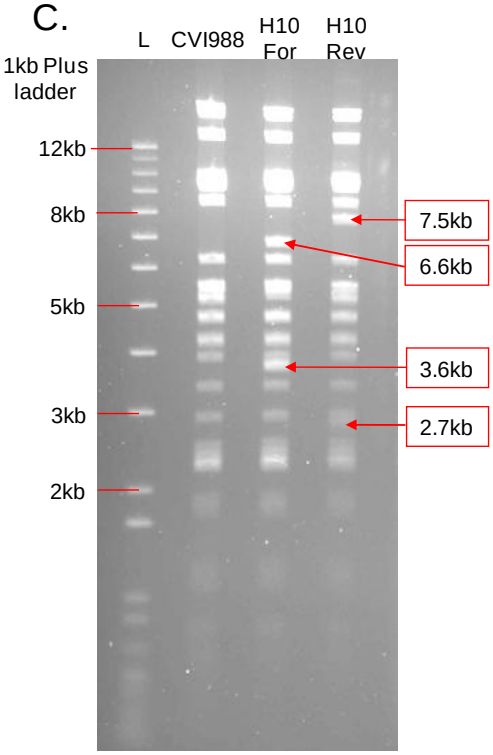


Figure 3.9 A) Agarose gel electrophoresis showing positive results from a colony PCR experiment designed to detect the H10 gene in the UL41(Rep) H10 BACs (Forward and Reverse). B) Table displaying the predicted digest fragments of the CVI988 BAC and UL41(Rep) H10 BACs following *Xho*I enzyme digestion. (DNA fragments of the same size and smaller than 2301 bp were not shown) C) A restriction enzyme digestion of the CVI988 BAC and the UL41(Rep) H10 BACs using *Xho*I.

3.2.6. *In vitro* Characterisation of UL41(Rep) H10 BAC (Forward and Reverse)

CEF cells were transfected with the CVI988 BAC and the UL41(Rep) H10 BACs (Forward and Reverse), as described in Section 2.3.7. Three wells of a six-well dish were transfected for each BAC. After 7 days, 10-15 plaques were visible in each of the three wells that had been transfected with MDV CVI988 BAC DNA. No plaques were visible in CEF cells transfected with either the UL41(Rep) H10(F) BAC or UL41(Rep) H10(R) BAC DNA.

3.2.7. Summary

In vitro analysis demonstrated that the CVI988 BAC DNA induced focal CPE in the form of plaques when transfected into CEF cells. The UL41(Rep) H10 (F and R) BACs, where the H10 gene had replaced the UL41 gene, did not form plaques in CEF cells. It was shown that UL41(Rep) H10(F) BAC transfected cells were expressing H10 mRNA and weakly expressing MDV mRNA, but the absence of plaques indicated that viable infectious virus particles were not being produced. By sequencing the UL41 flanking sequences and re-amplifying the flanking sequence B, the possibility of errors being introduced into the UL41(Rep) H10(F) BAC by mutagenesis was eliminated. Finally, the orientation of the introduced H10 gene was not a factor, as neither UL41(Rep) H10(F) BAC nor UL41(Rep) H10(R) BAC produced CPE.

To resolve this problem, several new viral BAC constructs were designed: -

a) UL41 Deleted (Del) BAC

Previous research demonstrating that the UL41 gene was not essential (Section 3.1.1) could be confirmed by creating a UL41 (Del) BAC where the UL41 gene was deleted and not replaced with any gene. Deleting the UL41 gene could be achieved

by performing BAC mutagenesis with a shuttle plasmid that contained only the UL41 flanking sequences, FSA and FSB.

b) UL41(Rep) H10 Truncated BAC

The inserted influenza gene may be the reason why plaques did not form when the UL41(Rep) H10 BACs were transfected into CEF cells. Once expressed, the insertion of the N-terminal polypeptide of the HA protein into adjacent CEF cellular membranes may lead to destabilisation of those membranes. As MDV is highly cell associated, this membrane disruption and subsequent cell lysis would prevent the formation of CPE. This could be prevented by removing the transmembrane domain from the end of the H10 gene, thereby truncating it and preventing the H10 protein from interacting with the CEF cell membrane.

c) UL41(Rep) GFP BAC

Replacing the UL41 gene with GFP would serve to help identify if it was the H10 gene insertion that was preventing the formation of plaques. In addition, transfected cells and plaques would be easier to observe if the GFP protein was produced.

3.3. UL41 (Del) BAC

3.3.1. UL41pST76kFSAB

The plasmid UL41pTOPFSAB (#2) (Section 3.2.2) was digested with *Kpn*I and *Pac*I in preparation for cloning. The digested FSAB DNA fragment was gel purified (Section 2.1.9) and ligated into the digested and purified pST76k plasmid (Section 3.1.10) to create the plasmid, pST76kFSAB (Figure 3.10). Competent *E. coli* cells were transformed with the ligation reaction as described in Section 2.2.2 and plasmid DNA was isolated (Section 2.2.5). Previously, shuttle plasmids were digested with the restriction enzymes, *Kpn*I and *Pac*I, to confirm that the ligation was successful.

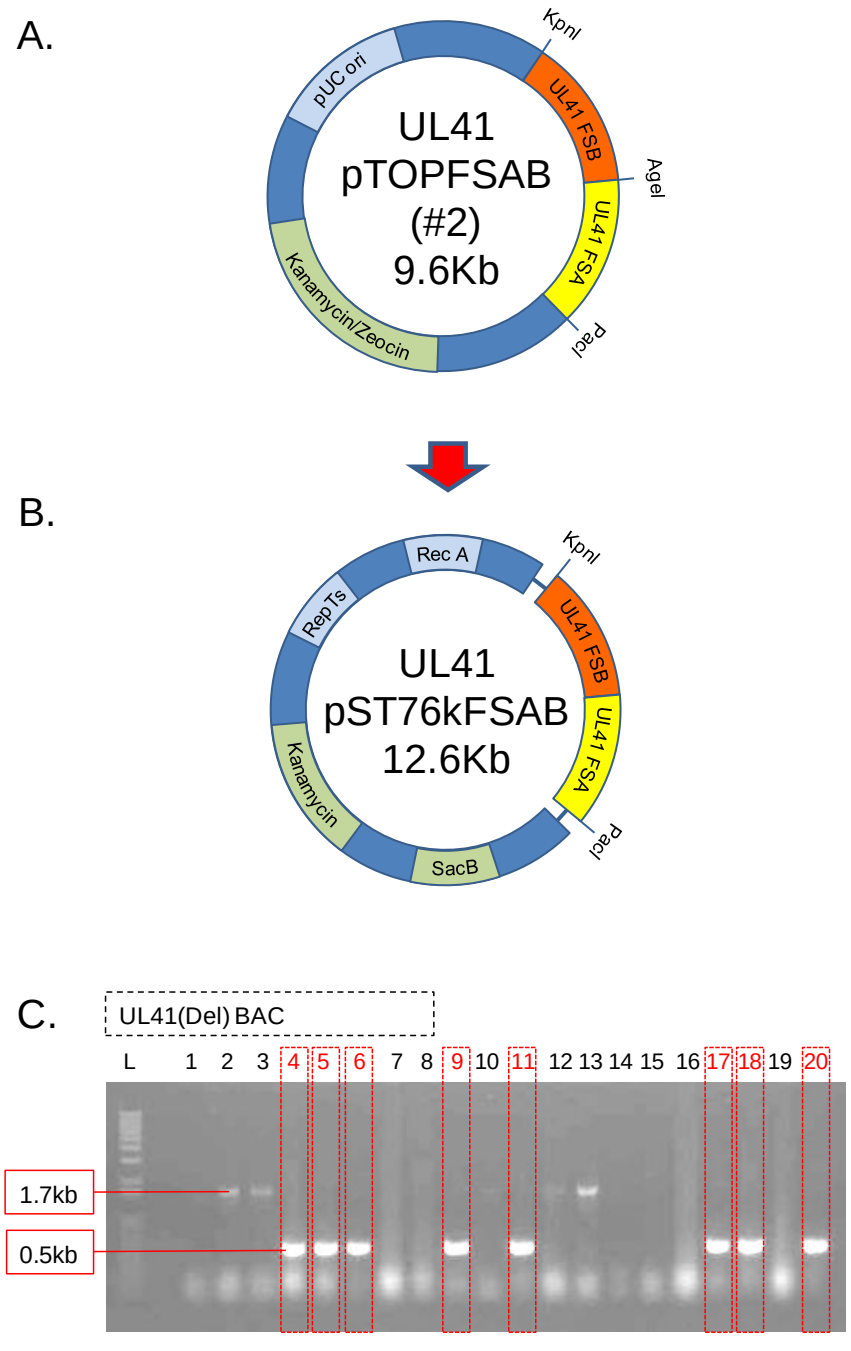


Figure 3.10 A) A diagrammatic representation of the created plasmid UL41pTOPFSAB (#2). B) A diagrammatic representation of the plasmid UL41pST76kFSAB, created by cloning the FSAB DNA fragment into the shuttle plasmid, pST76k. The plasmid was used to delete the UL41 by BAC mutagenesis. C) Agarose gel electrophoresis showing positive results from a colony PCR experiment designed to detect UL41(Del) BACs where the UL41 gene had been deleted.

However, in this case, the pST76k vector and the FSAB insert were both 6.3 kb in size so the isolated plasmids were instead digested with *Xho*I. All plasmids displayed the expected digest pattern indicating that the plasmid, UL41pST76kFSAB, had been successfully created.

3.3.2. BAC Mutagenesis using UL41pST76kFSAB

BAC mutagenesis was performed, as described in Section 2.2.9. DH5-α bacteria containing the MDV CVI988 BAC were made competent (Section 2.2.8) and transformed with 10 ng of the shuttle plasmid UL41pST76kFSAB, using electroporation. Kanamycin-sensitive colonies containing UL41(Del) BACs, where the UL41 gene had been deleted, were identified using colony PCR (Section 2.1.1 and Appendix A.1.4.1). Primers were designed to lie either side of the UL41 gene (Appendix A.1.3.1). By amplifying that region using PCR, the detection of a small product (507 bp) meant that the UL41 gene had been successfully deleted, whereas a larger product (1731 bp) meant that the UL41 gene was still present (Appendix A.1.3.1). 8 colonies out of the 20 colonies tested produced an approximately 500 bp product, indicating that the UL41 gene had been deleted by BAC mutagenesis (Figure 3.10). Two UL41(Del) BAC colonies were selected and isolated, as described in Section 2.2.7.

3.3.3. *In vitro* Characterisation of UL41(Del) BAC

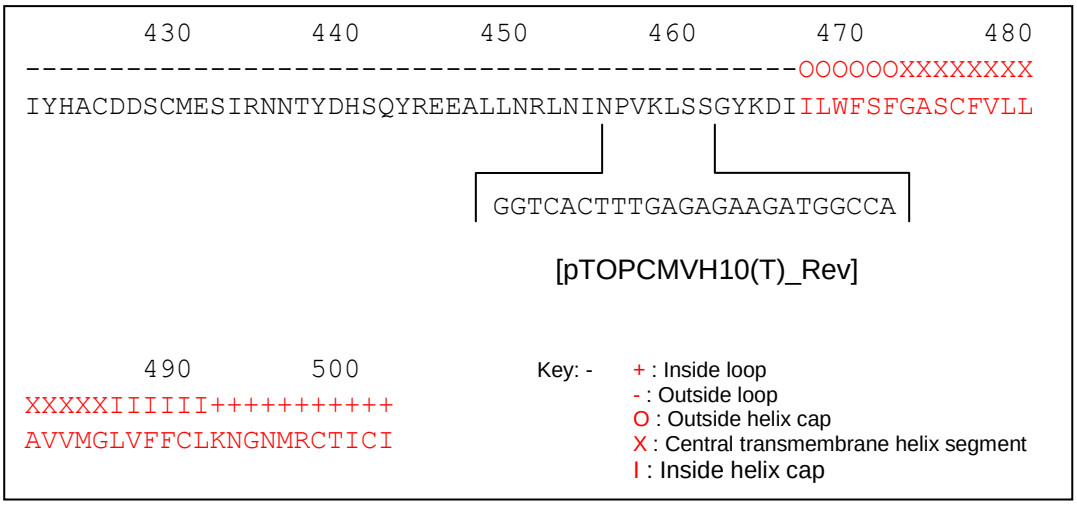
CEF cells, seeded in six-well dishes, were transfected with MDV CVI988 BAC DNA and both UL41(Del) BAC clones, as described in Section 2.3.7. Three wells of a six-well dish were transfected for each BAC. After 7 days, 10-15 plaques were visible in the wells transfected with the CVI988 BACs. CEF cells transfected with the UL41(Del) BACs displayed approximately 5-10 plaques per well. The *in vitro* analysis confirmed that the UL41 gene is not essential as the UL41(Del) BAC mutants were capable of producing CPE.

Therefore, the problem was not with the UL41 gene deletion, but with the insertion of the foreign H10 DNA. To resolve if the haemagglutinin gene was having a toxic effect, the UL41 gene was replaced with a truncated H10 gene and a GFP gene.

3.4. UL41(Rep) H10 (Truncated) BAC

The predicted site of the transmembrane domain in the H10 gene was located using the website www.psipred.com (UCL Bioinformatics group). The DNA sequence of the H10 was translated to reveal the predicted amino acid sequence of the gene. The transmembrane domain was situated at the COOH-terminal end, and is characterised by a predominance of hydrophobic non-polar amino acids (See Figure 3.11). Truncating the H10 gene was achieved by amplifying the CMVH10 DNA sequence from pTOPCMVH10 (Section 3.1.8) using PCR, placing the reverse primer upstream of the transmembrane domain (pTOPCMVH10(T)_Rev).

Figure 3.11 COOH-terminal end of H10, with the predicted transmembrane domain sequence



Primers (pTOPCMVHA10T_For and pTOPCMVH10T_Rev) that incorporated the unique restriction site, *Age*I, were designed to amplify the CMV-H10 (Truncated) DNA fragment (Appendix A.1.3.1). Using pTOPCMVH10 as a template (20 ng), the CMVH10(t) DNA fragment was successfully amplified as described in Section 2.1.1 and Appendix A.1.4.1. The amplified CMV-H10(t) DNA was cloned first into Blunt II TOPO (Section 2.1.2) to create pTOPCMVH10(t) and subsequently into UL41pTOPFSAB, in both orientations. As described previously, the FSABH10(t) DNA insert was then ligated into pST76k to create the shuttle plasmids, pST76kH10(t) (F) and pST76kH10(t) (R) (Figure 3.12).

BAC mutagenesis was performed using these shuttle plasmids and the UL41(Rep) BACs, UL41(Rep) H10(t) (F) BAC and UL41(Rep) H10(t) (R) BAC, were created and isolated (Section 2.2.7).

In vitro analysis was performed. CEF cells were transfected with MDV CVI988 BAC DNA and the UL41(Rep) H10(t) BACs, as described in Section 2.3.7. After 7 days, 10-15 plaques were visible in each of the three wells that had been transfected with the CVI988 BAC. No plaques were visible for either the UL41(Rep) H10(t) (F) BAC or the UL41(Rep) H10(t) (R) BAC.

These results suggested that the intact H10 protein was not the cause of the lack of CPE as without a transmembrane domain, it reduced the possibility of cell membrane lysis occurring.

3.5. UL41(Rep) GFP BAC

Due to the presence of an *Age*I restriction enzyme site between the CMV promoter and the GFP gene in the plasmid pEGFP-N1, the GFP DNA fragment was amplified with the primers (pGFP_For and pGFP_Rev) incorporating the unique enzyme restriction sites *Nhe*I and *Acc*I (Appendix A.1.3.1). Using site directed mutagenesis, the forward primer destroyed the *Age*I restriction enzyme site.

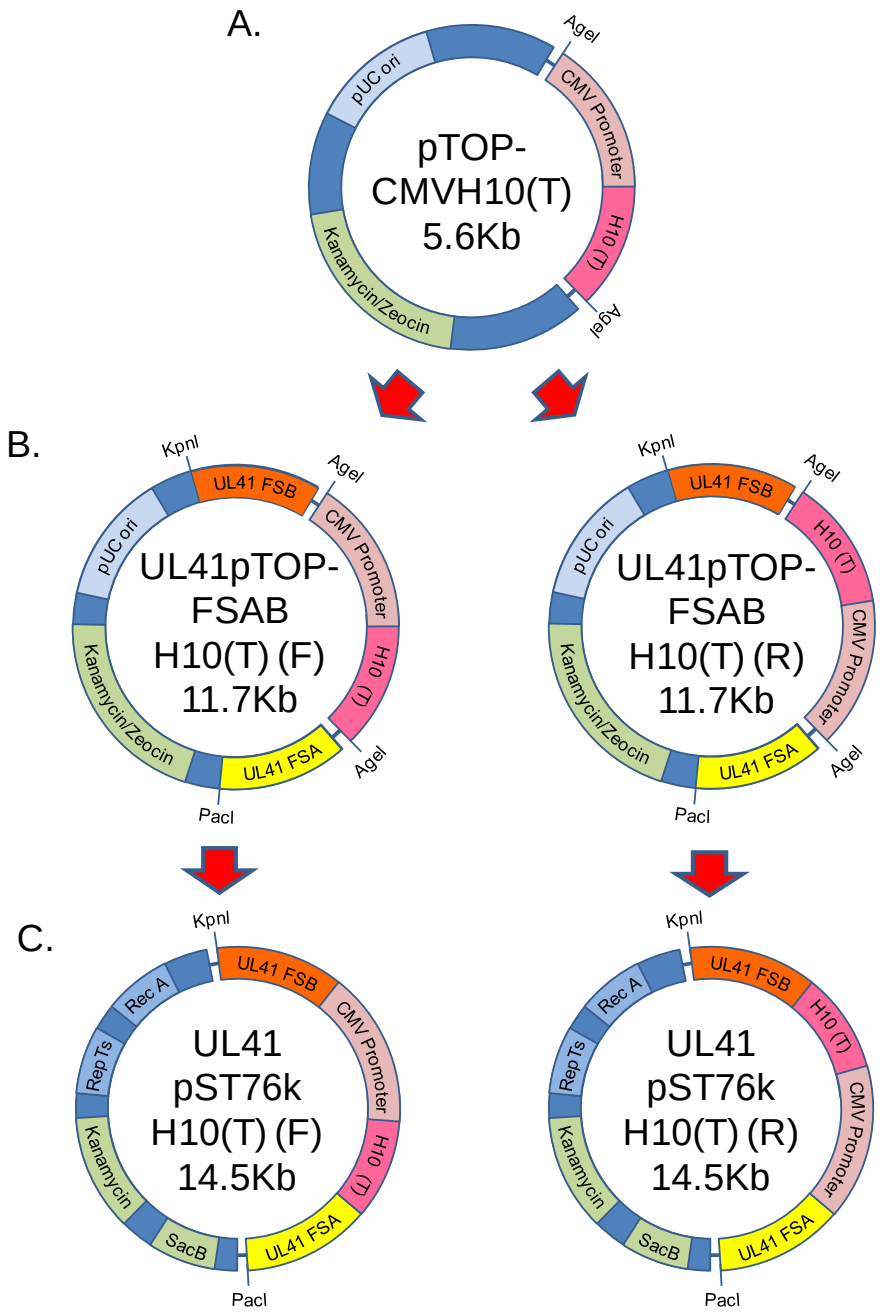


Figure 3.12 A) A diagrammatic representation of the plasmid pTOPCMVH10(t), created by cloning the PCR amplified CMV-H10(t) DNA fragment into the TOPO vector. B) The plasmids UL41pTOPFSABH10(t) (Forward and Reverse) were created by cloning the CMV-H10 DNA fragment between the flanking sequences FSA and FSB, in the plasmid UL41pTOPFSAB (#2). C) In the final cloning step, the FSABCMVH10(t) DNA fragments were transferred to the shuttle plasmid, pST76k, to create the plasmids UL41pST76kH10(t) (Forward and Reverse).

The GFP gene was successfully amplified (Section 2.1.1. and Appendix A.1.4.1) and cloned into Blunt II TOPO (Section 2.1.2) to create pTOPGFP. Subsequently, the GFP was isolated using restriction enzyme digestion (*Nhe*I and *Acc*I) and purified before being cloned downstream of the CMV promoter to create pTOPCMVGFP. The CMV-GFP DNA fragment was isolated using restriction enzyme digestion (*Age*I) and purified before being cloned between the flanking sequences of UL41pTOPFSAB (Section 3.2.2). As described previously, the FSABGFP DNA insert was then ligated into pST76k to create pST76kGFP(F) (Figure 3.13). BAC mutagenesis was carried out and the UL41(Rep) GFP(F) BAC was created and isolated according to the protocol in Section 2.2.7.

CEF cells were transfected with the CVI988 BAC and the UL41(Rep) GFP(F) BAC, as described in Section 2.3.7. After 7 days, 10-15 plaques were visible in each of the three wells that had been transfected with CVI988. Unfortunately, no plaques were visible in CEF cells transfected with the UL41(Rep) GFP(F) BAC.

3.6. The UL41 open reading frame

To summarise, bibliographic research and subsequent *in vitro* analysis has demonstrated that the UL41 gene may be deleted from the MDV CVI988 BAC genome, without affecting the ability of the BAC to produce CPE once it has been transfected into CEF cells. The introduction of any foreign DNA into the UL41 open reading frame, however, seems to disrupt the production of infectious virus particles, preventing plaque formation. Truncating the H10 gene by removing the transmembrane region or replacing the H10 gene with GFP did not produce positive results. The hypothesis that it was simply not possible to insert foreign DNA into this area of the MDV genome was feasible and this could be tested by attempting the process with a new, non-essential MDV gene other than UL41.

However, in a final attempt to resolve why the insertion of non-MDV DNA into the UL41 gene site was producing such a detrimental effect to the parental MDV CVI988 BAC, the BAC transfections of CEF cells were repeated.

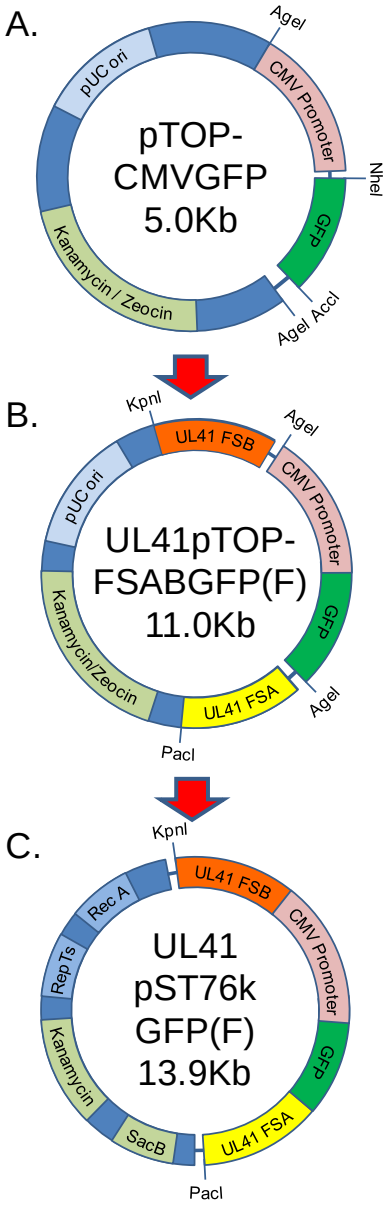


Figure 3.13 A) A diagrammatic representation of the plasmid pTOPCMVGFP, created by cloning the GFP DNA fragment downstream of the CMV promoter. B) The plasmid UL41pTOPFSABGFP(F) was created by cloning the CMV-GFP DNA fragment into the plasmid UL41pTOPFSAB (#2), between the flanking sequences FSA and FSB. C) In the final cloning step, the FSABCMVGFP DNA fragment was transferred to the shuttle plasmid, pST76k, to create the plasmid UL41pST76kGFP(F).

It was hypothesised that, if transfecting 1 µg of CVI988 BAC DNA into one well of a six-well dish of CEF cells resulted in the formation of only 15 plaques, then the detrimental effect caused by the introduction of foreign DNA may reduce this to zero. Increasing the transfection efficiency so that the MDV CVI988 BAC produced more plaques would potentially offset this detrimental effect, revealing a small number of plaques in CEF cells transfected with the UL41(Rep) BAC DNA. Previous research by Morgan *et al* (1990) found that the transfection of CEFs with MDV DNA is greatly improved if DNA is introduced at the seeding stage, rather than once the CEF monolayer is formed. This method was referred to as a 'Reverse' transfection.

Furthermore, two more set of primers were designed to analyse cDNA extracted from the BAC transfected CEF cells (Appendix A.1.3.1). These primers were specific for UL42 and UL44 and were selected due to their proximity to the deleted UL41 gene and the fact that they are deemed essential.

3.6.1. BAC transfections

CEF cells were simultaneously transfected with CVI988 BAC DNA, UL41(Del) BAC DNA and UL41(Rep) GFP(F) BAC DNA, as described in Section 2.3.8. After 7 days, cytopathic effect (CPE) in the form of plaques was visible. In CEF cells that had been transfected with the CVI988 BAC, there was an average of 77 plaques in each of the three wells. In CEF cells that had been transfected with the UL41(Del) BAC, there was an average of 38 plaques in each of the three wells. No plaques were visible in the CEF cells transfected with UL41(Rep) GFP(F) BAC DNA, although there were individual cells expressing GFP. This suggested that the transfection was successful, and that GFP mRNA was being expressed, but no virus replication was taking place.

RNA was extracted from the BAC transfected cells as described in Section 2.1.14. The RNA was isolated and, using random primers, approximately 3 µg of the extracted DNase-treated RNA was converted to complementary DNA (cDNA) by reverse transcription (Section 2.1.15).

3.6.2. CVI988/UL41(Rep) BAC cDNA PCR

PCR was carried out as described in Section 2.1.1 and Appendix A.1.4.1. The cDNA created from the CVI988 BAC and the UL41(Del) BAC transfected cells was tested, as well as cDNA created from untransfected CEF cells. In addition, cDNA previously created from the UL41(Rep) H10 BACs (Forward and Reverse) was tested. Four sets of primers specific to MDV (MDV UL18, vIL-8, UL42 and UL44) were used along with primers for H10 and GFP (Appendix A.1.3.1).

Following PCR, products were analysed using agarose gel electrophoresis on a 1% gel (Section 2.1.4) (Figure 3.14). PCR analysis of cDNA created from untransfected CEF cells revealed, as expected, no expression of MDV or H10 mRNA. The analysis of cDNA created from the CVI988 BAC and UL41(Del) BAC transfected cells demonstrated that all four sets of MDV genes, but not H10 or GFP, were expressed. cDNA that had been made from cells transfected with UL41(Rep) H10 (F and R) BAC DNA were shown to express H10 mRNA but not GFP mRNA. UL41(Rep) H10(F) BAC cDNA tested positive for all four sets of MDV primers, however, the UL41(Rep) H10(R) BAC cDNA was negative for UL42 and UL44. In this orientation, these essential genes are adjacent to the CMV promoter of the CMVH10 DNA insert.

3.6.3. CMV promoter

PCR analysis of the cDNA extracted from UL41(Rep) H10(R) BAC transfected cells has revealed that the mRNA of two essential genes, adjacent to the CMV promoter, was not being expressed. It was observed that the introduction of foreign DNA into the UL41 open reading frame affected the ability of the MDV BAC to form plaques upon transfection into CEF cells. However, it may be that it was not the genes that were causing this detrimental effect, but the CMV promoter itself. To test this hypothesis, it was decided that a different promoter would be used.

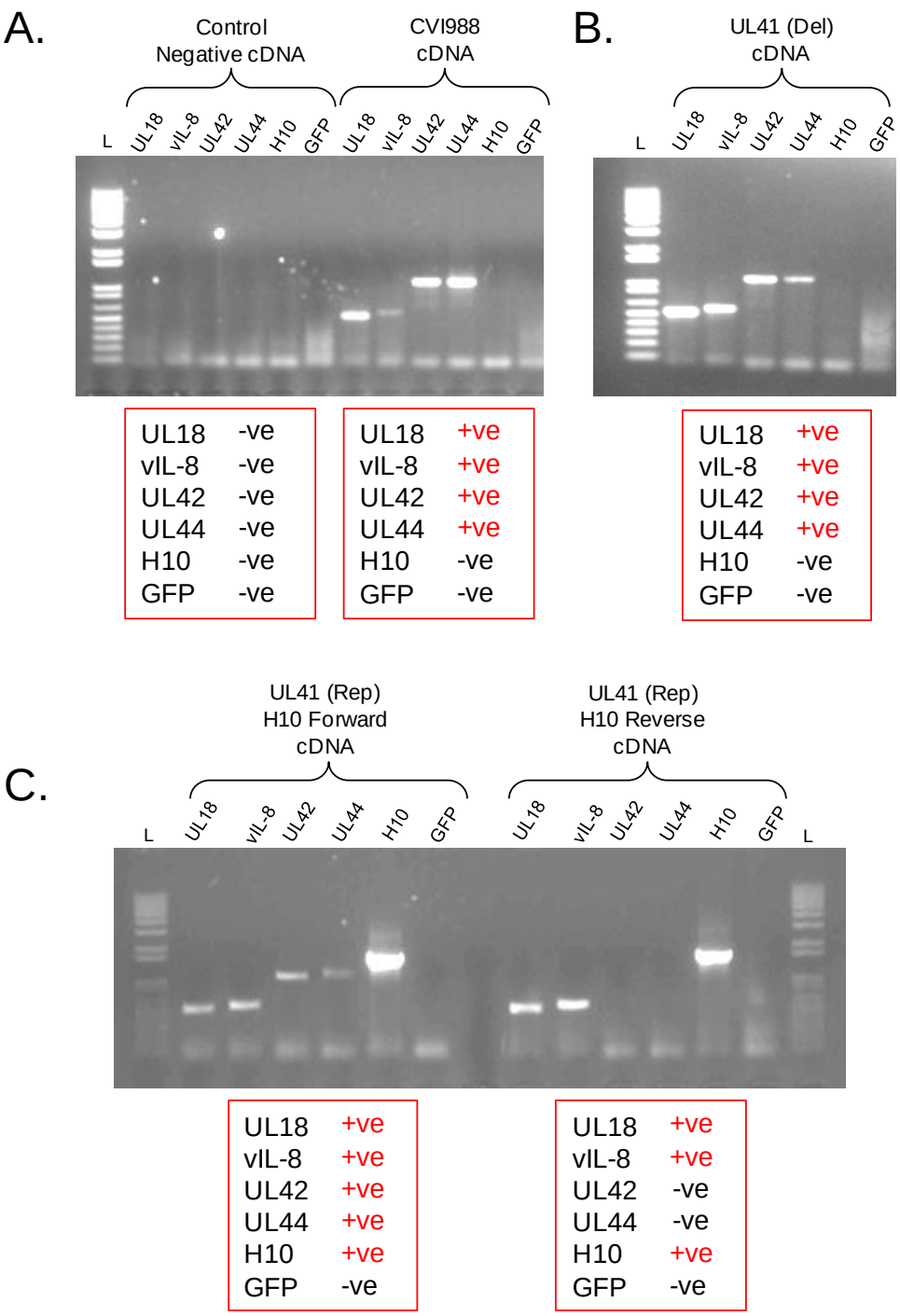


Figure 3.14 A) PCR products from cDNA created from untransfected CEF cells and MDV CVI988 transfected cells B) PCR products from cDNA created from the UL41(Del) BAC transfected cells. C) PCR products from cDNA made from cells transfected with the UL41(Rep) H10 BACs (Forward and Reverse).

The murine PGK-1 promoter can efficiently drive high levels of expression but it was found to work less efficiently in CEF cells than the CMV promoter (Prof. H. Sang, personal communication). It was speculated that, if the powerful CMV promoter was inhibiting other promoters or essential genes in the region, then a less powerful promoter like PGK-1 might not have the same antagonistic effect. The following sections describe the construction of these UL41(Rep) PGK BAC constructs.

3.7. UL41(Rep) GFP BAC – PGK-1 Promoter

3.7.1. pTOPPGKGFP

The plasmid, pEGFP-1 PGK, that contained the murine PGK-1 promoter upstream of the GFP gene, was kindly provided by Professor H. Sang (The Roslin Institute, Edinburgh). In this plasmid, the PGK-1 promoter is situated upstream of the GFP gene (Figure 3.15). Using *Hin*dIII and *Not*I, the PGKGFP DNA fragment was removed by restriction enzyme digestion (Section 2.1.6) and gel purified in preparation for cloning (Section 2.1.9). The plasmid vector, pTOPCMVH10 (Section 3.1.8), was digested with *Hin*dIII and *Not*I to remove the CMVH10 DNA fragment and the plasmid backbone was gel purified (Section 2.1.9). The digested and purified PGKGFP fragment was ligated into the plasmid backbone, as described in Section 2.1.10. Competent *E. coli* cells were transformed with the ligation mixture (Section 2.2.2) and plasmid DNA was isolated from small cultures (Section 2.2.5). The successfully ligated plasmid, pTOPPGKGFP (Figure 3.15), was confirmed using restriction enzyme digestion with *Hin*dIII and *Not*I.

Zero Blunt II TOPO primers, M13 forward and reverse, were used to sequence the PGK promoter and the GFP gene (Appendix A.1.3.1). Sequence results indicated complete homology with the published sequences for both the PGK promoter and the downstream GFP gene. Subsequently, pTOPPGKGFP plasmid DNA was prepared according to the protocol in Section 2.2.6.

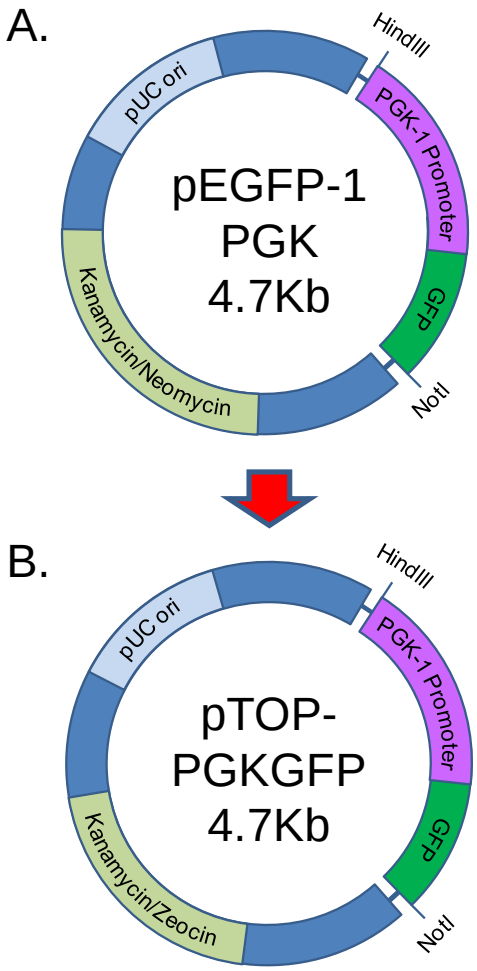


Figure 3.15 A) The PGK-1 promoter is situated upstream of the GFP gene in the plasmid, pEGFP-1 PGK. B) Using the restriction enzymes, *Hin*dIII and *Not*I, the PGKGFP fragment was digested, purified and ligated into the TOPO vector to create pTOPPGKGFP.

3.7.2. UL41pTOPFSAB (#2)

In previous work (Section 3.1.5 and Section 3.2.2), the *Age*I restriction enzyme was used to digest the plasmid UL41pTOPFSAB in preparation for cloning with the appropriate gene insert. Unfortunately, there was an *Age*I site located in the PGK-1 promoter sequence so this was no longer possible. With no other unique restriction enzymes sites available between the two flanking sequences, it was decided that the PGKGFP gene insert should be blunt cloned into the plasmid UL41pTOPFSAB (#2).

In preparation for blunt cloning, the plasmid UL41pTOPFSAB (#2) (Section 3.2.2) was digested with *Age*I-High Fidelity (HF) enzyme. Following digestion, the 5' overhangs of UL41pTOPFSAB (#2) plasmid DNA (5 µg) were converted to 5' phosphorylated, blunt-ended DNA, as described in Section 2.1.7, and dephosphorylated according the protocol in Section 2.1.8.

3.7.3. UL41pTOPFSABPGKGFP

In preparation for cloning, pTOPPGKGFP was digested with *Eco*RV and *Sna*BI to produce 'blunt' ends. The digested PGKGFP fragment was gel purified (Section 2.1.9) and ligated into the *Age*I digested, blunted and AP treated UL41pTOPFSAB (#2) (Section 3.7.2). XL-Gold Ultracompetent *E. coli* cells were transformed with the ligation reaction as described in Section 2.2.3 and plasmid DNA was isolated from small cultures (Section 2.2.5). The plasmids were digested with *Pst*I and *Xho*I, to confirm that the PGKGFP insert had ligated with the vector successfully and to ascertain the orientation of the insert. Two plasmids were found to contain the PGKGFP insert in the forward orientation, and five in the reverse orientation (Figure 3.16).

To confirm the successful ligation of PGKGFP, both plasmids, UL41pTOPFSABPGKGFP (F and R), were sequenced using the primers pCMVHA ID_For and pTOPFSABHA_Rev6 (Appendix A.1.3.1). Sequencing results indicated that for both plasmids, the PGKGFP insert had been correctly inserted between the flanking sequences.

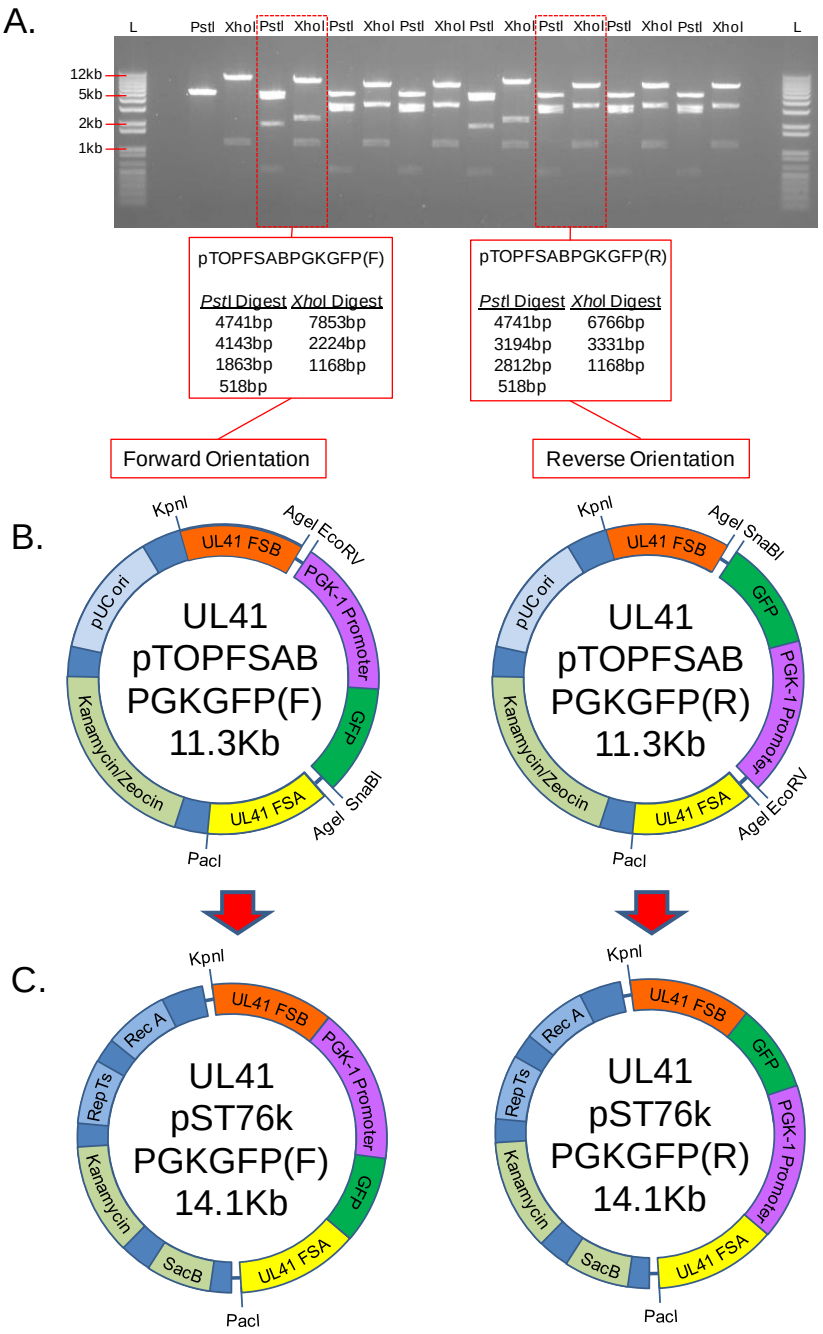


Figure 3.16 A) Agarose gel electrophoresis showing restriction enzyme digests of the plasmids pTOPFSABPGKGFP (F and R), using the enzymes *Pst*I and *Xho*I. B) A diagrammatic representation of the plasmids UL41pTOPFSABPGKGFP (F and R). The PGK-GFP DNA fragment was cloned into the plasmid UL41pTOPFSAB (#2), between the flanking sequences FSA and FSB. C) In the final cloning step, the FSABPGKGFP DNA fragments were transferred to the shuttle plasmid, pST76k, to create the plasmids UL41pST76kPGKGFP (F and R).

3.7.4. UL41pST76kPGKGFP (F and R)

The plasmids, UL41pTOPFSABPGKGFP (F and R), were digested with *Kpn*I and *Pac*I in preparation for cloning with the shuttle plasmid, pST76k. The digested FSABPGKGFP DNA fragments were gel purified (Section 2.1.9) and ligated into the digested and purified pST76k plasmid (Section 3.1.10) to create the plasmids UL41pST76kPGKGFP (F and R) (Figure 3.16). Competent *E. coli* cells were transformed with the ligation mixture (Section 2.2.2) and plasmid DNA was isolated (Section 2.2.5). The plasmids were digested with the restriction enzymes, *Kpn*I and *Pac*I, to confirm that the ligation was successful. For both constructs, UL41pST76kPGKGFP (F and R), the enzyme digests produced the expected digest pattern.

3.7.5. BAC Mutagenesis using the plasmids UL41pST76kPGKGFP(F) and UL41pST76kPGKGFP(R)

BAC mutagenesis was successfully carried out using the constructs, UL41pST76kPGKGFP(F) and UL41pST76kPGKGFP(R) (Section 2.2.9). DH5-α bacteria containing the MDV CVI988 BAC were made competent (Section 2.2.8) and transformed, by electroporation, with 10 ng of the shuttle plasmids. Kanamycin-sensitive colonies containing UL41(Rep) BACs, where the UL41 gene had been replaced with the PGKGFP DNA insert, were identified using colony PCR (Section 2.1.1 and Appendix A.1.4.1). The primers, peGFP_For and peGFP_Rev3 (Appendix A.1.3.1) were kindly provided by Dr B. M. Dutia and used to identify the PGKGFP insert. pEGFP-N1 plasmid DNA (25 ng) (Section 3.1.7) was included as a positive control, which produced expected results. BAC mutagenesis that had utilised the UL41pST76kPGKGFP(F) construct produced five colonies which were positive for the PGKGFP insert. BAC mutagenesis carried out using the UL41pST76k PGKGFP(R) shuttle plasmid, produced five colonies positive for the PGKGFP insert (Figure 3.17). A bacterial colony containing the UL41(Rep) PGKGFP(F) BAC and a

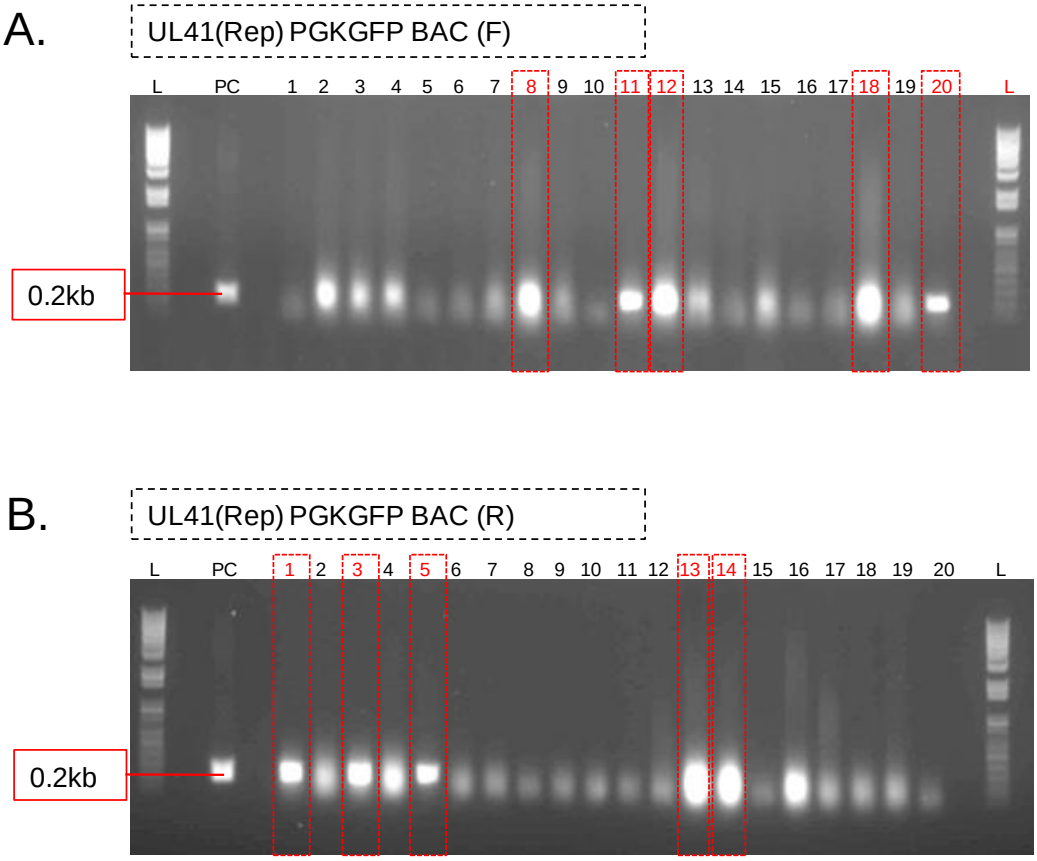


Figure 3.17 A) An agarose gel electrophoresis photograph showing positive results from a colony PCR experiment designed to detect a 0.2 kb DNA fragment of the GFP gene in the UL41(Rep) GFP BAC (Forward). B) Agarose gel electrophoresis showing positive results from a colony PCR experiment designed to detect a 0.2 kb DNA fragment of the GFP gene in the UL41(Rep) GFP BAC (Reverse).

UL41(Rep) PGKGFP(R) BAC was selected and the BAC DNA was isolated according to the protocol in Section 2.2.7.

To confirm the successful insertion of PGKGFP into the CVI988 BAC, the constructs were sequenced using the primers pCMVHAID_For and pTOPFSABHA_ Rev6 (Appendix A.1.3.1). Sequencing results indicated that for both UL41(Rep) BACs, the PGKGFP insert had successfully replaced the UL41 gene, in both orientations.

3.8. UL41(Rep) H10 BAC – PGK-1 Promoter

As with GFP, the H10 gene was cloned downstream of the PGK-1 promoter. In anticipation of *in vitro* analysis, the H10 gene was linked to a Myc-HIS tag, so that in lieu of a specific H10 antibody, the H10 protein could be detected if expressed in CEF cells. The following sections describe the construction this shuttle plasmid and the creation of the UL41(Rep) H10myc BAC.

3.8.1. pcDNA3.1H10myc

The plasmid, pcDNA3.1/myc-HIS(-)A, contains the myc-HIS tag downstream of a multiple cloning site (Figure 3.18). Using the restriction enzymes *Xho*I and *Hin*dIII, the H10 gene could be cloned upstream of this tag, in the same frame. Primers (pH10Rpt_For and pH10Rpt_Rev) that incorporated these unique restriction enzyme sites were designed to amplify the H10 gene (Appendix A.1.3.1). The H10 PCR product was inserted the same distance from the end of the promoter as the original GFP (13 bp). Using pTOPCMVH10 (Section 3.1.8) as a template (20 ng), the H10 gene was successfully amplified (Section 2.1.1 and Appendix A.1.4.1) and gel purified (Section 2.1.6). It was subsequently ligated into Blunt II TOPO (Section 2.1.2) to create the plasmid pTOPH10 (Figure 3.18). Following transformation (Section 2.2.2), the plasmid DNA was isolated (Section 2.2.5) and the successfully ligated plasmid was confirmed using restriction enzyme digestion with *Xho*I and *Hin*dIII.

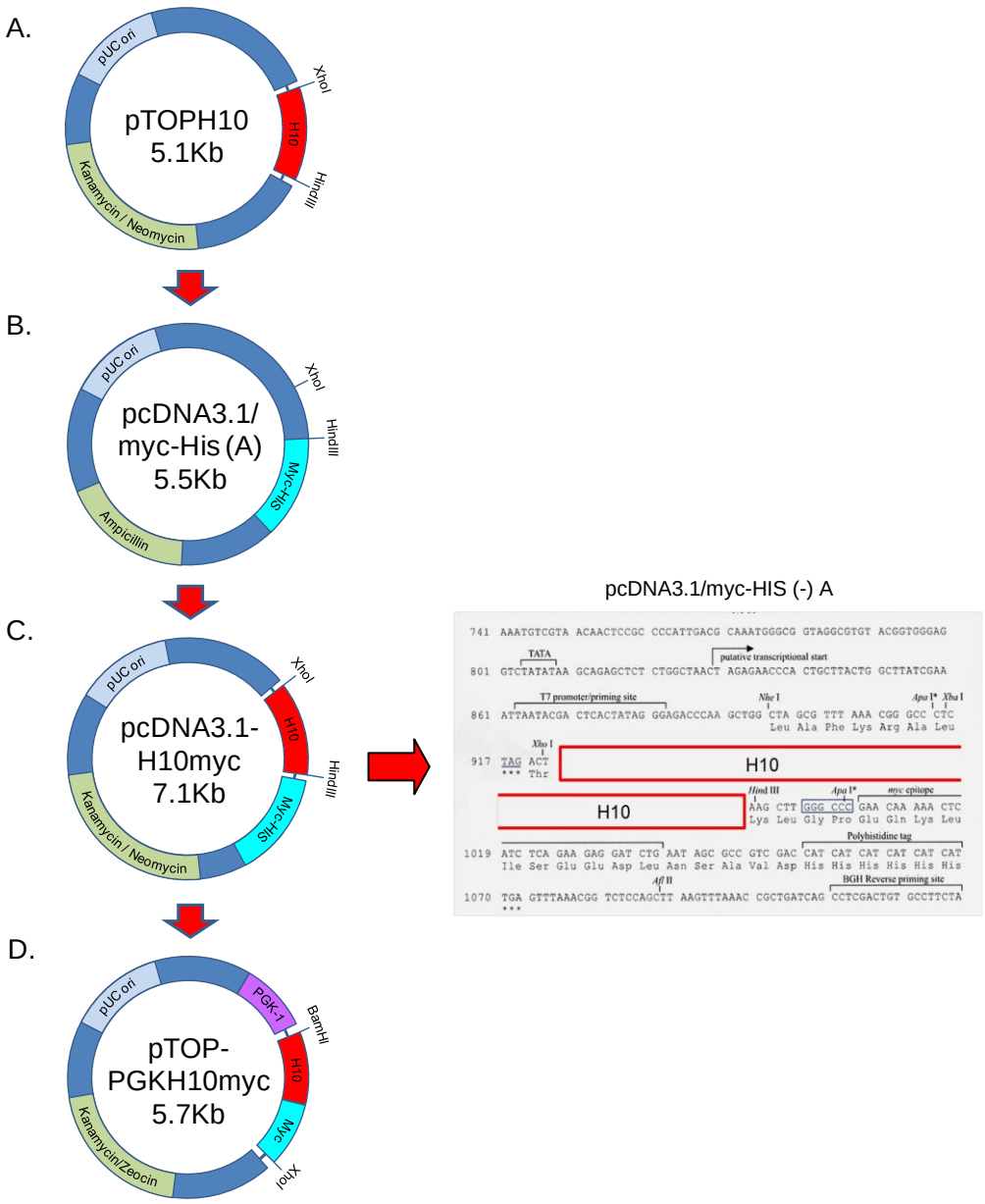


Figure 3.18 A) The H10 gene was PCR amplified with primers incorporating the restriction enzyme sites, *Xho*I and *Hin*dIII, and cloned into Blunt II TOPO to create pTOPH10. B) The plasmid, pcDNA3.1/myc-HIS(-) A, contained the myc-HIS tag downstream of a multiple cloning site. C) Using the restriction enzymes *Xho*I and *Hin*dIII, the H10 DNA fragment was cloned upstream of the myc-HIS tag to create the plasmid pcDNA3.1H10myc. D) The H10myc-HIS DNA fragment was amplified with primers that incorporated the restriction enzyme sites, *Bam*HI and *Xho*I, and ligated downstream of the PGK-1 promoter to create the plasmid pTOPPGKH10myc.

Using *Xho*I and *Hin*dIII, the H10 DNA fragment was removed by enzyme digestion and gel purified in preparation for cloning (Section 2.1.9). The plasmid vector, pcDNA3.1/myc-HIS was also digested with *Xho*I and *Hin*dIII and gel purified (Section 2.1.9). The digested and purified vector and insert were ligated, as described in Section 2.1.10, to create the plasmid pcDNA3.1H10myc (Figure 3.18). Competent *E. coli* cells were transformed with the ligation mix according to the protocol in Section 2.2.2, and plasmid DNA was isolated from small cultures (Section 2.2.5). The plasmid, pcDNA3.1H10myc, was identified by digestion with *Ahd*I.

3.8.2. pTOPPGKH10myc

Primers (pH10myc_For and pH10myc_Rev) which incorporated the unique restriction sites, *Bam*HI and *Xho*I, were designed to amplify the H10myc-HIS DNA fragment (Appendix A.1.3.1). Using pcDNA3.1H10myc (Section 3.8.1) as a template (20 ng), the H10myc gene was amplified (Section 2.1.1 and Appendix A.1.4.1), gel purified (Section 2.1.9) and subsequently ligated into Blunt II TOPO (Section 2.1.2) to create pTOPH10myc. Competent *E. coli* cells were transformed with the ligation reaction (Section 2.2.2) and, following plasmid DNA isolation (Section 2.2.5), the successfully ligated plasmid was confirmed using restriction enzyme digestion with *Bam*HI and *Xho*I.

Using *Bam*HI and *Xho*I, the H10myc DNA fragment was removed by enzyme digestion and gel purified (Section 2.1.9). The plasmid vector, pTOPPGKGFP, was digested with *Bam*HI and *Xho*I to remove the GFP gene and the vector backbone was gel purified (Section 2.1.9). The digested and purified vector and H10myc insert were ligated, as described in Section 2.1.10, to create the plasmid pTOPPGKH10myc (Figure 3.18). Following transformation (Section 2.2.2), plasmid DNA was isolated from small cultures (Section 2.2.5). The plasmid, pTOPPGKH10myc, was successfully identified using restriction enzyme digestion with *Bam*HI and *Xho*I, and *Hin*dIII.

The primers, M13 forward and reverse and pTOPCMVHA_For 3, were used to sequence the PGK promoter and H10myc DNA (Appendix A.1.3.1). Sequence

results indicated complete homology to the published sequences for both the PGK promoter and the downstream H10 gene and myc-HIS tag.

3.8.3. UL41pTOPFSABPGKH10myc

In preparation for cloning, pTOPPGKH10myc was digested with *Eco*RV and *Sna*BI. The digested PGKH10myc fragment was gel purified (Section 2.1.9) and ligated into the *Age*I digested, blunted and phosphatased UL41pTOPFSAB (Section 3.7.2). XL-Gold Ultracompetent *E. coli* cells were transformed with the ligation reaction (Section 2.2.3) and plasmid DNA was isolated, as described in Section 2.2.5. To confirm the successful ligation of the PGKH10myc insert into the UL41pTOPFSAB plasmid vector, the isolated plasmid DNA was digested with *Pst*I and *Xho*I. This digest also demonstrated the orientation of the PGKH10myc insert. Four plasmids were identified that contained the PGKH10myc insert in the forward orientation, and two in the reverse orientation (Figure 3.19).

To confirm the successful ligation of PGKH10myc, both of the plasmids, UL41pTOPFSABPGKH10myc (F) and UL41pTOPFSABPGKH10myc (R), were sequenced using the primers pCMVHAID_For and pTOPFSABHA_Rev6 (Appendix A.1.3.1). For both orientations, sequencing results indicated that the PGKH10myc insert had been correctly inserted between the flanking sequences.

3.8.4. UL41pST76kPGKH10myc (F and R)

In preparation for cloning, the plasmids, UL41pTOPFSABPGKH10myc (F and R), were digested with *Kpn*I and *Pac*I. The digested FSABPGKH10myc fragments were gel purified (Section 2.1.9) and ligated into the digested and purified pST76k plasmid (Section 3.1.10), creating the plasmids UL41pST76kPGKH10myc (F and R) (Figure 3.19). Following transformation of competent *E. coli* cells (Section 2.2.2), plasmid DNA was isolated (Section 2.2.5) and digested with *Kpn*I and *Pac*I to confirm that the ligation was successful.

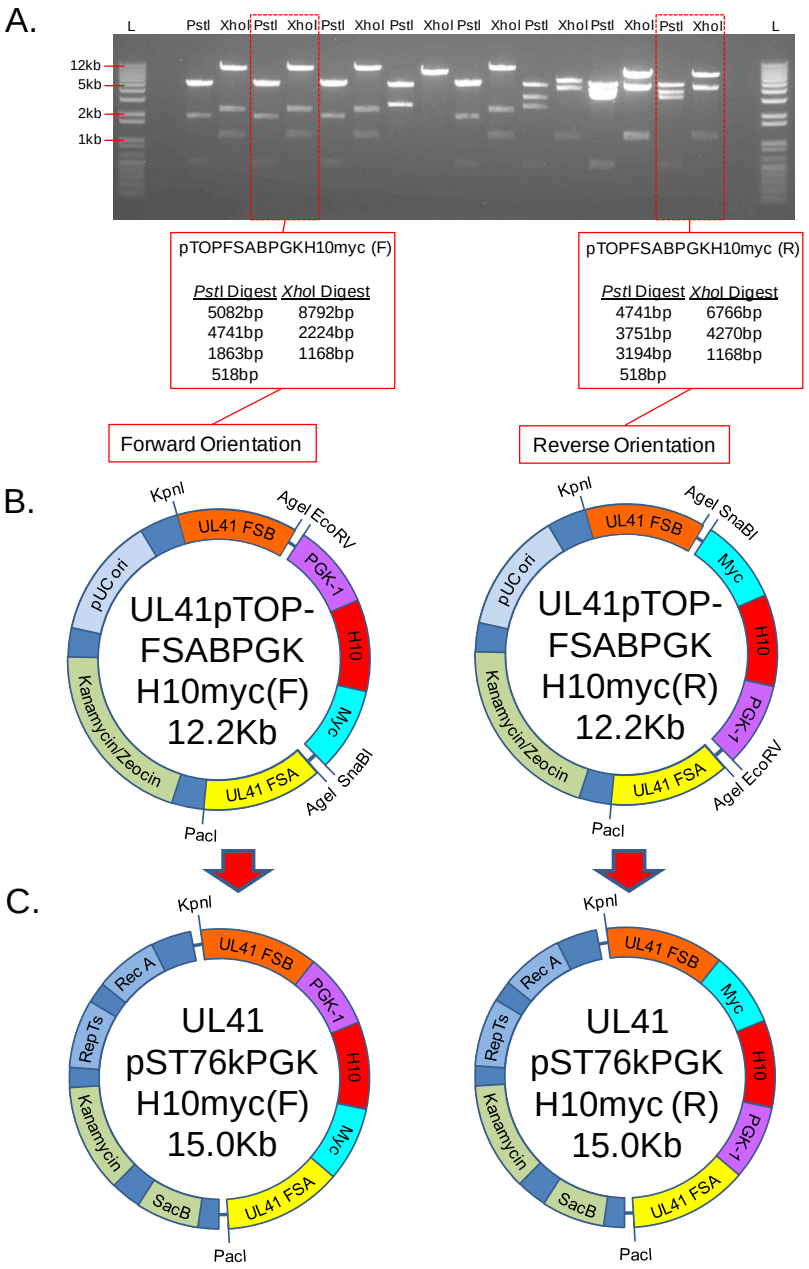


Figure 3.19 A) Agarose gel electrophoresis showing restriction enzyme digests of the plasmids pTOPFSABPGKH10myc (F and R), using the enzymes *Pst*I and *Xho*I. B) A diagrammatic representation of the plasmids UL41pTOPFSABPGKH10myc (F and R). The PGK-H10myc DNA fragment was cloned into the plasmid UL41pTOPFSAB (#2), between the flanking sequences FSA and FSB. C) In the final cloning step, the FSABPGKH10myc DNA fragments were transferred to the shuttle plasmid, pST76k, to create the plasmids UL41pST76kPGKH10myc (F and R).

For both constructs, UL41pST76kPGKH10myc (F and R), the enzyme digests produced the expected digest pattern.

3.8.5. BAC Mutagenesis using the plasmids UL41pST76kPGKH10myc (F) and UL41pST76kPGKH10myc (R)

BAC mutagenesis, using the constructs UL41pST76kPGKH10myc(F) and UL41pST76kPGKH10myc(R) was performed (Section 2.2.9). DH5-α bacteria containing the MDV CVI988 BAC were made competent (Section 2.2.8) and transformed with 10 ng of the shuttle plasmids using electroporation. Kanamycin-sensitive colonies containing UL41(Rep) BACs, where the UL41 gene had been replaced with H10myc gene, were identified using colony PCR (Section 2.1.1 and Appendix A.1.4.1). The primers, pH10myc_For and pH10myc_Rev (Appendix A.1.3.1) were used to identify the 1.7 kb H10myc insert. The pTOPH10myc plasmid (Section 3.8.2) was included as a positive control, which produced the expected results. BAC mutagenesis performed with the UL41pST76kPGKH10myc(F) shuttle plasmid yielded four colonies which were positive for the PGKH10myc insert. BAC mutagenesis carried out using the UL41pST76kPGKH10myc(R) construct, produced five colonies which were positive for the PGKH10myc insert (Figure 3.20). Subsequently, the BAC DNA of a UL41(Rep) PGKH10myc(F) BAC and a UL41(Rep) PGKH10myc(R) BAC construct was isolated, as described in Section 2.2.7.

Using the primers pCMVHAID_For and pTOPFSABHA_Rev6 (Appendix A.1.3.1), sequencing was used to confirm the successful insertion of PGKH10myc into the MDV CVI988 BAC. Sequencing results indicated that for both UL41(Rep) BACs, the PGKH10myc insert had successfully replaced the UL41 gene, in both orientations

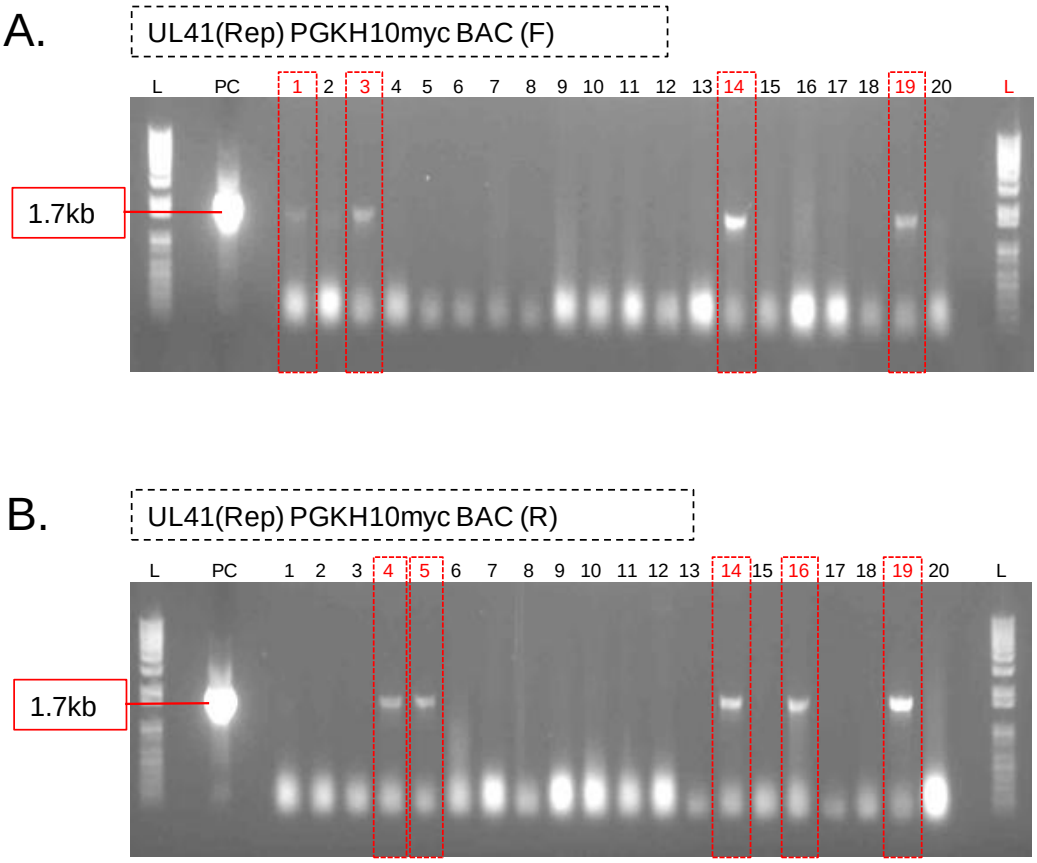


Figure 3.20 A) Agarose gel electrophoresis photograph showing positive results from a colony PCR experiment designed to detect an approximately 1.7 kb H10myc DNA fragment in the UL41(Rep) H10myc BAC (F). B) Agarose gel electrophoresis showing positive results from a colony PCR experiment designed to detect an approximately 1.7 kb H10myc DNA fragment in the UL41(Rep) H10myc BAC (R).

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3.9. *In vitro* Characterisation of the UL41(Rep) PGKGFP/H10myc BACs

3.9.1. BAC transfections

BAC transfections were carried out using the 'reverse' transfection method (Section 3.6). CEF cells were seeded at a density of 5.0 x 10^5 cells per well in six-well dishes and simultaneously transfected with the following BACs, according to the protocol in Section 2.3.8: -

- CVI988 - 6 wells
- UL41(Rep) PGKGFP (F) - 2 wells
- UL41(Rep) PGKGFP (R) - 2 wells
- UL41(Rep) PGKH10myc (F) - 8 wells
- UL41(Rep) PGKH10myc (R) - 8 wells

After 7 days, CPE in the form of plaques was visible in all wells. The number of plaques in each well was counted and documented in Table 3.1. The presence of plaques in the CEF cells, following transfection with all the UL41(Rep)PGK GFP/H10myc BAC constructs, indicated that viable recombinant viruses were being produced and were spreading through the CEF cell sheet. The hypothesis that it was the CMV promoter preventing the UL41(Rep) BACs from forming CPE can now be regarded as valid. By disrupting essential genes in the UL41 region, or other MDV promoters in the UL41(Rep) CMV BACs, the presence of the CMV promoter inhibited the formation of infectious virus particles and subsequently prevented plaque formation in CEF cells.

Table 3.1 Plaque counts following transfection

Construct	Well	Plaque Number	Next step
CVI988	Well 1	74	Virus propagation
	Well 2	53	
	Well 3	102	Protein Quantification
	Well 4	61	
	Well 5	79	Myc/HIS Immunohistochemistry
	Well 6	88	
UL41(Rep) PGKGFP(F)	Well 1	122	Immunofluorescence and virus propagation
	Well 2	105	
UL41(Rep) PGKGFP(R)	Well 1	99	Immunofluorescence
	Well 2	109	
UL41(Rep) PGKH10myc(F)	Well 1	24	Virus propagation
	Well 2	16	
	Well 3	12	
	Well 4	14	
	Well 5	10	
	Well 6	10	
	Well 7	13	Myc/HIS Immunohistochemistry
	Well 8	7	
UL41(Rep) PGKH10myc(R)	Well 1	75	Virus propagation
	Well 2	67	
	Well 3	72	
	Well 4	62	Protein Quantification
	Well 5	60	
	Well 6	44	
	Well 7	52	Myc/HIS Immunohistochemistry
	Well 8	64	

The CEF cells that had been transfected with the UL41(Rep) PGKGFP BACs developed CPE. As the PGK-1 promoter was successfully driving the expression of the GFP gene, these plaques exhibited bright green fluorescence. However, to fulfil the primary objective of this study, which was to incorporate a haemagglutinin gene into the MDV viral genome, it needed to be proved that the PGK-1 promoter was also driving the expression of the H10 gene. This was carried out using immunohistochemistry and western blot techniques, utilising antibodies specific to the myc and HIS tag, which would demonstrate the presence of the H10myc protein.

3.9.2. Immunohistochemistry

CEF cells that were transfected with UL41(Rep) PGKH10myc (F and R) BAC DNA demonstrated extensive plaque formation. To determine if the PGK-1 promoter was working effectively and H10myc protein was being manufactured, CEF cells were analysed using immunohistochemistry, utilising α-myc and α-HIS antibodies (Section 2.3.13).

Mock-transfected CEF cells were analysed, as controls, and stained using α-myc, α-HIS and HB3 (α-gB) antibodies. Non-infected cells that were stained with α-myc and α-HIS antibodies were photographed using UV light; cells stained with the HB3 antibody and unstained cells were photographed using white light. As expected, the primary α-myc and HB3 antibodies did not bind to the non-infected CEF cells, due to the lack of myc and MDV gB protein, respectively, and no fluorescence was detected. However, α-HIS antibody did bind non-specifically to non-infected CEF cells, producing some background fluorescence (Figure 3.21).

MDV CVI988 BAC-transfected CEF cells displaying CPE were analysed and plaques were stained using α-myc, α-HIS and HB3 (α-gB) antibodies. Plaques stained with the α-myc antibody did not fluoresce, as expected. Plaques stained with α-HIS antibody displayed some background florescence and this would have been regarded as a false positive. Finally, plaques stained with the HB3 antibody

displayed the distinctive bright red stain under white light. Stained plaques were photographed using white and UV light (Figure 3.22).

CEF cells transfected with the UL41(Rep) PGKGFP(F and R) BAC constructs did not require antibody staining but were prepared as described in Section 2.3.13. Infected CEF cells within the plaque, expressing GFP, displayed bright green fluorescence when viewed using UV light. Plaques were also photographed using white light to observe the individual plaques and combined UV and white light to observe both simultaneously (Figure 3.23).

Used separately, staining with either the α-myc or the α-HIS antibodies resulted in plaques on the CEF cell sheet that displayed bright green fluorescence. The plaques were photographed using white light to observe the individual plaques and UV light to observe the fluorescence (Figure 3.24). Non-infected CEF cells, that separated the plaques on the cell sheet, did not display fluorescence when using the α-myc antibody. Non-infected CEF cells that were stained with α-HIS antibody, however, did display some background fluorescence indicating that this antibody bound non-specifically to CEF cells (Figure 3.21).

The immunohistochemistry results confirmed that the myc-HIS tagged H10 protein was being produced by CEF cells infected with the MDV mutants. This provided conclusive proof that the haemagglutinin gene had successfully been incorporated into the MDV genome and that the PGK-1 promoter was efficiently driving the expression of the H10 gene. To complete the *in vitro* work, the final step was to demonstrate the presence of the H10myc protein using western blot analysis.

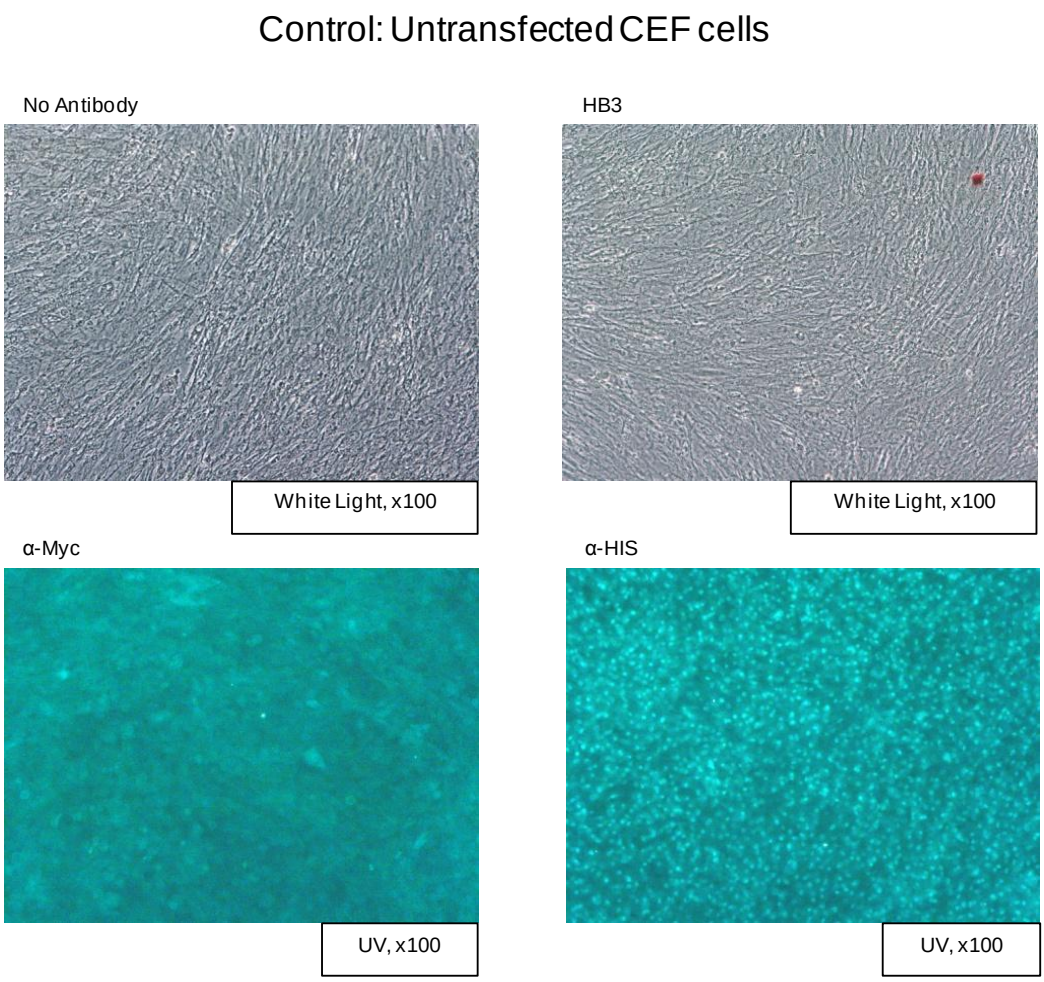


Figure 3.21 Immunohistochemistry of untransfected CEF cells using α-Myc, HB3 (α-gB) and α-HIS antibodies. Cells were photographed using white and UV light.

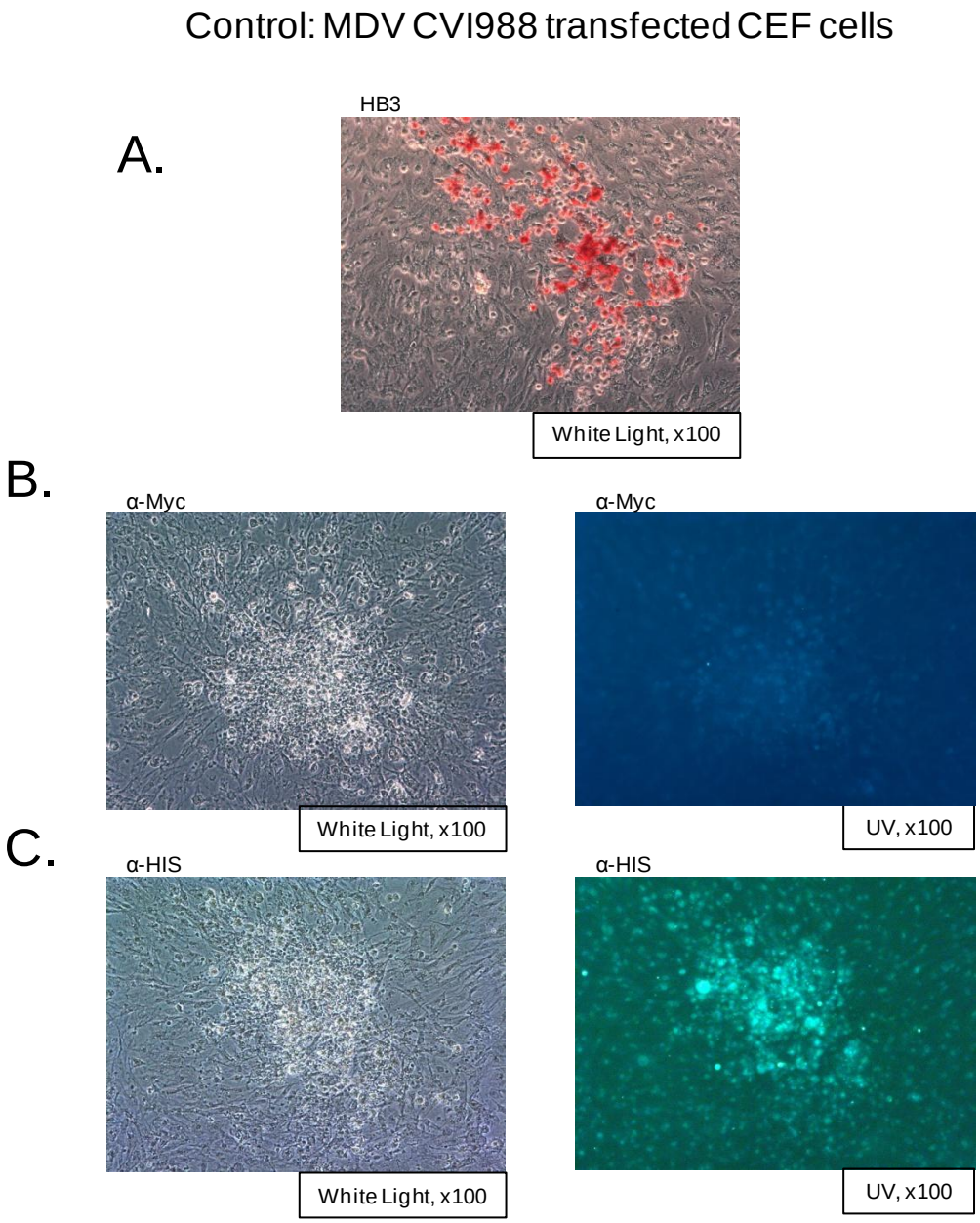


Figure 3.22 A) Immunohistochemistry of a single plaque developing on CEF cells transfected with the MDV CVI988 BAC and immunostained using HB3 (α-gB) antibody. B) Immunohistochemistry of MDV CVI988 transfected CEF cells using α-myc antibody. C) Immunohistochemistry of MDV CVI988 transfected CEF cells using α-HIS antibody.

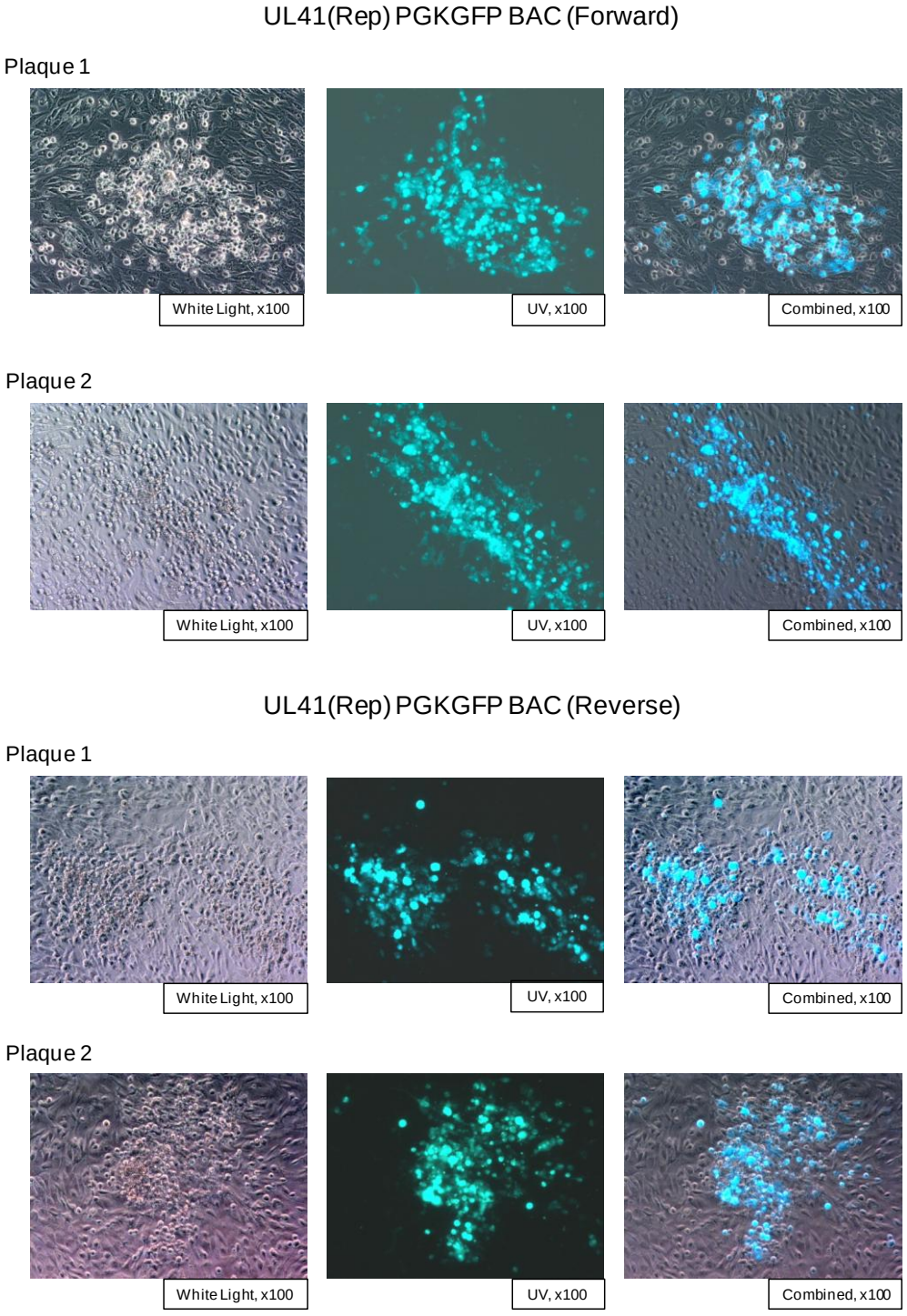


Figure 3.23 Chicken Embryo Fibroblast cells, transfected with the UL41 (Rep) PGKGFP BACs, displaying cytopathic effect in the form of plaques. The plaques were photographed using white light, UV light and both combined to observe the MDV infected CEF cells and the subsequent expression of the GFP protein.

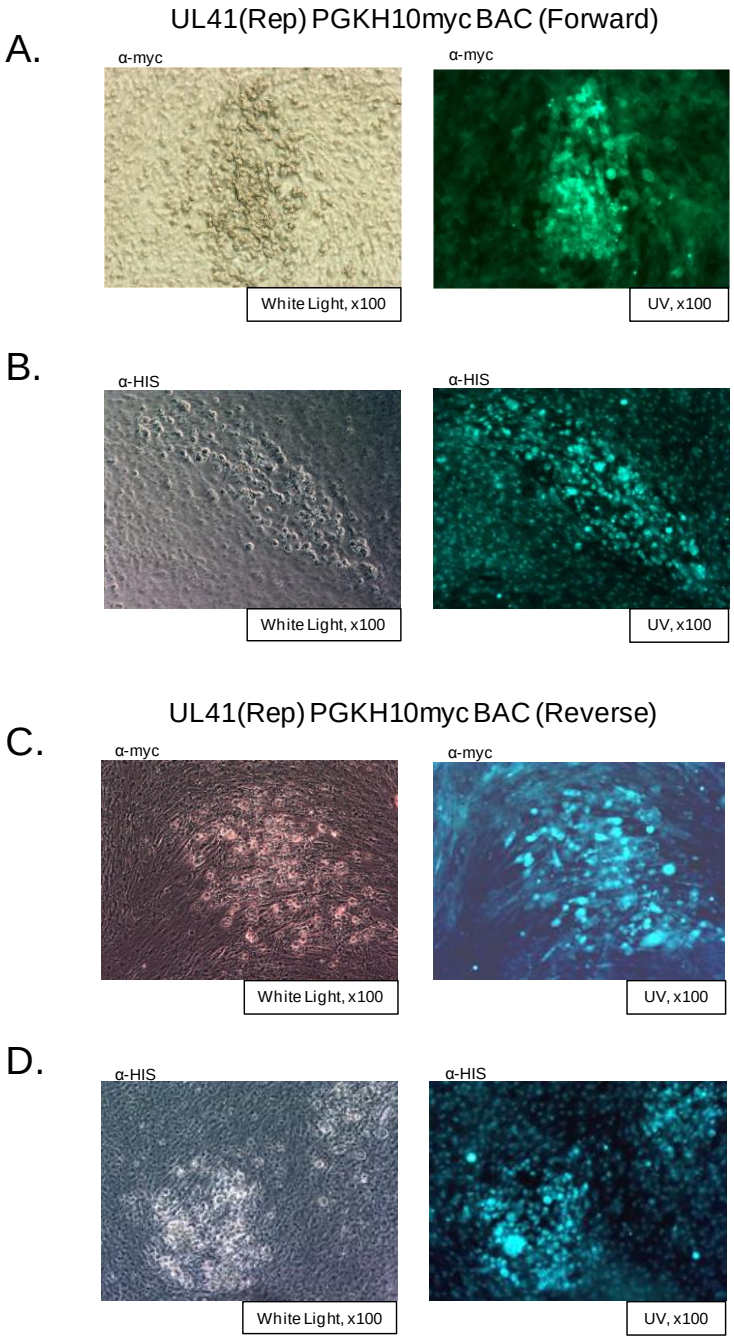


Figure 3.24 A) Immunohistochemistry of a single plaque developing on CEF cells transfected with the UL41(Rep) PGKH10myc BAC (Forward) and immunostained using the α-myc antibody. B) Immunohistochemistry of the UL41(Rep) PGKH10myc BAC (Forward) transfected CEF cells using α-HIS antibody. C) Immunohistochemistry of the UL41(Rep) PGKH10myc BAC (Reverse) transfected CEF cells using α-myc antibody. D) Immunohistochemistry of the UL41(Rep) PGKH10myc BAC (Reverse) transfected CEF cells using α-HIS antibody.

3.9.3. Western blot analysis

Western blot analysis was performed on protein samples extracted from CEF cells transfected with the following BACs: -

- CVI988
- UL41(Rep) PGKH10(R) BAC

Once extensive CPE was observable, total protein was extracted using lysis buffer containing Pefabloc, a protease inhibitor (Section 2.4.1). In addition, protein was extracted from untransfected CEF cells to be used as a negative control. Once extracted, the protein solution was diluted 1:100 (v:v) in lysis buffer and quantified using the Pierce BCA protein assay kit (Section 2.4.2). Table 3.2 displays the calculated protein concentration of each of the samples and the volume required to load 20 µg of protein into one well of an SDS-PAGE gel.

Table 3.2 Protein sample concentration (µg/ml)

Sample	Concentration	Volume for 20 µg
CEF	8153 µg/ml	2.45 µl
CVI988	8967 µg/ml	2.25 µl
UL41 H10	8804 µg/ml	2.25 µl

SDS-PAGE was performed, according to the protocol in Section 2.4.4. The protein samples were loaded into the wells, as below: -

Blank	Ladder	CEF	CVI988	UL41H10	Ladder	Blank

The SDS-PAGE was repeated five times, producing five replicates. Following electrophoresis, proteins of each of the five gels were transferred to a nitrocellulose

membrane (Section 2.4.5). These five membranes were incubated with one of the following combinations (Section 2.4.6): -

- 1^o α-Myc, 2^o biotinylated antibody and 3^o streptavidin AP
- 1^o α-HIS, 2^o biotinylated antibody and 3^o streptavidin AP
- 1^o HB3 (α-gB), 2^o biotinylated antibody and 3^o streptavidin AP
- 2^o biotinylated antibody and 3^o streptavidin AP
- 3^o streptavidin AP

Two membranes were stained without using the primary antibodies (1^o α-Myc, α-HIS or HB3) to assess if the 2^o and 3^o antibodies bound non-specifically to proteins on the membrane. Incubating a membrane with the secondary biotinylated anti-mouse IgG combined with the 3^o Streptavidin AP conjugate, or only the 3^o Streptavidin AP conjugate, should have produced a negative result. Without the presence of the primary antibodies, α-Myc, α-HIS or HB3, the 2^o biotinylated antibody and 3^o streptavidin AP should not bind.

Bound antibody was detected using the Sigmafast BCIP/NBT stain (Section 2.4.6). Protein sizes were predicted using the ExPASy proteomics server (Section 2.4.3). The H10myc-HIS protein was predicted to be approximately 80 kDa in size; the MDV gB glycoprotein was predicted to be 110 kDa in size. Proteins of this size were not detected by the HB3, α-myc or α-HIS antibodies. The 2^o biotinylated antibody and 3^o streptavidin AP both bound non-specifically. The same non-specific binding occurred for all protein samples regardless of whether they were the UL41 H10 sample or the CEF and CVI988 control protein samples that did not possess a myc-HIS tag (Figure 3.25).

Unfortunately, due to time limitations this could not be resolved, but the next step would have been to select a different 2^o biotinylated antibody and 3^o streptavidin AP that do not bind, non-specifically, to CEF cells.

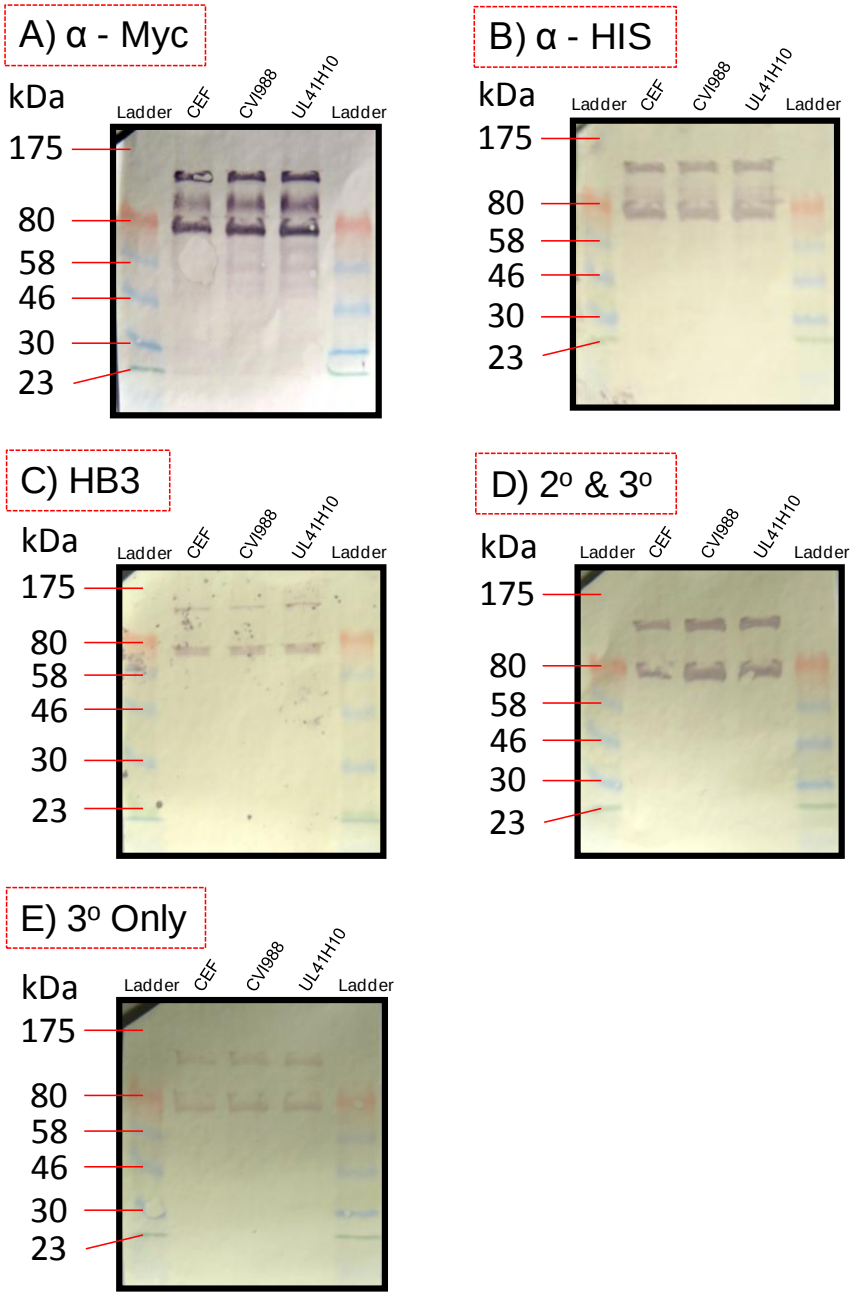


Figure 3.25 Western blot analysis was performed on protein samples extracted from untransfected CEF cells and CEF cells transfected with CVI988 and the UL41(Rep) H10 BAC. Following SDS-PAGE, proteins were transferred to nitrocellulose membranes and immunostained with: A) α-myc B) α-HIS C) HB3 D) 2^{o} biotinylated antibody and 3^{o} streptavidin AP only E) 3^{o} streptavidin AP only.

3.9.4. Recombinant virus propagation

The formation of plaques, following transfection with each of the UL41(Rep) PGK BACs, demonstrated that the viruses were replicating successfully. To prepare master stocks for each of the recombinant viruses, MDV-PGKH10 and MDV-PGKGFP, as well as the parental MDV CVI988 strain, infected CEF cells that had been transfected with BAC DNA (Section 3.9.1) were subcultured for two passages, as described in Section 2.3.9, to allow the viruses to grow to sufficient titres. Virus stocks were created for the following:-

- CVI988
- UL41(Rep)PGKGFP (F)
- UL41(Rep)PGKH10myc (F)
- UL41(Rep)PGKH10myc (R)

For each virus, two T175cm^2 flasks of infected CEF cells (Pass #2) were resuspended in 2 ml freezing solution (90% v/v FCS, 10% v/v dimethyl sulphoxide) and aliquoted into 0.2 ml quantities (Section 2.3.9). All cryovials were frozen at -80^{o}C to act as master stocks.

The infectivity titre, in PFU/ml, of these frozen stocks of the cell-associated MDV CVI988 strain and MDV mutants was determined using plaque assays, as described in Section 2.3.10. After 6 days, plaques had developed to a sufficient size and the CEF cells were fixed using ice cold 1:1 acetone-methanol. To aid visualisation of the CPE, the CEF cells were stained using an antibody specific for the MDV gB glycoprotein (Section 2.3.12). The number of plaques in each well was counted and used to determine the virus titre in Plaque Forming Unit (PFU)/ml (Table 3.3). In a commercially available MD vaccine, the standard required PFU of MD per 0.2 ml chicken dose is 1.0 x 10^3 PFU (Landman and Verschuren, 2003). The infectivity titres of the frozen stocks of MDV CVI988 strain and recombinant virus mutants were greatly in excess of this figure.

Table 3.3 Plaque numbers following a plaque assay

Construct / Titre	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	PFU/ml
CVI988	TMTC	305	93	11	0	0	$9.4x10^5$
UL41(Rep) PGKGFP (F)	232	82	12	0	0	0	$1.2x10^5$
UL41(Rep) PGKH10myc (F)	248	138	19	0	0	0	$1.9x10^5$
UL41(Rep) PGKH10myc (R)	216	74	8	1	0	0	$8.0x10^4$

3.10. Future Work

The success of the project dismissed the hypothesis that the haemagglutinin gene was toxic. As the source of the disruption was the CMV promoter, and not the H10 gene, more genes may now be incorporated into the MDV CVI988 genome. For this purpose, the neuraminidase (NA) gene and the haemagglutinin (HA) gene from the highly pathogenic strain, A/chicken/Ivory Coast/1787-35 H5N1, were kindly supplied by Dr Haas who acquired them from the Institute Zooprofilattico Sperimentale delle Venezie (Padua, Italy). Unfortunately, time constraints prevented the successful creation of MDV mutants containing these genes; however, the initial work to clone them into plasmids has been successfully achieved.

3.10.1. pTOPH5

The H5 gene was amplified using the primers, pDONRH5_For and pDONRH5_Rev, which incorporated the unique enzyme restriction sites *Xho*I and *Hin*dIII (Appendix A.1.3.1). These sites would allow the H5 gene to be cloned upstream of the myc tag in the plasmid pCDNA3.1Myc-HIS. In addition, using site directed mutagenesis, the H5 stop codon (TAG) was removed by the reverse primer. The H5 gene was successfully amplified (Section 2.1.1 and Appendix A.1.4.1) and cloned into Blunt II

TOPO (Section 2.1.2). Following transformation (Section 2.2.2), plasmid DNA was isolated from small cultures (Section 2.2.5). The successfully ligated plasmid, pTOPH5 (Figure 3.26), was confirmed using restriction enzyme digestion with *Hin*dIII and *Xho*I and *Hin*dIII.

The primers, M13 forward and reverse, were used to sequence the PGK promoter and H5 gene (Appendix A.1.3.1). Sequencing results indicated that the H5 gene had been successfully incorporated into TOPO and the site directed mutagenesis to remove the stop codon was successful.

In addition, primers were designed to amplify H5myc, once the H5 gene had been cloned upstream of the myc tag. These primers, pH5myc(rpt)_For and pH10myc_Rev, incorporated the restriction sites, *Bam*HI and *Xho*I, that would allow the gene to be cloned downstream of the PGK-1 promoter.

3.10.2. pTOPNA

Using the primers, pNA_For (Fixed) and pNA_Rev, that incorporated the unique enzyme restriction sites *Nhe*I and *Xho*I (Appendix A.1.3.1), the NA gene was successfully amplified (Section 2.1.1 and Appendix A.1.4.1). The gene was subsequently cloned into Blunt II TOPO (Section 2.1.2), competent *E. coli* cells were transformed with the ligation mix (Section 2.2.2) and plasmid DNA was isolated (Section 2.2.5). The successfully ligated plasmid, pTOPNA (Figure 3.26), was confirmed using restriction enzyme digestion with *Nhe*I and *Xho*I.

3.11. Conclusion

The primary objective in this study was to incorporate a haemagglutinin gene into the MDV viral genome. This has now been achieved and confirmed using immunohistochemistry. Attempts to insert the H10 or GFP gene, under the control of the CMV promoter, were not successful, as the promoter appears to inhibit virus gene expression, resulting in the failure of UL41(Rep) BACs to induce plaque

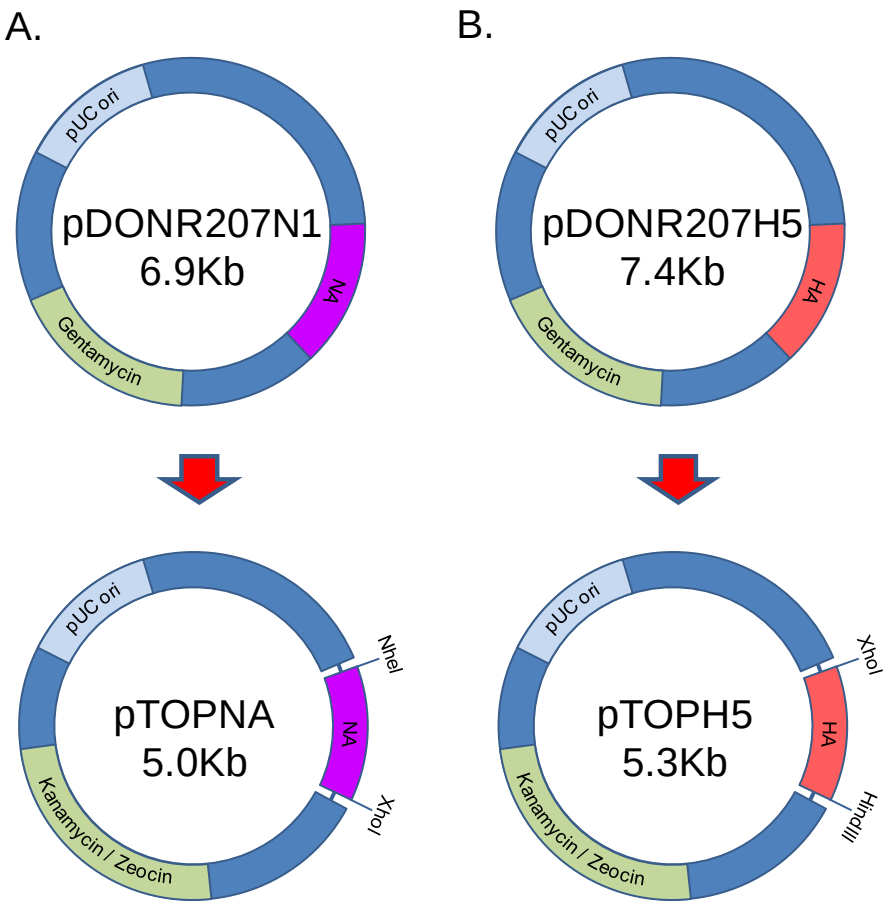


Figure 3.26 A) A diagrammatic representation of the created plasmid pTOPNA, starting with the plasmid pDONR207N1. The amplified NA gene was cloned into the TOPO vector to create pTOPNA. B) The H5 gene was amplified from the plasmid pDONR207H5, and cloned into the TOPO vector to create pTOPH5.

formation in CEF cells. Further investigation into the detrimental effect of the CMV promoter on the MDV genome could be carried out, but lies outside the scope of this current research.

MDV recombinant mutants have been propagated by passage in CEF cells and virus stocks were frozen at -80^{o}C. In collaboration with the Istituto Zooprofilattico Sperimentale delle Venezie in Italy, these will be tested in future *in vivo* studies to determine if they induce a protective level of antibody response in chickens to the H10 protein, while insuring adequate protection to MDV challenge. If so, the creation of a vaccine that allows simultaneous vaccination against both pathogens would be a major achievement.

To follow on from the successful research to introduce the H10 and GFP genes into the UL41 site of the MDV CVI988 genome, the next step will focus on the identification of a second non-essential gene site. Additional sites would allow multiple HA genes to be introduced or combinations of HA and NA genes. The next site identified as the target for the insertion of HA genes was the US10 open reading frame.

Chapter 4: The Construction and Characterisation of recombinant CVI988 Bacterial Artificial Chromosomes (BACs) expressing Haemagglutinin and GFP within the US10 Open Reading Frame

4.1. US10 Deleted BAC Construct

4.1.1. Aims and approach

Following on from the successful research to introduce the H10 and GFP genes into the UL41 site of the MDV CVI988 genome, additional sites were identified so that, in future, genes could be introduced into multiple locations. Again, as with the UL41 open reading frame, the focus was on the identification and replacement of a non-essential gene, so that unmapped and intragenic promoters could be avoided.

Initial work involved the identification and deletion of a second non-essential gene, which would allow the insertion of a novel gene (H10 or GFP) into the CVI988 genome. As in Section 3.3, the goal was to initially delete the proposed non-essential MDV gene by creating a shuttle plasmid that contained only the gene flanking sequences, thus confirming the previously published deletion studies. Once the gene was deleted, the subsequent *in vitro* analysis would reveal if the identified MDV gene was essential for virus growth in tissue culture, determined by the ability of the CVI988 (Del) BAC to form CPE in CEF cells. If the identified MDV gene was confirmed to be non-essential, then the H10 and GFP gene would be incorporated into the open reading frame. All genes inserted into the MDV viral genome would be under the control of the PGK-1 promoter.

4.1.2. Identification of the non-essential gene US10

In Section 3.1.2, several genes that had been deemed to be non-essential in the MDV genome were identified using previously published research. The US10 open reading frame was identified as a site that could potentially be targeted (Jones *et al,* 1991; Parcells *et al*, 1994; Sakaguchi *et al*, 1994). Sakaguchi *et al* (1994) demonstrated that the US10 gene, a minor virion protein of MDV-1, was an effective site for the insertion of foreign genes from which to construct a polyvalent live vaccine for poultry. They incorporated the *E. coli* lacZ gene into the US10 open reading frame of the MDV-1 genome by homologous recombination. When tested *in vitro,* the recombinant virus replicated as well as the parental strain, indicating that the US10

gene was non-essential for viral growth in tissue culture. *In vivo*, the deletion of this gene did not affect vaccine-induced immunity. Therefore, it was decided that the US10 open reading frame of MDV CVI988 would be the next target for the insertion of genes into the MDV viral genome.

4.1.3. MDV US10 gene flanking sequences

Primers were designed to amplify approximately 2 kb of the DNA sequence on either side of the US10 gene (Appendix A.1.3.2). The MDV CVI988 BAC was isolated, as described in Section 2.2.7, and used as the template. To facilitate downstream cloning, the unique restriction enzyme sites, *Kpn*I, *Pac*I and *Bam*HI, were incorporated into the primers. The flanking sequences (US10FSA and US10FSB) were successfully amplified as described in Section 2.1.1 and Appendix A.1.4.2. Both DNA fragments were gel purified (Section 2.1.9) and separately cloned into Zero Blunt II TOPO (Section 2.1.2) to create the plasmids US10pTOPFSA and US10pTOPFSB (Figure 4.1). Competent *E. coli* cells were transformed with the ligation reactions (Section 2.2.2) and plasmid DNA was isolated from small cultures (Section 2.2.5). US10pTOPFSA was shown by restriction enzyme digest analysis (*Bam*HI, *Bam*HI and *Pac*I, and *Ase*I) to contain an insert of the correct size, in the correct orientation. Digestion with *Eco*R1 demonstrated that the plasmid, US10pTOPFSB, contained an insert of the correct size (Sections 2.1.6).

4.1.4. US10pTOPFSAB

The plasmids, US10pTOPFSA and US10pTOPFSB, were sequentially digested with *Kpn*I and *Bam*HI in preparation for cloning. Following the *Kpn*I enzyme digest (Buffer 1), the DNA was ethanol precipitated (Section 2.1.5) and subsequently digested with *Bam*HI, in buffer 3. The digested fragments were gel purified according to the protocol in Section 2.1.9. The purified FSB fragment was ligated into US10pTOPFSA (Section 2.1.10) to create the plasmid, US10pTOPFSAB (Figure 4.1). Competent *E. coli* cells were transformed with the ligation mix (Section 2.2.2) and plasmid DNA was isolated according to the protocol in Section 2.2.5. The

successfully ligated plasmid was confirmed using restriction enzyme digestion with *Ase*I and *Pst*I (Section 2.1.6). Following confirmation, US10pTOPFSAB DNA was prepared according to the protocol in Section 2.2.6.

Primers were designed to sequence the US10pTOPFSAB plasmid (Appendix A.1.3.2). Sequencing results indicated that, when compared to the published sequence, no errors were present in the plasmid, US10pTOPFSAB.

4.1.5. US10pST76kFSAB

In preparation for cloning, the US10pTOPFSAB plasmid was digested with *Kpn*I and *Pac*I to isolate the FSAB insert. Digestion of US10pTOPFSAB with these enzymes, however, produced two DNA fragments very similar in size (3.6 kb and 3.5 kb) preventing gel purification. By digesting the DNA with a third enzyme, *Aat*II, this problem was resolved as it digested the smaller, vector DNA fragment into 2 kb and 1.5 kb fragments. The remaining 3.6 kb fragment (FSAB) was gel purified (Section 2.1.9) and ligated into the digested and purified pST76k plasmid (Section 3.1.10) to create the plasmid, US10pST76kFSAB (Figure 4.1). Following transformation (Section 2.2.2) the plasmid DNA was isolated (Section 2.2.5). To confirm that the ligation had been successful the isolated plasmids were digested with *Kpn*I and *Pac*I. Both colonies displayed the correct digest pattern (6.3 and 3.6 kb bands) indicating that the plasmid US10pST76kFSAB had been successfully created (Figure 4.1).

4.1.6. BAC Mutagenesis using US10pST76kFSAB

BAC mutagenesis was performed, as described in Section 2.2.9. DH5-α bacteria, that contained the MDV CVI988 BAC, were made competent (Section 2.2.8) and transformed with 10 ng of the shuttle plasmid US10pST76kFSAB, by electroporation. Kanamycin-sensitive colonies containing US10(Del) BACs, where the US10 gene had been deleted, were identified using colony PCR (Section 2.1.1 and Appendix A.1.4.2). Two sequencing primers, US10pTOPFSAB_F5 and

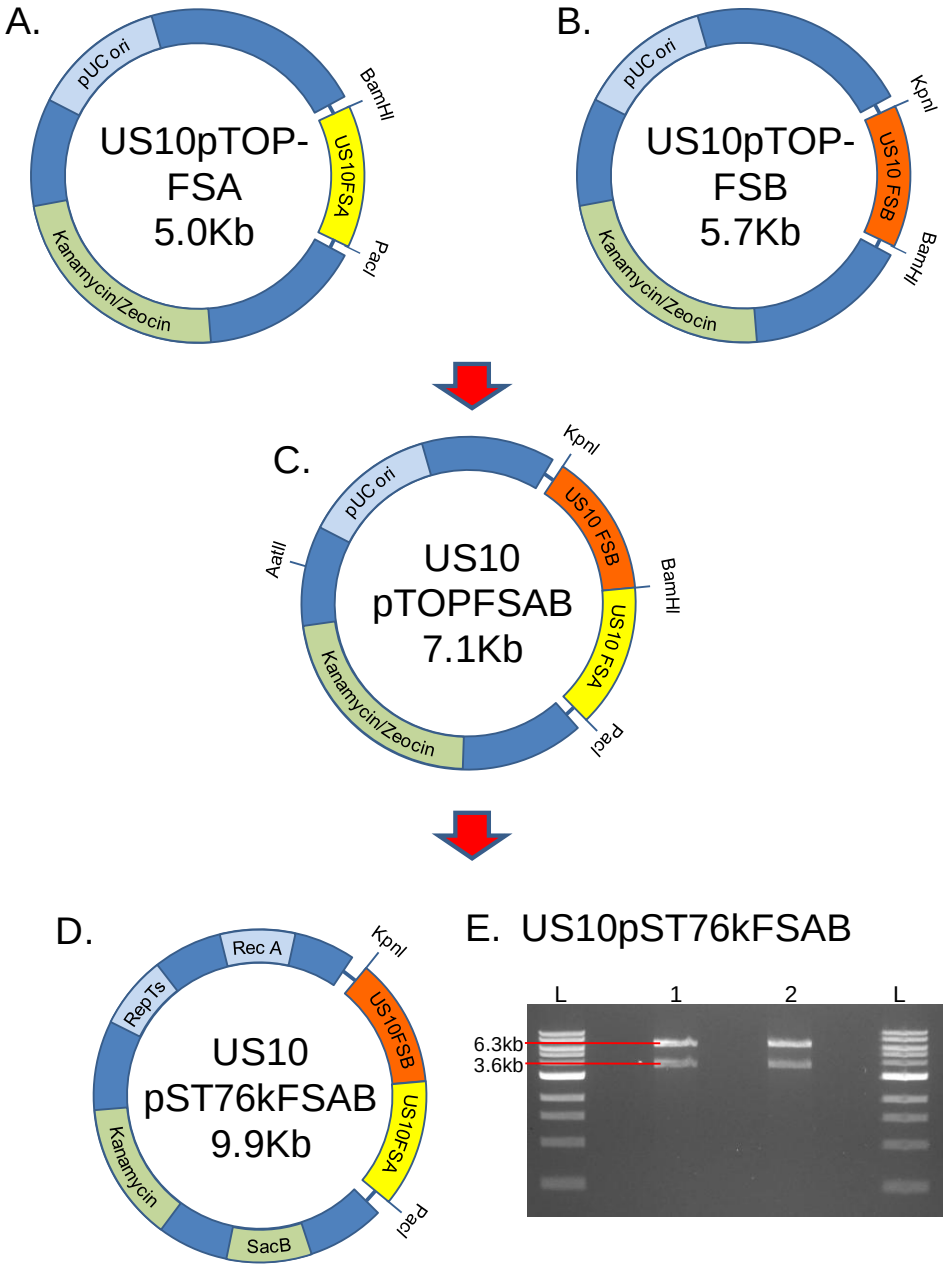


Figure 4.1 A) A diagrammatic representation of the created plasmid US10pTOPFSA. B) A diagrammatic representation of the created plasmid US10pTOPFSB C) The 2.1 kb US10FSB DNA fragment was ligated into US10pTOPFSA to create US10pTOPFSAB. D) A diagrammatic representation of the plasmid US10pST76kFSAB, created by cloning the FSAB DNA fragment into the shuttle plasmid, pST76k. E) A restriction enzyme digestion of the UL41pST76kFSAB plasmid using the enzymes, *Kpn*I and *Pac*I.

US10pTOPFSAB_R5, which lay either side of the US10 gene, were selected (Appendix A.1.3.2). By amplifying that region using PCR, the detection of a small product (854 bp) meant that the US10 gene had been successfully deleted, whereas a larger product (1301 bp) meant that the US10 gene was still intact. CVI988 BAC DNA (20 ng) was tested as a positive control and, as expected, produced an approximately 1.3 kb band. Nine colonies tested produced an approximately 0.9 kb product, indicating that the US10 gene had been deleted (Figure 4.2). A colony was selected, and the US10(Del) BAC DNA was isolated, as described in Section 2.2.7.

To confirm the successful deletion of the US10 gene, the US10(Del) BAC was sequenced with the primers US10pTOPFSAB_F5 and US10pTOPFSAB_R4. (Appendix A.1.3.2). Sequencing results indicated that the US10 gene had been effectively deleted, creating the US10(Del) BAC.

4.1.7. *In vitro* Characterisation of US10 (Del) BAC

CEF cells, seeded in six-well dishes, were transfected with MDV CVI988 BAC and the US10(Del) BAC DNA, as described in Section 2.3.8. CEF seeding and the BAC transfections were carried out simultaneously, using the 'reverse' transfection method (Section 2.3.8). For each BAC construct, three wells of a six-well dish were transfected. After 7 days, approximately 70 plaques were visible in all the wells transfected with the CVI988 and US10(Del) BAC (Figure 4.2). The presence of CPE demonstrated that viable recombinant viruses were being produced, following transfection with the US10(Del) BAC. This *in vitro* analysis confirmed the previous research, by Sakaguchi *et al* (1994), that the US10 gene was not essential for virus growth in cell culture.

Therefore, the next step was to introduce the GFP and the H10 gene into the US10 open reading frame. Previously, it had been found that the PGK promoter effectively drove the expression of the H10 and GFP genes, without disrupting the MDV CVI988 BAC. To ensure that the US10 gene site was suitable for the introduction of foreign genes, this work was repeated, inserting the GFP and H10 gene under the control of this promoter.

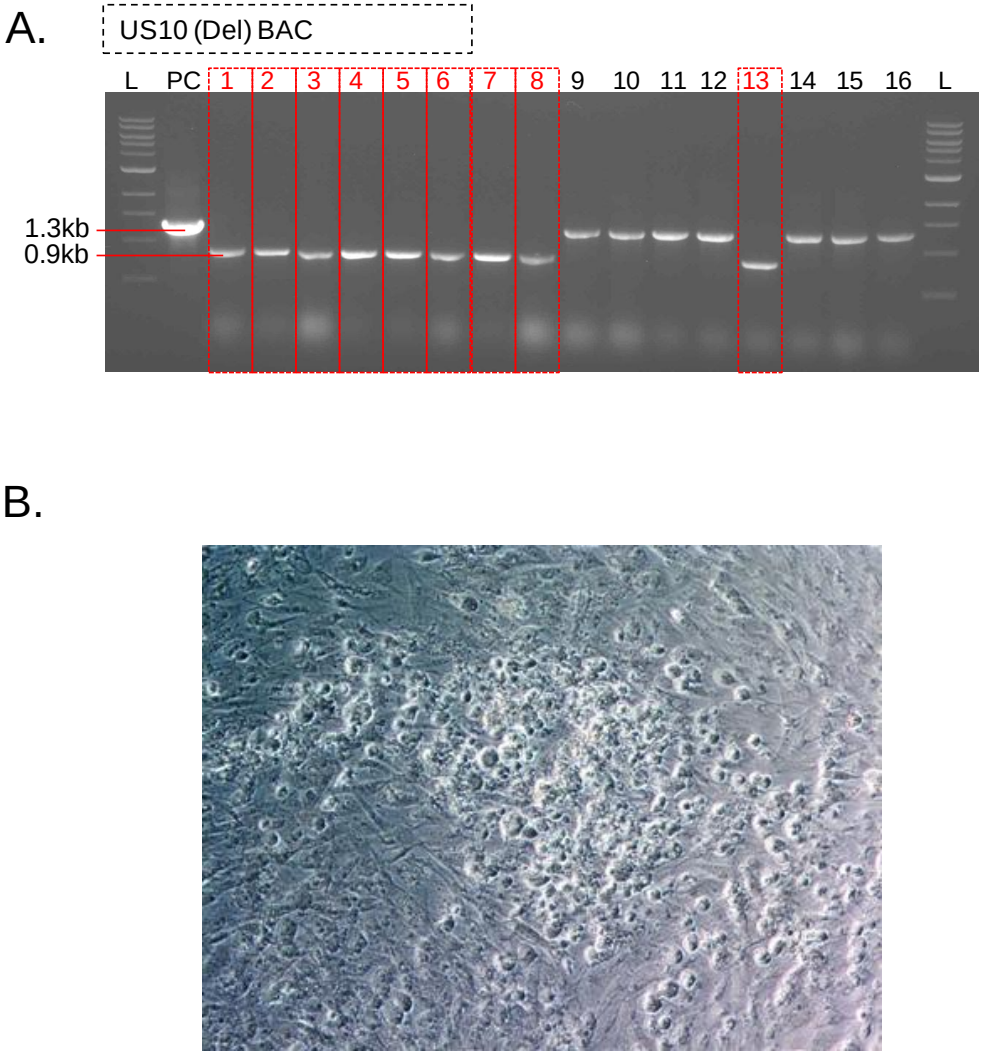


Figure 4.2 A) Agarose gel electrophoresis showing positive results from a colony PCR experiment designed to detect US10 (Del) BACs. A small product (0.9 kb) meant that the US10 gene had been successfully deleted; a large product (1.3 kb) meant that the US10 gene was still intact. B) A photograph displaying a single plaque caused by the transfection of the US10 (Del) BAC into CEF cells.

4.2. US10 (Rep) PGKGFP BAC

4.2.1. pTOPPGKGFP

This plasmid, containing the murine PGK-1 promoter upstream of the GFP gene, was created previously (Section 3.7.1 and Figure 3.14). In preparation for cloning, the PGKGFP DNA fragment had been isolated using the restriction enzymes, *Eco*RV and *Sna*BI, and gel purified (Section 3.7.3).

4.2.2. US10pTOPFSAB

In preparation for blunt cloning, the plasmid US10pTOPFSAB (Section 4.1.3) was digested with *Bam*HI. Following digestion, the 5' overhangs of the US10pTOPFSAB plasmid DNA (5 µg) were converted to 5' phosphorylated, blunt ended DNA (Section 2.1.7). Subsequently, the digested and blunted plasmid DNA was gel purified (Section 2.1.9) and treated with Antarctic Phosphatase (AP) (Section 2.1.8).

4.2.3. US10pTOPFSABPGKGFP

The digested and purified PGKGFP fragment (Section 4.2.1) was ligated into the *Bam*HI digested, blunted and AP-treated US10pTOPFSAB plasmid (Section 4.2.2). Competent *E. coli* cells were transformed with the ligation reaction (Section 2.2.2) and plasmid DNA was isolated from small cultures (Section 2.2.5). The plasmids were digested with *Pst*I and *Apa*I to confirm that the PGKGFP insert had ligated into US10pTOPFSAB successfully and to determine the orientation of the insert. Agarose gel electrophoresis revealed that five plasmids were found to contain the PGKGFP insert in the forward orientation, and two in the reverse orientation (Figure 4.3). To confirm the successful ligation of PGKGFP, both of the plasmids, US10pTOPFSABPGKGFP (F and R), were sequenced using the primers US10 pTOPFSAB_F5 and US10pTOPFSAB_R4 (Appendix A.1.3.2). Sequencing results

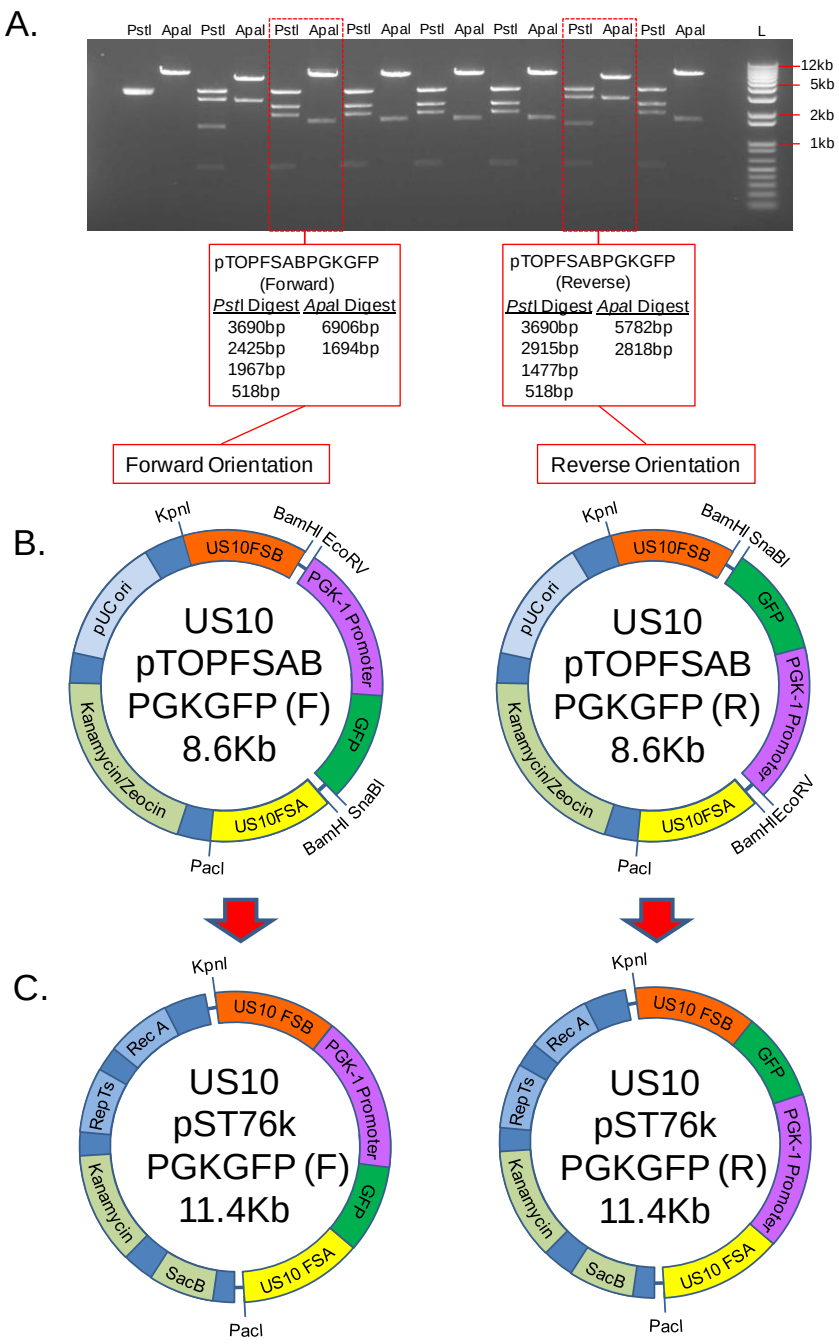


Figure 4.3 A) Agarose gel electrophoresis showing restriction enzyme digests of the plasmids pTOPFSABPGKGFP (F and R) using the enzymes *Pst*I and *Apa*I. B) A diagrammatic representation of the plasmids US10pTOPFSABPGKGFP (F and R). The PGK-GFP DNA fragment was blunt cloned into the plasmid US10pTOPFSAB, between the flanking sequences A and B. C) In the final cloning step, the FSABPGKGFP DNA fragments were transferred to the shuttle plasmid, pST76k, to create the plasmids US10pST76kPGKGFP (F and R).

indicated that for both plasmids, the PGKGFP insert had been correctly inserted between the flanking sequences.

4.2.4. US10pST76kPGKGFP (F and R)

The plasmids, US10pTOPFSABPGKGFP (F and R) were digested with *Kpn*I and *Pac*I and gel purified, in preparation for cloning with the shuttle plasmid, pST76k (Section 3.1.10). The digested FSABPGKGFP fragments were ligated into the digested and purified pST76k plasmid to create the plasmids US10pST76kPGKGFP(F and R) (Figure 4.3). Following transformation (Section 2.2.2), the plasmid DNA was isolated (Section 2.2.5). The plasmids were digested with *Bam*HI to confirm that the ligation was successful. With this enzyme, digestion of the plasmid US10pST76kPGKGFP (F) was expected to produce an 8.7 kb and a 2.7 kb DNA fragment; the plasmid US10pST76kPGKGFP (R) was expected to produce an 8.3 kb and a 3.1 kb DNA fragment. The correct digest pattern was displayed for both constructs.

4.2.5. BAC Mutagenesis using the plasmids US10pST76kPGKGFP (F) and US10pST76kPGKGFP (R)

BAC mutagenesis was successfully carried out for both plasmids (Section 2.2.9). DH5-α bacteria containing the MDV CVI988 BAC were made competent (Section 2.2.8) and transformed with 10 ng of the shuttle plasmids, US10pST76kPGKGFP (F and R), using electroporation. Kanamycin-sensitive colonies were tested using colony PCR (Section 2.1.1 and Appendix A.1.4.2) to identify those that had the US10 gene replaced with the GFP gene. The primers, p2AGFP_For and p2AGFP_Rev (Appendix A.1.3.2), were used to identify the GFP gene. pTOPGFP plasmid DNA (Section 6.2.2) was used as a positive control and produced expected results. BAC mutagenesis performed with the US10pST76kPGKGFP (F) shuttle plasmid produced 10 colonies that were positive for the GFP insert. BAC mutagenesis carried out with the US10pST76kPGKGFP (R) shuttle plasmid yielded 13 colonies which were positive for the GFP insert (Figure 4.4). Subsequently, the

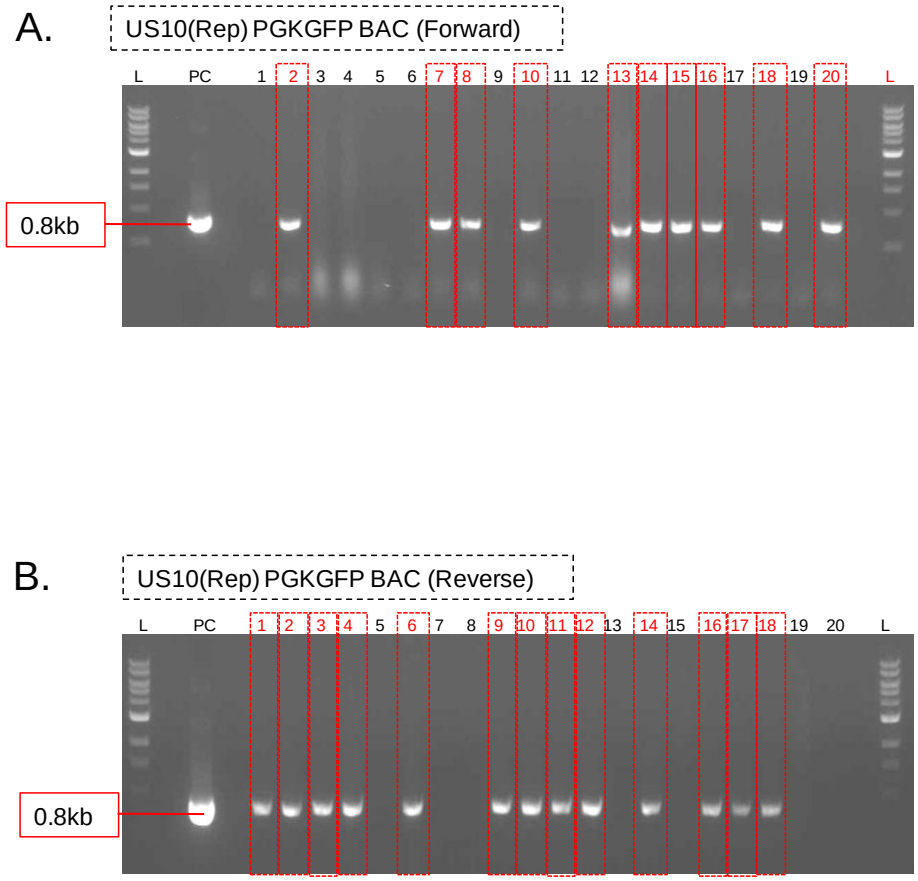


Figure 4.4 A) An agarose gel electrophoresis photograph showing positive results from a colony PCR experiment designed to detect the GFP gene in the US10(Rep) PGKGFP BAC (F). B) Agarose gel electrophoresis showing positive results from a colony PCR experiment designed to detect the GFP gene in the US10(Rep) PGKGFP BAC (R).

BAC DNA of one US10(Rep) PGKGFP(F) BAC and one US10(Rep) PGKGFP(R) BAC was selected and isolated as described in Section 2.2.7.

To confirm the successful insertion of PGKGFP into the MDV CVI988 genome, both US10(Rep) BACs were sequenced using the primers US10pTOPFSAB_F5 and US10pTOPFSAB_R4 (Appendix A.1.3.2). Sequencing results indicated that for both US10(Rep) PGKGFP (F and R) the PGKGFP insert had successfully replaced the US10 gene.

4.3. US10(Rep) PGKH10myc BAC

4.3.1. pTOPPGKH10myc

This plasmid was constructed previously (Section 3.8.2 and Figure 3.17). The H10 gene was initially cloned upstream of a Myc-HIS tag (Section 3.8.1). Subsequently, the H10myc DNA fragment was cloned downstream of the PGK-1 promoter to create the plasmid pTOPPGKH10myc. In preparation for cloning, the PGKH10myc DNA fragment was digested with the restriction enzymes, *Eco*RV and *Sna*BI, and gel purified (Section 3.8.3).

4.3.2. US10pTOPFSABPGKH10myc

The purified PGKH10myc fragment (Section 4.3.1) was ligated into the *Bam*HI digested, blunted and AP-treated US10pTOPFSAB plasmid (Section 4.2.2). Competent *E. coli* cells were transformed with the ligation mix (Section 2.2.2) and plasmid DNA was isolated from small cultures (Section 2.2.5). The plasmids were digested with *Pst*I and *Apa*I to confirm the ligation was successful and to ascertain the orientation of the insert. Three plasmids were found to contain the PGKH10myc insert in the forward orientation, and five plasmids with the insert ligated in the reverse orientation (Figure 4.5). The plasmids, US10pTOPFSABPGKH10myc (F and R), were sequenced to confirm the successful ligation of the PGKH10myc insert.

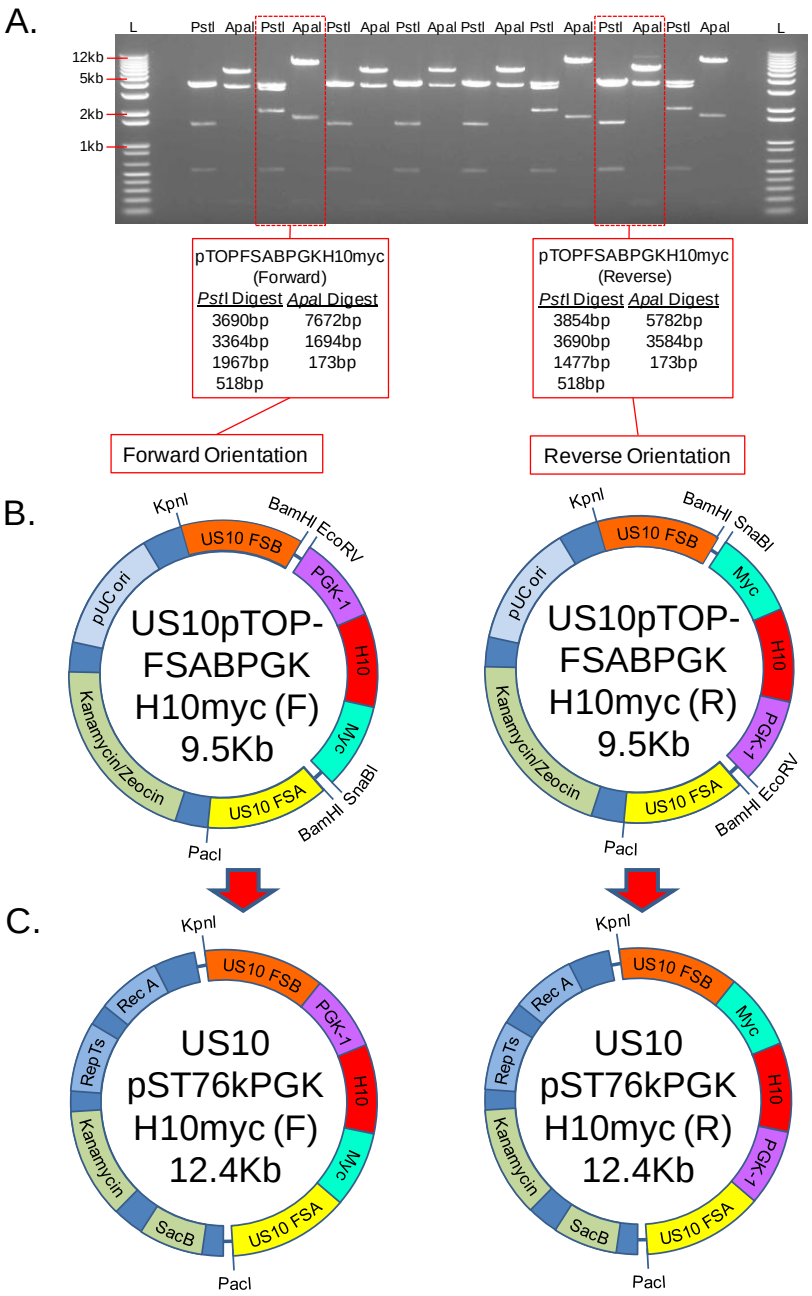


Figure 4.5 A) Agarose gel electrophoresis showing restriction enzyme digests of the plasmids pTOPFSABPGKH10myc (F and R), using the enzymes *Pst*I and *Apa*I. B) A diagrammatic representation of the plasmids US10pTOPFSABPGKH10myc (F and R). The PGK-H10myc DNA fragment was blunt cloned into the plasmid US10pTOPFSAB. C) In the final cloning step, the FSABPGKH10myc DNA fragments were transferred to the shuttle plasmid, pST76k, to create the plasmids US10pST76kPGKH10myc (F and R).

Sequencing was carried out using the primers US10pTOPFSAB_F5 and US10pTOPFSAB_R4 (Appendix A.1.3.2). Sequencing results indicated that for both plasmids, the PGKH10myc insert had been correctly inserted between the flanking sequences.

4.3.3. US10pST76kPGKH10myc (F and R)

The plasmids, US10pTOPFSABPGKH10myc (F and R), were digested with *Kpn*I and *Pac*I in preparation for cloning with the shuttle plasmid, pST76k. The digested FSABPGKH10myc fragments were gel purified (Section 2.1.9) and ligated into the digested and purified pST76k plasmid (Section 3.1.10), to create the plasmids US10pST76kH10myc (F and R) (Figure 4.5). Competent *E. coli* cells were transformed with the ligation mix, as described in Section 2.2.2 and plasmid DNA was isolated (Section 2.2.5). To confirm that the ligation was successful, the plasmids were digested with the restriction enzyme, *Bam*HI. With this enzyme, digestion of the plasmid US10pST76kH10myc(F) would produce a 9.6 kb and a 2.7 kb DNA fragment; the plasmid US10pST76kH10myc(R) was expected to produce an 8.3 kb and a 4.1 kb DNA fragment. For both constructs, US10pST76kH10myc (F and R), the enzyme digests produced the expected digest pattern.

4.3.4. BAC Mutagenesis using the plasmids US10pST76kPGKH10myc (F) and US10pST76kPGKH10myc (R)

BAC mutagenesis was carried out according to the protocol in Section 2.2.9. DH5-α bacteria containing the MDV CVI988 BAC were made competent (Section 2.2.8) and transformed with 10 ng of the shuttle plasmids, US10pST76kPGKH10myc (F and R). US10(Rep) BACs, where the US10 gene had been replaced with the H10myc gene, were identified using colony PCR (Section 2.1.1 and Appendix A.1.4.2). The primers, pH10myc_For and pH10myc_Rev (Appendix A.1.3.2) were used to identify the 1.7 kb H10myc insert. The plasmid, pTOPPGKH10myc (Section 3.8.2), was used as a positive control and produced expected results. BAC mutagenesis, performed with the US10pST76kPGKH10myc (F) shuttle plasmid, yielded one colony that was

positive for the H10myc insert. BAC mutagenesis carried out with the US10pST76k PGKH10myc(R) shuttle plasmid produced three colonies that were positive for the H10myc insert (Figure 4.6). Subsequently, the BAC DNA of a US10(Rep) PGKH10myc(F) BAC and a US10(Rep) PGKH10myc(R) BAC was selected and isolated as described in Section 2.2.7.

Using the primers US10pTOPFSAB_F5 and US10pTOPFSAB_R4 (Appendix A.1.3.2 and A.1.4.2), sequencing was used to confirm the successful insertion of PGKH10myc into the CVI988 BAC genome. Sequencing results indicated that for US10(Rep) PGKH10myc (F and R) the PGKH10myc insert had successfully replaced the US10 gene.

4.4. *In vitro* Characterisation of the US10(Rep) BACs

The *in vitro* analysis of the US10(Rep) BAC constructs was carried out in parallel with the studies of the UL41(Rep) PGK constructs. For clarity, the data arising from transfections using the MDV CVI988 BAC have also been included here. These data can also be found in Section 3.9.

BAC transfections were carried out using the 'reverse' transfection method described in Section 2.3.8. CEF cells were seeded at a density of 5.0 x10 ^{5}cell per well in six-well dishes and simultaneously transfected with the following BACs, according to the protocol in Section 2.3.8:-

- US10(Rep) PGKGFP (F) - 2 wells
- US10(Rep) PGKGFP (R) - 2 wells
- US10(Rep) PGKH10myc (F) - 7 wells
- US10(Rep) PGKH10myc (R) - 5 wells

After 7 days, cytopathic effect (CPE) in the form of plaques was visible in all wells. The number of plaques in each well was counted and documented in Table 4.1.

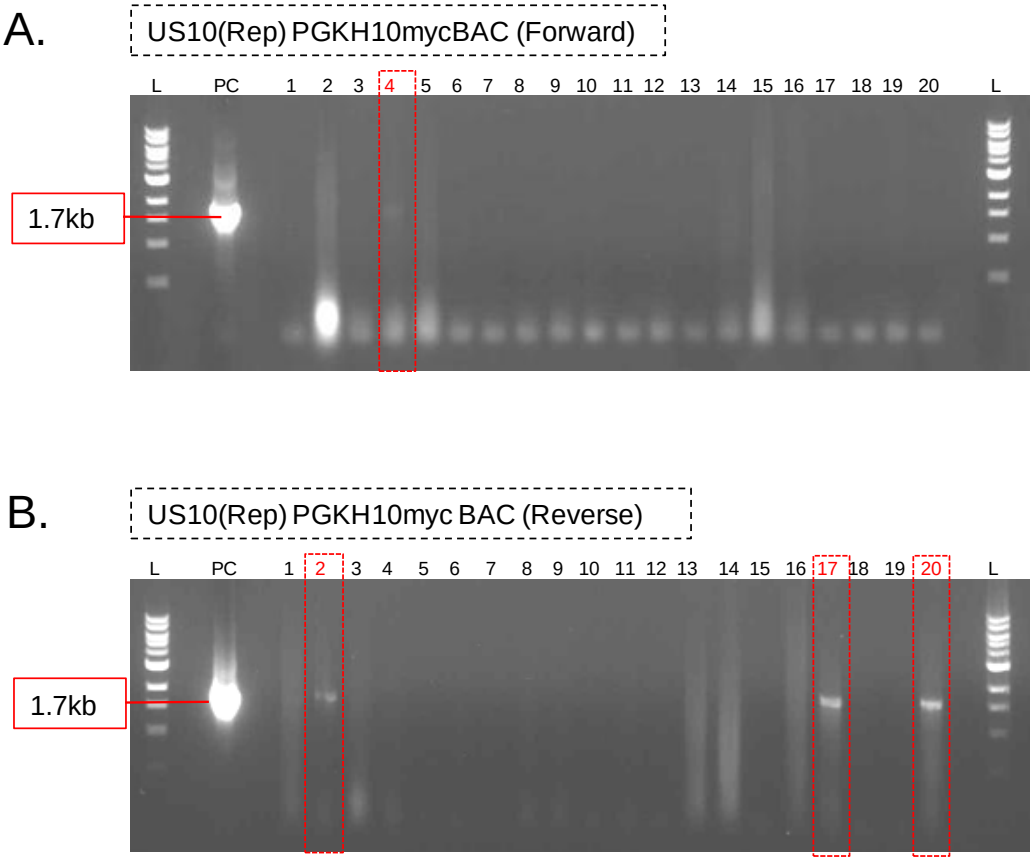


Figure 4.6 A) Agarose gel electrophoresis photograph showing positive results from a colony PCR experiment designed to detect an approximately 1.7 kb H10myc DNA fragment in the US10(Rep) PGKH10myc BAC (F). B) Agarose gel electrophoresis showing positive results from a colony PCR experiment designed to detect an approximately 1.7 kb H10myc DNA fragment in the US10(Rep) PGKH10myc BAC (R).

Table 4.1 Plaque counts following transfection

Construct	Well	Plaque Number	Next step
CVI988	Well 1	79	Virus Propagation
	Well 2	53	
	Well 3	102	Protein Quantification
	Well 4	61	
	Well 5	79	Myc/HIS Immunohistochemistry
	Well 6	88	
US10(Rep) PGKGFP (F)	Well 1	129	Immunofluorescence and virus propagation
	Well 2	141	
US10(Rep) PGKGFP (R)	Well 1	121	Immunofluorescence
	Well 2	101	
US10(Rep) PGKH10myc (F)	Well 1	139	Virus Propagation
	Well 2	128	
	Well 3	101	
	Well 4	109	Protein Quantification
	Well 5	146	
	Well 6	137	Myc/HIS Immunohistochemistry
	Well 7	144	
US10(Rep) PGKH10myc (R)	Well 1	138	Virus Propagation
	Well 2	151	
	Well 3	133	
	Well 4	78	Myc/HIS Immunohistochemistry
	Well 5	59	

The presence of CPE indicated that viable recombinant viruses were being produced following transfection with the US10(Rep) BACs. This indicated that it was possible to insert foreign genes, under the control of the PGK-1 promoter, into the US10 open reading frame of the MDV CVI988 genome.

The CEF cells that had been transfected with the US10(Rep) PGKGFP BAC DNA exhibited bright green fluorescence. This established that the PGK-1 promoter was successfully driving the expression of the GFP gene. In order to determine if the PGK-1 promoter was also prompting the expression of the H10myc gene in CEF cells transfected with the US10(Rep)PGKH10myc BAC constructs, western blot techniques and immunohistochemistry were used to demonstrate the presence of the H10myc protein.

4.4.1. Immunohistochemistry

CEF cells, demonstrating extensive CPE following transfection with the US10(Rep) PGKH10myc (F and R) BAC DNA were analysed using immunohistochemistry. The antibody, α-myc, was used to detect the presence of the H10myc protein and to determine if it was being produced by MDV infected cells. Previously, in Section 3.9.2, the α-HIS antibody had been found to bind non-specifically to CEF cells so this was not used again.

As controls, mock-transfected CEF cells were incubated with α-myc, α-HIS and HB3 (α-gB) antibodies. CEF cells that had been transfected with MDV CVI988 BAC DNA and which had subsequently developed CPE were also stained with α-myc, α-HIS and HB3 antibodies. These data can be seen in Section 3.9.2 and in Figures 3.21 and 3.22.

CEF cells transfected with the US10(Rep) PGKGFP constructs developed plaques that expressed GFP so did not require antibody staining. CEF cells transfected with US10(Rep) PGKGFP BAC DNA were prepared as described in Section 2.3.13.

Plaques were photographed using white light to observe the individual plaques and combined UV and white light to observe the cells and the GFP fluorescence simultaneously (Figure 4.7).

CEF cells, transfected with the US10(Rep) PGKH10myc BAC constructs, were incubated with the α-myc antibody (Section 2.3.13). This resulted in plaques on the CEF cell sheet that displayed bright green fluorescence when viewed using a fluorescent microscope. This was confirmation that the CEF cells, infected by the recombinant US10(Rep) MDV viruses, were also expressing the myc-tagged H10 protein. The plaques were photographed using white light to observe the individual plaques and UV light to observe the fluorescence (Figure 4.8).

The immunohistochemistry results confirm that the plaques of infected CEF cells, formed as a result of the transfection with US10(Rep) PGKH10myc BAC DNA, produced the myc-tagged H10 protein. This demonstrates that the PGK-1 promoter is successfully driving the expression of the H10 protein and confirms that it had been fully incorporated into the MDV viral genome. This also proves that foreign genes can be incorporated into two gene sites of the MDV genome, UL41 and US10. To complete the *in vitro* work, western blot analysis was used to try and determine the presence of the H10myc protein.

4.4.2. Western Blot analysis

Western blot analysis was performed on protein samples extracted from CEF cells transfected with the following BACs: -

- CVI988
- US10(Rep) PGKH10 BAC (F)

Total protein was extracted using lysis buffer containing Pefabloc (Section 2.4.1), once extensive CPE was observable. Protein samples from untransfected CEF cells and the CVI988 transfected CEF cells were prepared previously in Section 3.9.3.

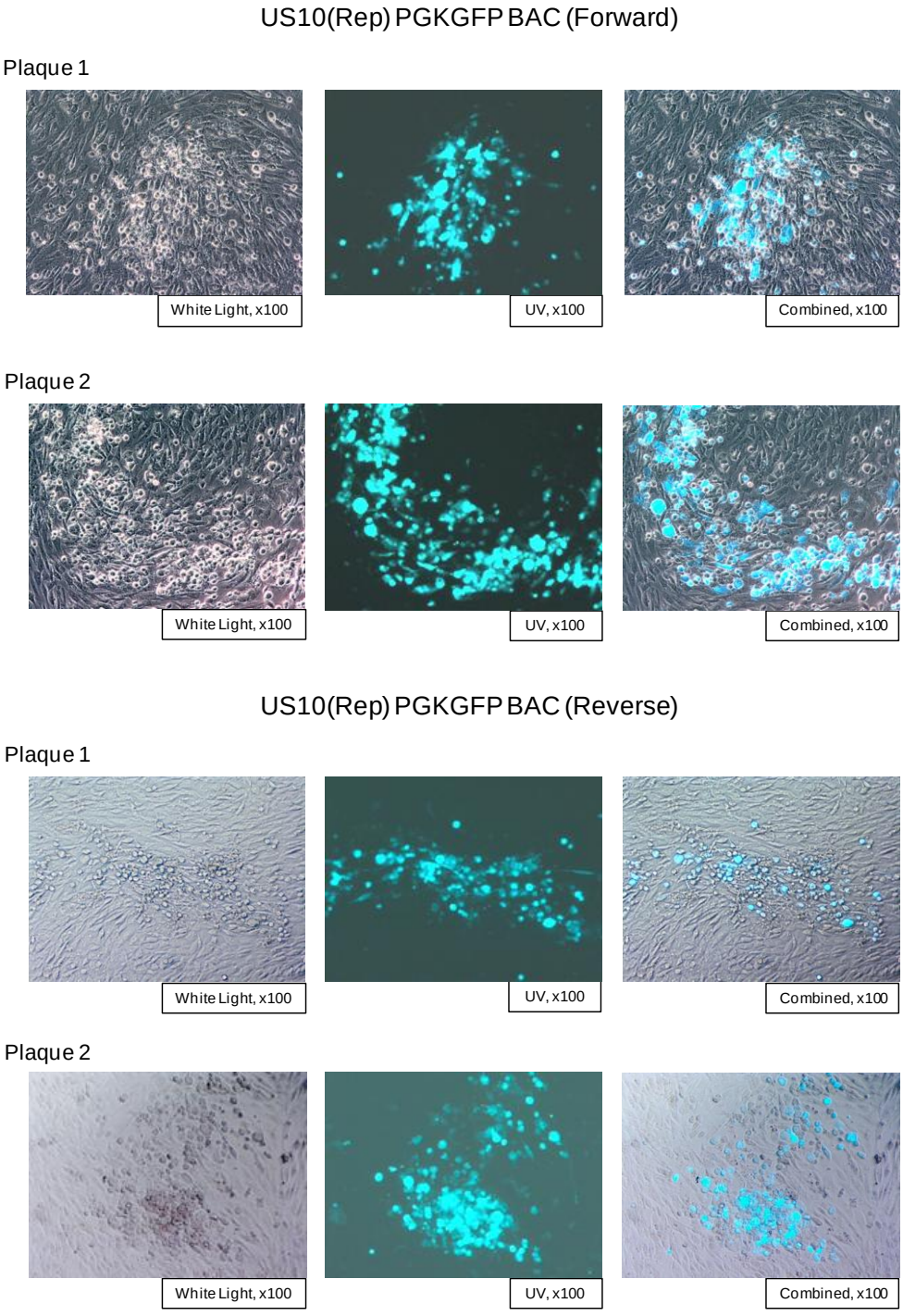
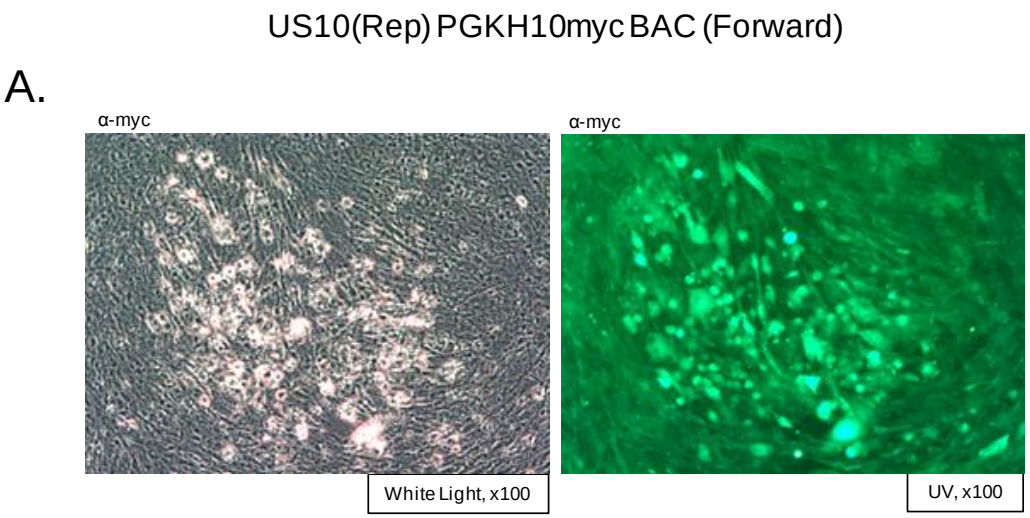


Figure 4.7 CEF cells, transfected with the US10(Rep) PGKGFP BACs, displaying cytopathic effect in the form of plaques. The plaques were photographed using white light, UV light and both combined to observe the MDV infected CEF cells and the subsequent expression of the GFP fluorescent protein.



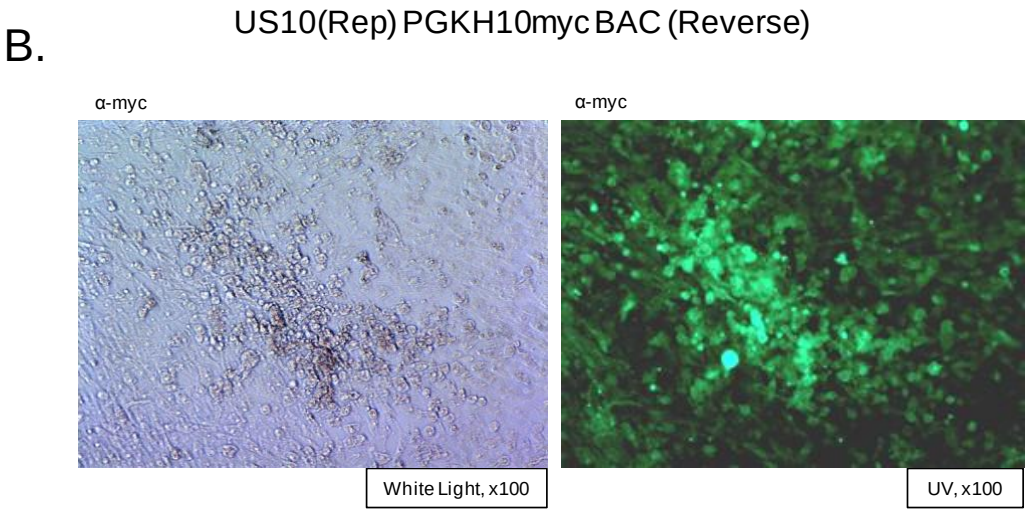


Figure 4.8 A) Immunohistochemistry of a single plaque developing on CEF cells transfected with the US10(Rep) PGKH10myc (F) BAC using the α-myc antibody. B) Immunohistochemistry of the US10(Rep) PGKH10myc (R) BAC transfected CEF cells using the α-myc antibody.

Once extracted, protein samples were diluted 1:100 (v:v) in lysis buffer and quantified using the Pierce BCA protein assay kit (Section 2.4.2). Table 4.2 displays the calculated concentrations of each of the samples and the volume required to load 20 µg of protein into one well of an SDS-PAGE gel. For clarity, the protein concentrations of the previously calculated CEF only and CVI988 samples are included (Section 3.9.3, Table 3.2): -

Table 4.2 Protein sample concentration (µg/ml)

Sample	Concentration	Volume for 20 µg
CEF	8153 µg/ml	2.45 µl
CVI988	8967 µg/ml	2.25 µl
US10 H10	9292 µg/ml	2.15 µl

SDS-PAGE was performed, according to the protocol in Section 2.4.4. The protein samples (20 µg) were loaded into the wells, as below: -

Blank	Ladder	CEF	CVI988	US10H10	Ladder	Blank

The SDS-PAGE was repeated five times, producing five replicate gels. Following electrophoresis, proteins of each of the five gels were transferred to a nitrocellulose membrane (Section 2.4.5). These five membranes were incubated with one of the following combinations (Section 2.4.6): -

- 1^o α-Myc, 2^o biotinylated antibody and 3^o streptavidin AP
- 1^o α-HIS, 2^o biotinylated antibody and 3^o streptavidin AP
- 1^o HB3 (α-gB), 2^o biotinylated antibody and 3^o streptavidin AP
- 2^o biotinylated antibody and 3^o streptavidin AP
- 3^o streptavidin AP

As in Section 3.9.3, two membranes were stained with the secondary biotinylated anti-mouse IgG combined with the 3^{o} Streptavidin AP conjugate, or only the 3^{o} Streptavidin AP conjugate only. By not incubating the membranes with the primary antibodies, α-Myc and α-HIS, it could be determined if the 2^{o} biotinylated antibody and/or 3^{o} streptavidin AP bound non-specifically to the nitrocellulose membranes.

As determined previously in Section 3.9.3, the H10myc-HIS protein was expected to be approximately 80 kDa in size; the MDV gB glycoprotein was predicted to be 110 kDa in size. Bound antibody was detected using the Sigmafast BCIP/NBT stain (Section 2.4.6). As was the case with the protein samples extracted from CEF cells, transfected with the UL41(Rep) PGKH10myc BAC DNA, western blots failed to detect proteins of appropriate size using the HB3, α-myc or α-HIS antibodies. In addition, the 2^{o} biotinylated antibody and 3^{o} streptavidin AP both bound non-specifically (Figure 4.9). Due to time limitations, this could not be resolved but future work would involve the selection of a different 2^{o} biotinylated antibody and 3^{o} streptavidin AP that did not bind non-specifically.

4.4.3. Recombinant virus propagation

To prepare master stocks of each of the recombinant viruses, US10MDV-PGKH10 and US10MDV-PGKGFP, infected CEF cells that had been transfected with the US10 (Rep) BAC constructs were subcultured for two passages (Section 2.3.9) using fresh CEF cells, to allow the viruses to grow to sufficient titres. Once widespread CPE was visible in the T175cm^2 flasks, virus stocks were created for the following: -

- US10(Rep) PGKGFP (F)
- US10(Rep) PGKH10myc (F)
- US10(Rep) PGKH10myc (R)

In each case, two T175cm^2 flasks of infected CEF cells (Pass #2) were resuspended in 2 ml freezing solution (90% v/v FCS, 10% v/v DMSO) and aliquoted in 200 µl quantities (Section 2.3.9). All cryovials were frozen at -80^{o}C to act as master stocks.

To determine the infectivity titre of the frozen stocks of cell-associated MDV US10(Rep) viruses, plaque assays were performed according to the protocol in Section 2.3.10. Six days after the CEF cell-associated MDV US10(Rep) viruses were introduced to the fresh CEF monolayer, plaques had developed to a sufficient size. Using ice cold 1:1 acetone-methanol, the CEF cells were fixed and stained using the HB3 antibody (Section 2.3.12). The number of plaques in each well was counted, to determine the virus titre in PFU/ml (Table 4.3). The infectivity titres of the frozen stocks of recombinant US10(Rep) viruses were all in excess of the commercial standard of $1.0x10^3$ PFU per 0.2 ml chicken vaccine dose.

Table 4.3 Plaque numbers following a plaque assay

Construct / Titre	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	PFU/ml
US10(Rep) PGKGFP (F)	251	116	59	3	0	0	$5.9x10^5$
US10(Rep) PGKH10myc (F)	199	86	16	2	0	0	$1.6x10^5$
US10(Rep) PGKH10myc (R)	TMTC	224	69	1	0	0	$6.9x10^5$

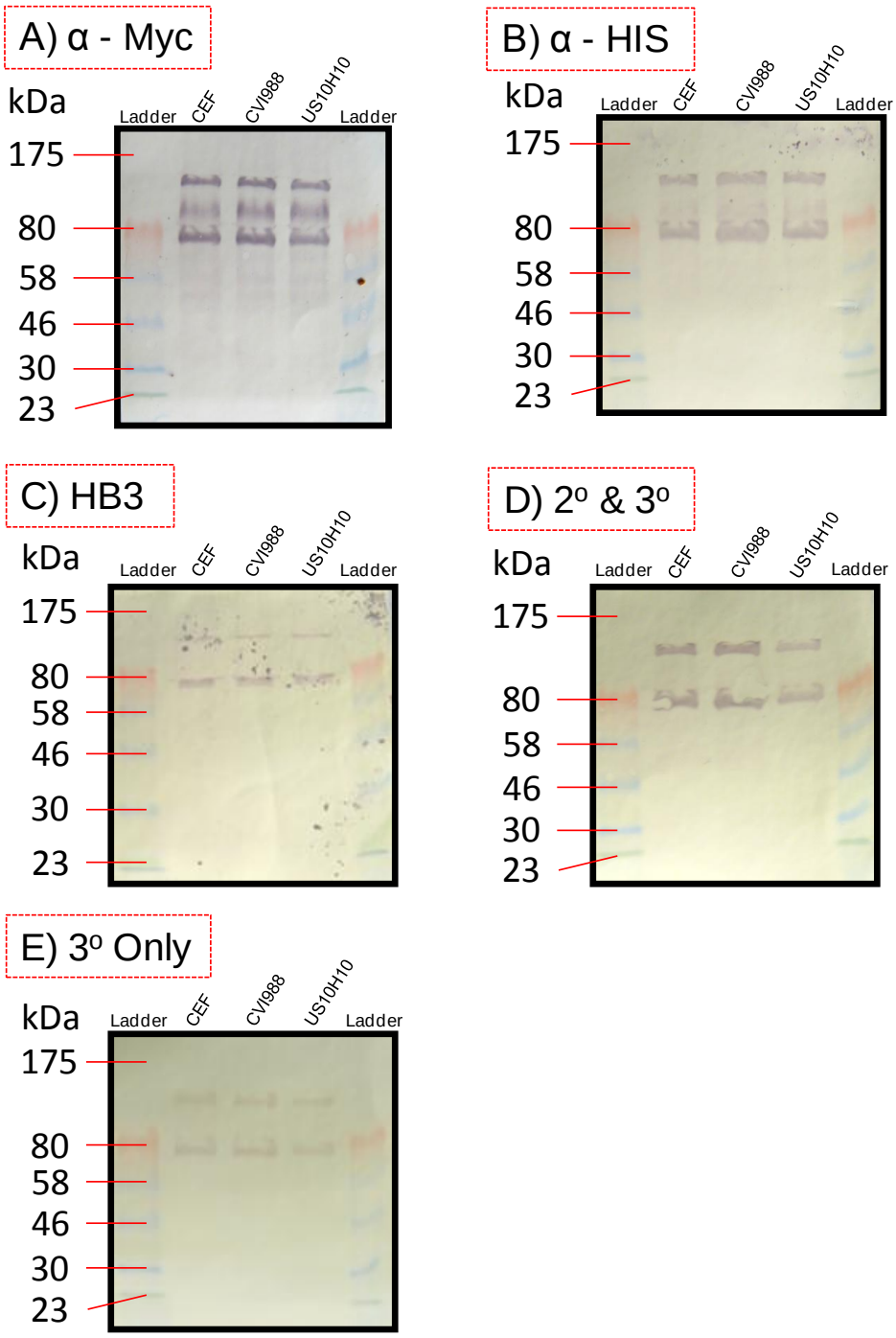


Figure 4.9 Western blot analysis was performed on protein samples extracted from untransfected CEF cells and CEF cells transfected with CVI988 and the mutant US10 H10 BAC. Following SDS-PAGE, proteins were transferred to nitrocellulose membranes and immunostained with: A) α-myc B) α-HIS C) HB3 D) 2^{o} biotinylated antibody and 3^{o} streptavidin AP only E) 3^{o} streptavidin AP only.

4.5. Conclusion

The H10 gene has now been successfully incorporated into two sites of the MDV genome, under control of the PGK-1 promoter, although work must continue to prove by western blot that the haemagglutinin protein is being produced by CEF cells transfected and infected by the recombinant viruses. The US10(Rep) recombinant viruses have been propagated to sufficient titre by passage in CEF cells and frozen at -80^{o}C. As with the UL41 recombinant viruses, they will be tested in future *in vivo* studies to determine if they induce a protective level of antibody response to MDV and influenza.

Finally, the presence of two non-essential gene sites in the MDV genome means that a permanent selective marker such as GFP may be introduced for further *in vitro* analysis. In addition, it opens up the possibility of incorporating multiple haemagglutinin strains or a haemagglutinin and a neuraminidase gene (Section 3.10). To conclude this work to identify non-essential gene sites in the MDV genome, and to introduce foreign genes, the final gene site to be targeted was the UL50 open reading frame.

Chapter 5: Analysis of the MDV CVI988 UL50 Open Reading Frame

5.1. UL50 Deleted (Del) BAC Construct

5.1.1. Identification of the non-essential gene UL50

The third, and final, MDV gene site target identified was the UL50 gene. The UL50 gene product is the ubiquitous enzyme, dUTPase (deoxyuridine triphosphatase), which is required during dTTP synthesis and for the prevention of uracil-incorporation into DNA (Fuchs *et al*, 2000). It has been studied in several other alphaherpesviruses including HSV-1, VZV and ILTV. It is dispensable for normal viral replication in HSV (Fisher and Preston, 1986). In VZV, deletion of viral dUTPase had no effect on growth and syncytia formation *in vitro* (Ross *et al*, 1997). Fuchs *et al* (2000) determined that, in ILTV, a UL50 deletion virus propagated like wild-type ILTV in cell culture proving that the gene was non-essential for virus replication. To date, the impact of deleting the UL50 gene in MDV has not been studied. The first objective was to assess if this gene can be deleted from the MDV genome to establish if it is essential for virus propagation. If the growth of the UL50 MDV deletion mutant was comparable to that of the parental MDV CVI988 BAC, then the effect of inserting foreign genes into this open reading frame could be assessed.

5.1.2. MDV UL50 gene flanking sequences

Using the MDV CVI988 BAC as the template, approximately 3 kb of the DNA sequence on either side of the UL50 gene was amplified, as described in Section 2.1.1 and Appendix A.1.4.3. To facilitate downstream cloning, the unique restriction enzyme sites *Kpn*I, *Pac*I and *Age*I, were incorporated into the primers (Appendix A.1.3.3). Due to the presence of a naturally occurring *Age*I restriction enzyme site at the end of the UL50 gene, however, there was no need to incorporate this site into the primer, pUL50FSA_For (Appendix A.1.3.3). Once amplified, the flanking sequences were gel purified (Section 2.1.9), and cloned separately into Zero Blunt II TOPO (Section 2.1.2) to create the plasmids UL50pTOPFSA and UL50pTOPFSB (Figure 5.1).

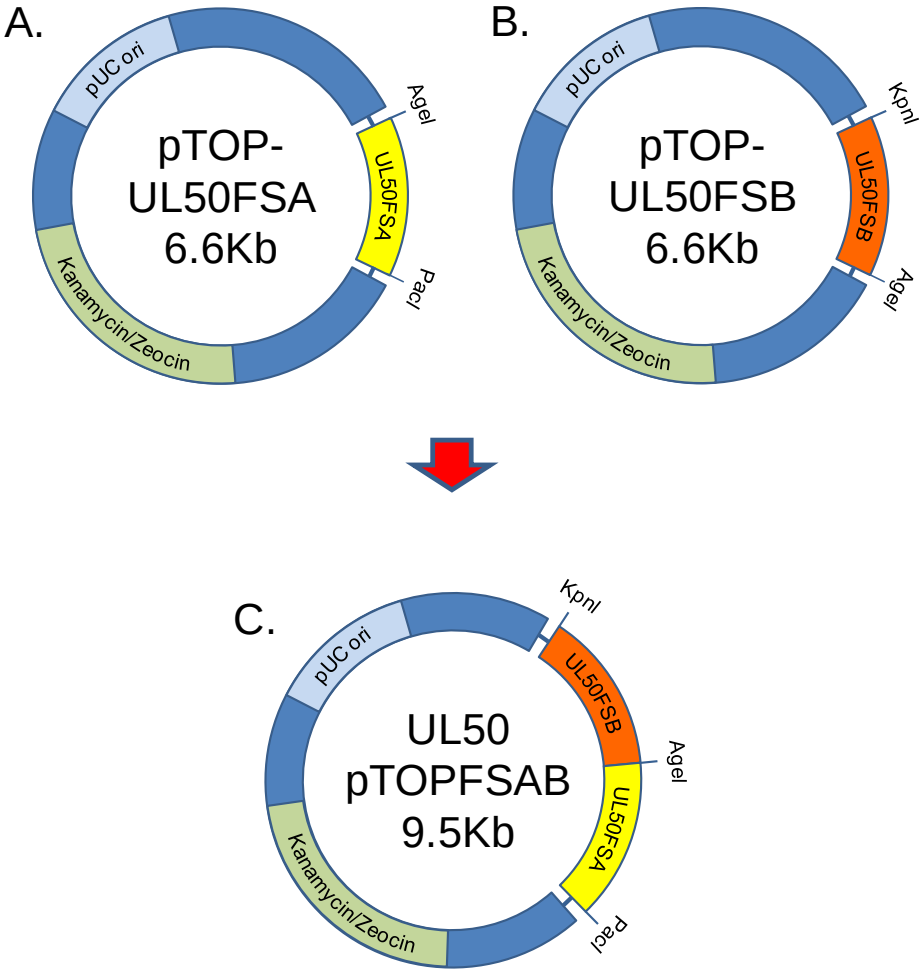


Figure 5.1 A) A diagrammatic representation of the created plasmid UL50pTOPFSA. B). A diagrammatic representation of the created plasmid UL50pTOPFSB. C) A diagrammatic representation of the plasmid UL50pTOPFSAB, created by cloning the FSB DNA fragment into the plasmid, UL50pTOPFSA.

Competent *E. coli* cells were transformed with the ligated plasmids (Section 2.2.2) and plasmid DNA was isolated from small cultures according to the protocol in Section 2.2.5. UL50pTOPFSA was correctly identified using *Bam*HI; UL50pTOPFSB was identified using *Xho*I (Section 2.1.6).

5.1.3. UL50pTOPFSAB

In preparation for cloning, the UL50pTOPFSA and UL50pTOPFSB plasmids were digested with *Kpn*I and *Age*I, and the resulting DNA fragments were gel purified (Section 2.1.9). To create the plasmid UL50pTOPFSAB, the purified FSB fragment was ligated into UL50pTOPFSA (Section 2.1.10) (Figure 5.1). Following transformation (Section 2.2.2), the plasmid DNA was isolated (Section 2.2.5). The successfully ligated plasmid was confirmed using restriction enzyme digestion with *Apa*I (Section 2.1.6). Following confirmation, UL50pTOPFSAB plasmid DNA was prepared according to the protocol in Section 2.2.6.

Primers were designed to sequence the UL50pTOPFSAB plasmid (Appendix A.1.3.3). Sequencing results indicated that, when compared to the published sequence, no errors were present.

5.1.4. UL50pST76kFSAB

Using *Kpn*I and *Pac*I, the UL50pTOPFSAB plasmid was digested to isolate the FSAB insert in preparation for cloning with the shuttle plasmid, pST76k. The 6.0 kb FSAB DNA insert was gel purified (Section 2.1.9) and ligated into the digested and purified pST76k plasmid (Section 3.1.10), to create the UL50pST76kFSAB plasmid (Figure 5.2). Competent *E. coli* cells were transformed with the ligation reaction (Section 2.2.2) and four plasmid DNA samples were isolated from small cultures (Section 2.2.5). As the pST76k vector was 6.3 kb in size and the FSAB insert was 6.0 kb, the isolated plasmids were digested with *Xho*I to confirm that the ligation was successful. All plasmids displayed the correct digest pattern indicating that the UL50pST76kFSAB plasmid had been successfully created (Figure 5.2).

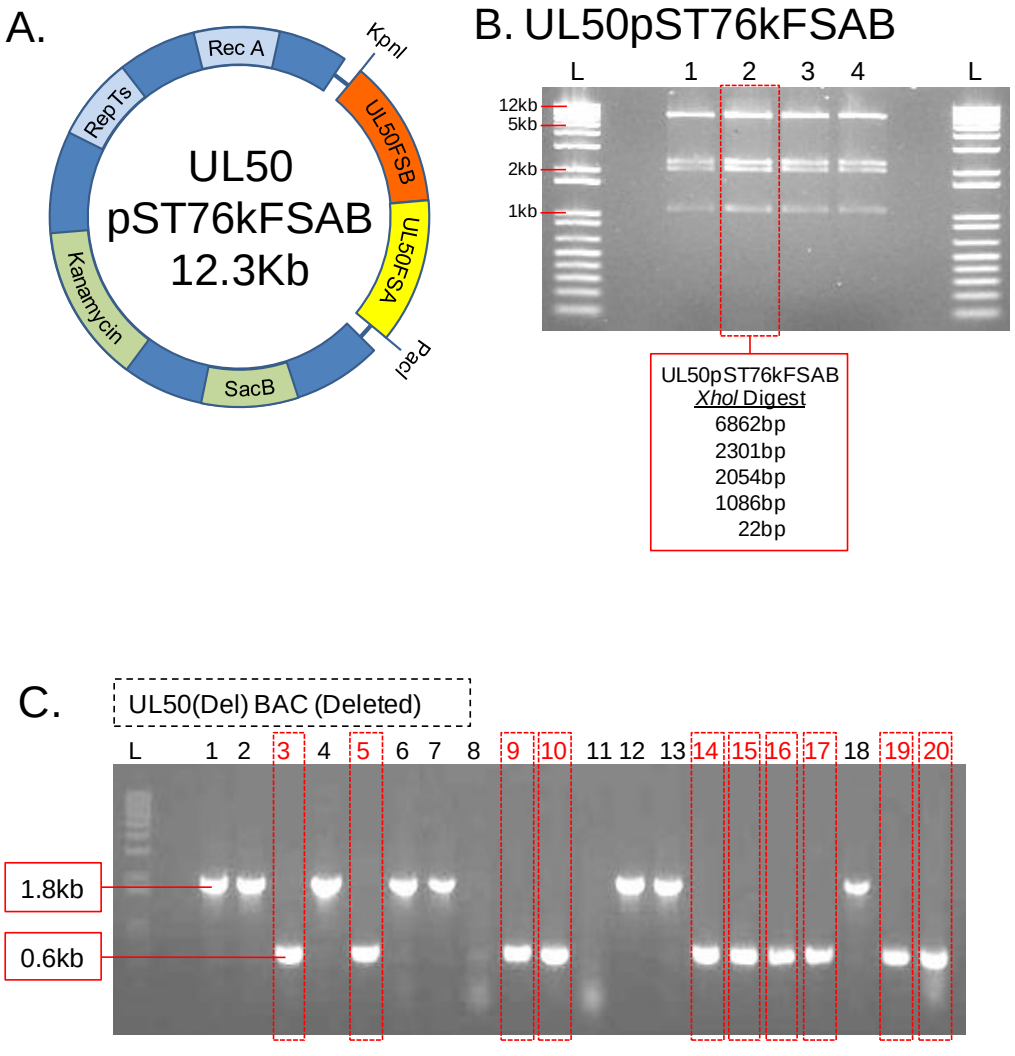


Figure 5.2 A) In the final cloning step, the FSAB DNA fragment was transferred to the shuttle plasmid, pST76k, to create the plasmid UL50pST76kFSAB. B) A restriction enzyme digestion of the UL50pST76kFSAB plasmid using the enzyme, *Xho*I. C) Agarose gel electrophoresis showing positive results from a colony PCR experiment designed to detect UL50(Del) BACs where the UL50 gene had been deleted.

5.1.5. BAC Mutagenesis using UL50pST76kFSAB

BAC mutagenesis was performed, as described in Section 2.2.9. DH5-α bacteria containing the MDV CVI988 BAC were made competent (Section 2.2.8) and transformed with 10 ng of the shuttle plasmid, UL50pST76kFSAB, using electroporation. Kanamycin-sensitive colonies containing UL50(Del) BACs, where the UL50 gene had been deleted, were identified using colony PCR (Section 2.1.1 and Appendix A.1.4.3). The primers, UL50MutBAC_For and UL50MutBAC_Rev, were designed to lie either side of the UL50 gene (Appendix A.1.3.3). By amplifying that region using PCR, the detection of a small product (594 bp) would mean that the UL50 gene had been successfully deleted. A larger product (1821 bp) would mean that the UL50 gene was still intact (Appendix A.1.3.3). Ten colonies tested produced an approximately 0.6 kb product, indicating that the UL50 gene had been successfully deleted (Figure 5.2). A colony was selected, and the UL50(Del) BAC DNA was isolated, as described in Section 2.2.7.

5.1.6. *In vitro* Characterisation of UL50(Del) BAC

CEF cells, seeded in six-well dishes, were transfected with the CVI988 and the UL50(Del) BAC (Section 2.3.7). Three wells of a six-well dish were transfected for each BAC. After 7 days, approximately 15 plaques were visible in the CEF cells that were transfected with the CVI988 BAC. No plaques were visible in the CEF cells that had been transfected with the UL50(Del) BAC. Two more UL50(Del) BAC clones were isolated and the *in vitro* analysis was repeated (Section 2.2.7). Again, *in vitro* analysis failed to demonstrate the ability of these BAC constructs to form CPE in the form of plaques.

These results seemed to indicate that the UL50 gene is essential for normal viral replication of MDV in CEF cells. To determine if this was the case, revertant viruses were created where the UL50 gene was re-inserted into the UL50(Del) BAC. If the UL50 gene was essential to the formation of CPE, then the re-insertion of this gene would restore the plaque forming ability of the virus.

5.2. UL50 re-insertion

The re-insertion of the UL50 gene was achieved by finding compatible, unique and naturally occurring enzyme restriction sites on either side of the UL50 gene. This would allow the insertion of the UL50 gene between the flanking sequences of the plasmid, UL50pTOPFSAB (Section 5.1.3). In addition, as the enzyme sites already exist in the MDV CVI988 BAC, no new DNA would be introduced. The following sections describe the construction the shuttle plasmid, containing the UL50 gene, and its subsequent re-insertion using BAC mutagenesis.

5.2.1. UL50

Using the CVI988 BAC DNA as a template, the UL50 gene was amplified, as described in Section 2.1.1 and Appendix A.1.4.3. The primers, pUL50_For and pUL50_Rev, were designed to amplify the region of DNA that included the UL50 gene, as well as the flanking enzyme restriction sites, *Sac*II and *Cla*I (Appendix A.1.3.3). The amplified sequence was gel purified (Section 2.1.9) and cloned into Zero Blunt II TOPO (Section 2.1.2), to create the pTOPUL50 plasmid (Figure 5.3). Following transformation (Section 2.2.2), plasmid DNA was isolated (Section 2.2.5). The successfully ligated plasmid, pTOPUL50, was confirmed using restriction enzyme digestion with *Sac*II and *Cla*I (Section 2.1.6).

5.2.2. UL50pTOPFSABUL50

The restriction enzyme, *Cla*I, is methylation sensitive so will not cut methylated DNA effectively. Dam^{-} competent cells were transformed (Section 2.2.2) with the plasmids, UL50pTOPFSAB (Section 5.1.3) and pTOPUL50 (Section 5.2.1), and isolated according to the protocol in Section 2.2.5. In preparation for cloning, the unmethylated UL50pTOPFSAB plasmid and the UL50 DNA fragment were digested with *Sac*II and *Cla*I and gel purified (Section 2.1.9). The digested and purified

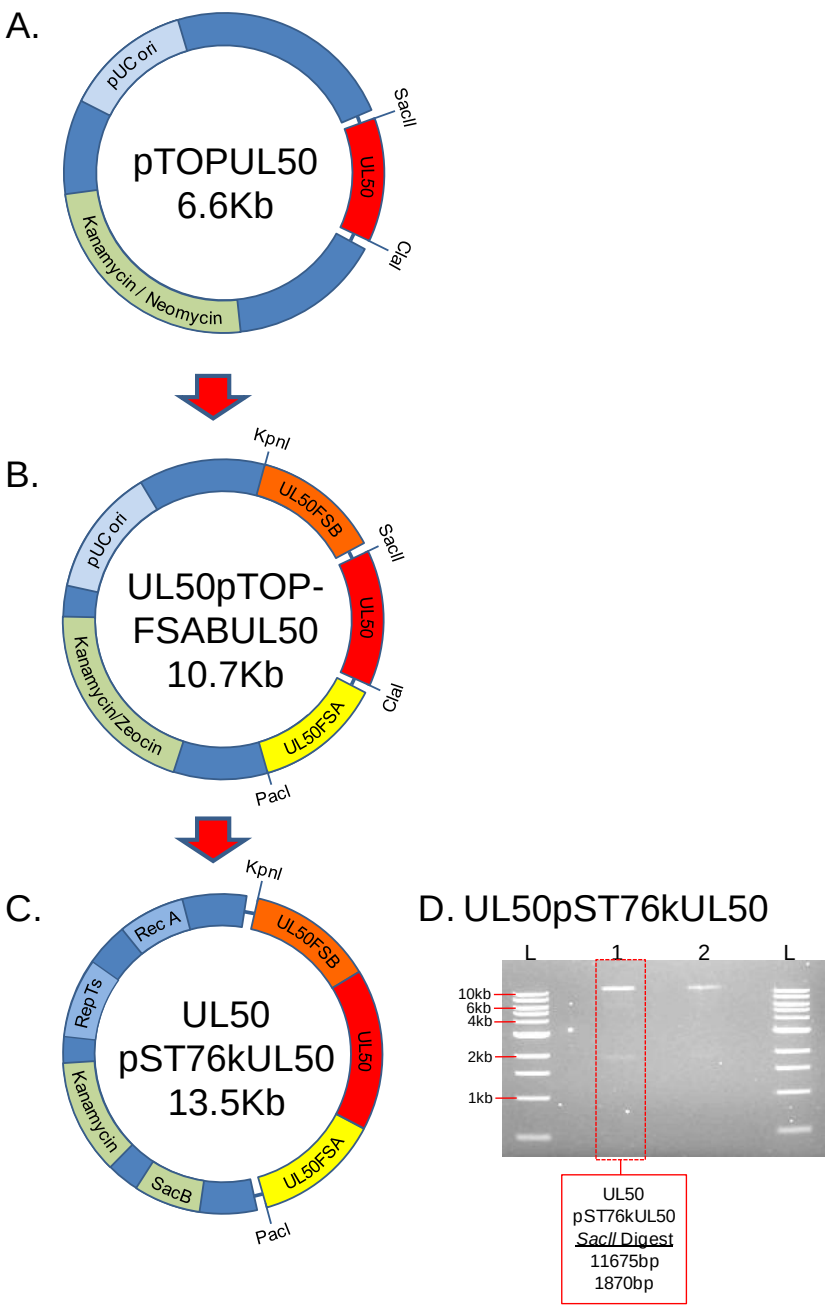


Figure 5.3 A) A diagrammatic representation of the created plasmid pTOPUL50 B). Using the restriction enzyme sites, *Sac*II and *Cla*I, the UL50 gene was cloned between the flanking sequences of UL50pTOPFSAB to create the plasmid, UL50pTOPFSABUL50. C) The FSABUL50 DNA fragment was cloned into the shuttle plasmid, pST76k, to create the plasmid UL50pST76kUL50. D) A restriction enzyme digestion of the UL50pST76kUL50 plasmid using the enzyme, *Sac*II.

vector and insert were ligated (Section 2.1.10) to create the plasmid UL50pTOPFSABUL50 (Figure 5.3). Competent *E. coli* cells were transformed (Section 2.2.2) and plasmid DNA was isolated (Section 2.2.5). The plasmid, UL50pTOPFSABUL50, was confirmed using restriction enzyme digestion with *Bam*HI.

This plasmid was sequenced using the UL50pTOPFSAB sequencing primers pUL50TOPFSAB_For 3-6, as well as two extra sequencing primers, pTOPFSAB UL50_For1 and 2 (Appendix A.1.3.3). Sequencing results indicated that, compared to the published sequence, there was a single point mutation present where a thymidine had been replaced with a cytosine. However, this did not result in an amino acid change (leucine) so was disregarded.

5.2.3. UL50pST76kUL50

The plasmid, UL50pTOPFSABUL50, was digested with *Kpn*I and *Pac*I in preparation for cloning with the shuttle plasmid. The digested FSABUL50 fragment was gel purified (Section 2.1.9) and ligated into the digested and purified pST76k plasmid (Section 3.1.10), creating the plasmid UL50pST76kUL50 (Figure 5.3). Competent *E. coli* cells were transformed with the ligation reaction as described in Section 2.2.2 and plasmid DNA was isolated (Section 2.2.5).

As the pST76k vector was 6.3 kb in size and the FSABUL50 insert was 7.2 kb, the isolated plasmids were digested with the restriction enzyme, *Sac*II, to confirm that the ligation was successful. All plasmids displayed the correct digest pattern indicating that the plasmid UL50pST76kFSABUL50 had been successfully constructed (Figure 5.3).

5.2.4. BAC Mutagenesis using the plasmid UL50pST76kUL50

A glycerol stock of DH5-α bacteria, containing the UL50(Del) BAC, was plated onto to a LB agar plate that contained chloramphenicol (20 µg/ml). Previously this clone had failed to form CPE when transfected into CEF cells (Section 5.1.6). The bacteria

cells were made competent (Section 2.2.8) and transformed with 10 ng of the shuttle plasmid, UL50pST76kUL50, by electroporation. BAC mutagenesis was successfully performed (Section 2.2.9) and kanamycin sensitive colonies containing UL50(Rep) BACs, where UL50 gene had been re-introduced, were identified using colony PCR (Section 2.1.1 and Appendix A.1.4.3).

The primers, UL50MutBAC_For and UL50MutBAC_Rev, were designed previously to identify colonies that contained the UL50(Del) BAC (Appendix A.1.3.3). With these same primers, the detection of a large product (1821 bp) would indicate that the UL50 gene had successfully been re-inserted into the MDV BAC genome. A smaller product (594 bp) meant that the UL50 gene had not been restored. CVI988 BAC DNA was tested as a positive control and produced expected results. Three colonies produced an approximately 1.8 kb product, indicating that the UL50 gene had been successfully re-inserted (Figure 5.4). A colony was selected and the UL50(Rep) BAC was isolated using as described in Section 2.2.7.

To confirm the successful insertion of the UL50 gene into the UL50(Del) BAC, sequencing was carried out using the primers pUL50TOPFSAB_For6 and pTOPFSABHA_Rev6 (Appendix A.1.3.3). Sequencing results indicated that UL50 gene had been successfully re-inserted.

5.2.5. *In vitro* Characterisation of the UL50(Rep) BAC

BAC transfections were carried out using the ‘reverse’ transfection method (Section 2.3.8). CEF cells were seeded at a density of 5.0 x 10^5 cells per well in six-well dishes and simultaneously transfected with the following BACs, according to the protocol in Section 2.3.8 –

- CVI988 - 3 wells
- UL50(Del) BAC - 2 wells
- UL50(Rep) BAC - 3 wells

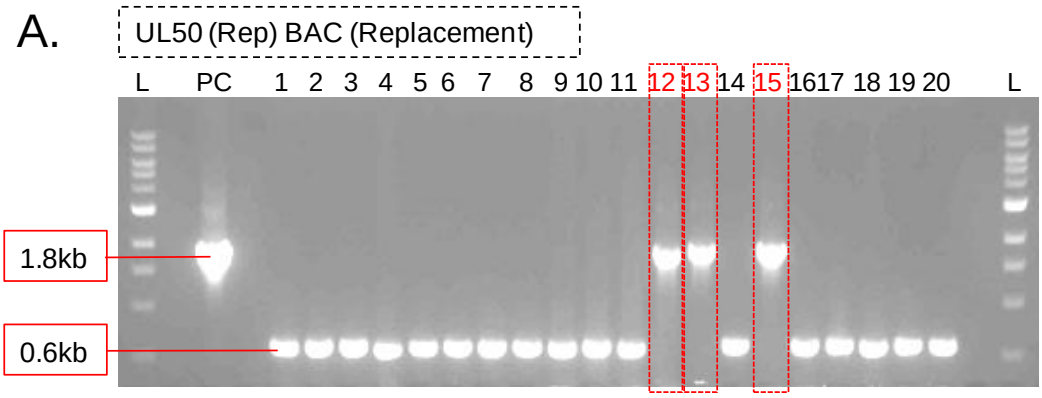


Figure 5.4 A) Agarose gel electrophoresis showing positive results from a colony PCR experiment designed to detect mutant BACs where the UL50 gene had been re-inserted into the UL50 (Del) BAC. A large product (1.8 kb) meant that the UL50 gene had been successfully re-inserted; a small product (0.6 kb) meant that the UL50 gene was not present.

After 7 days, cytopathic effect (CPE) in the form of plaques was visible. The CEF cells were fixed using ice cold 1:1 acetone-methanol and to aid plaque visualisation, the CEF cells were stained using the HB3 antibody (Section 2.3.12). The number of plaques in each well was counted (Table 5.1) and the stained plaques were photographed (Figure 5.5).

Table 5.1 Plaque counts following transfection

Construct	Well	Plaque Number
CVI988	Well 1	102
	Well 2	97
	Well 3	89
UL50 (Del) BAC	Well 1	14
	Well 2	4
UL50 (Rep) BAC	Well 1	91
	Well 2	72
	Well 3	76

The most surprising result was that transfection with the UL50(Del) BAC resulted in the formation of a small number of plaques. This can be explained by the fact that, using the reverse transfection method, the transfection efficiency was sufficiently increased to allow the formation of CPE. Previous *in vitro* analysis (Section 5.1.5) had been carried out according to the protocol in Section 2.3.7, where transfections had been performed using previously seeded CEF cells that had already formed a monolayer. The formation of CPE indicated that the UL50 gene is not essential, however, its deletion severely reduced the number of plaques formed per well. Replacement of the UL50 gene fully restored the ability of the BAC to form CPE, and plaque numbers were comparable to that of the parental strain, the MDV CVI988 BAC.

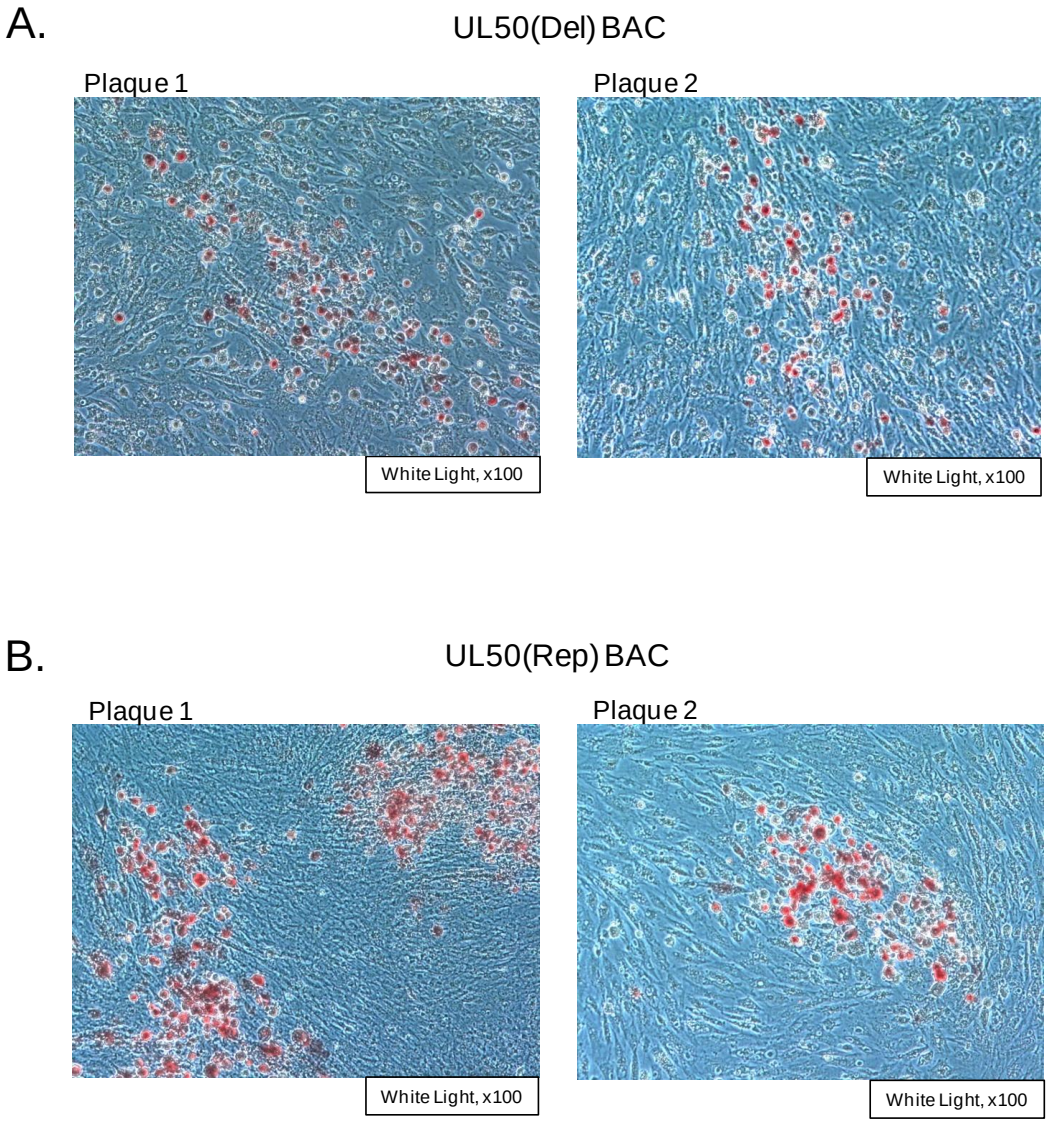


Figure 5.5 A) Visualisation of two plaques, using immunohistochemistry, resulting from the transfection of the UL50(Del) BAC. The plaque was stained with 1^{o} HB3 antibody, specific for the MDV gB glycoprotein, 2^{o} Horseradish peroxidise-conjugated goat anti-mouse IgG and 3-amino-9-ethylcarbazole (AEC) developing solution. B) Immunohistochemistry of two plaques, developing on CEF cells, which were transfected with the UL50(Rep) BAC and stained using the HB3 antibody.

5.3. Conclusion

The work to establish if the UL50 gene was dispensable for normal viral replication in MDV CVI988 has confirmed that this is not the case. Without the presence of the enzyme, dUTPase, the recombinant virus failed to replicate well *in vitro* and did not produce CPE that was comparable to the parental strain. Re-inserting the gene completely restored this ability of the BAC to cause plaque formation when transfected into CEF cells, confirming that the UL50 gene is important for successful virus replication. The results confirmed that the UL50 open reading frame would not be an appropriate site for the introduction of the influenza haemagglutinin gene.

Chapter 6: The 2A Polypeptide

6.1. The 2A polypeptide

In collaboration with the University of St Andrews, work was undertaken to develop a co-expression system, based upon the short oligopeptide sequence, 2A. The 2A polypeptide, derived from the foot and mouth virus, mediates a co-translational cleavage at its own COOH-terminus by manipulating the ribosome into skipping the synthesis of a specific peptide bond, producing a discontinuity in the peptide backbone (Felipe and Ryan, 2004). The translation of downstream genes is not disrupted. By joining together genes using 2A, a single open reading frame would be created which would prevent unequal expression or gene silencing. This means that multiple discrete genes could be expressed from the same promoter.

This research was undertaken because it would be advantageous to be able to join together multiple haemagglutinin genes with 2A. Using this system, multiple HA genes, or combinations of haemagglutinin and neuraminidase genes, could be introduced into the same non-essential gene site of the MDV genome. The genes would be expressed using the same PGK-1 promoter. To assess the potential of 2A to produce discrete proteins, the GFP and H10 genes were cloned and linked by this unique polypeptide.

6.1.1. JC3

The JC3 plasmid, previously created in St Andrews, contains a GFP and a DsRED gene, linked by the 2A polypeptide (Figure 6.1). Both genes were expressed by the CMV IE promoter. When the JC3 plasmid DNA (400 ng/µl) was transfected into Vero cells (Section 2.3.6), cells subsequently expressed both red and green fluorescence simultaneously (Figure 6.1). This indicated that the GFP and DsRed proteins were being co-expressed as discrete proteins from the same promoter, confirmation that the 2A protein was working as expected. As controls, Vero cells were also transfected with plasmids containing the GFP (pEGFP-N1) and DsRED (pDsRed) genes individually (Figure 6.1).

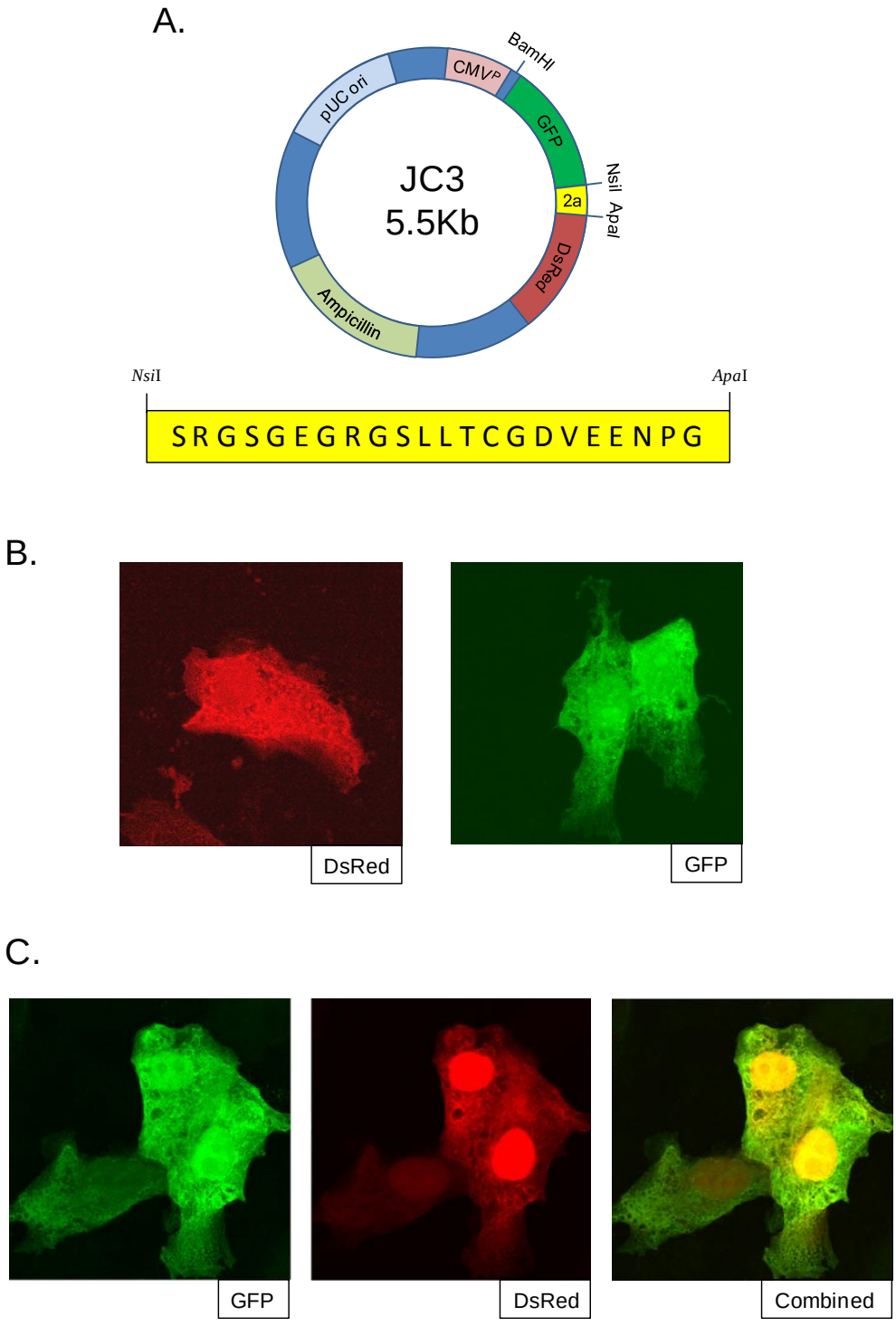


Figure 6.1 A diagrammatic representation of the plasmid JC3, acquired from the University of St Andrews. The plasmid contains the GFP gene and the DsRed gene, linked by the polypeptide, 2A (protein sequence shown). B) Photographs displaying Vero cells transfected with the control plasmids, pEGFP-N1 and pDsRed. C) Three photographs of the same Vero cell, transfected with JC3, simultaneously expressing GFP and DsRed proteins.

6.2. pTOPPGKGFP2AH10myc

The creation of the plasmid, pTOPPGKGFP2AH10myc, would determine if the GFP and the myc-HIS tagged H10 gene could be linked by the 2A polypeptide. Upon transfection, the production of the individual H10 and GFP proteins, detectable using immunofluorescence and western blots, would establish if this is was the case. If successful, work could progress towards introducing these genes, linked by 2A, into a single open reading frame of the MDV CVI988 BAC (UL41 or US10).

6.2.1. pTOPPGKGFP2A

The first step in creating the plasmid, pTOPPGKGFP2AH10myc, was to clone the GFP2A DNA fragment downstream of the PGK promoter. The plasmids, JC3 and pTOPPGKGFP (Section 3.9.1), were sequentially digested with *Bam*HI and *Apa*I in preparation for cloning. Following digestion with *Apa*I at 25^{o}C, the reaction temperature was raised to 37^{o}C and the DNA was subsequently digested with *Bam*HI (Section 2.1.6). The digested fragments were gel purified according to the protocol in Section 2.1.9. The purified 830 bp GFP2A fragment was ligated into pTOPPGK-vector backbone, as described in Section 2.1.10. Competent *E. coli* cells were transformed with the ligation reaction (Section 2.2.2) and plasmid DNA was isolated from small cultures (Section 2.2.5). The successfully ligated plasmid, pTOPPGKGFP2A (Figure 6.2), was confirmed using restriction enzyme digestion with *Eco*RI and *Sma*I.

The primers, peGFP_For and peGFP_Rev3, were used to sequence the GFP gene (Appendix A.1.3.4). Sequence results indicated that that there were several mutations present in the GFP gene that differed from the published GFP sequence. The decision was made to re-amplify the GFP gene, cloned and sequenced previously (Section 3.7.1), and ligate it upstream of the 2A region in the plasmid, pTOPPGKGFP2A.

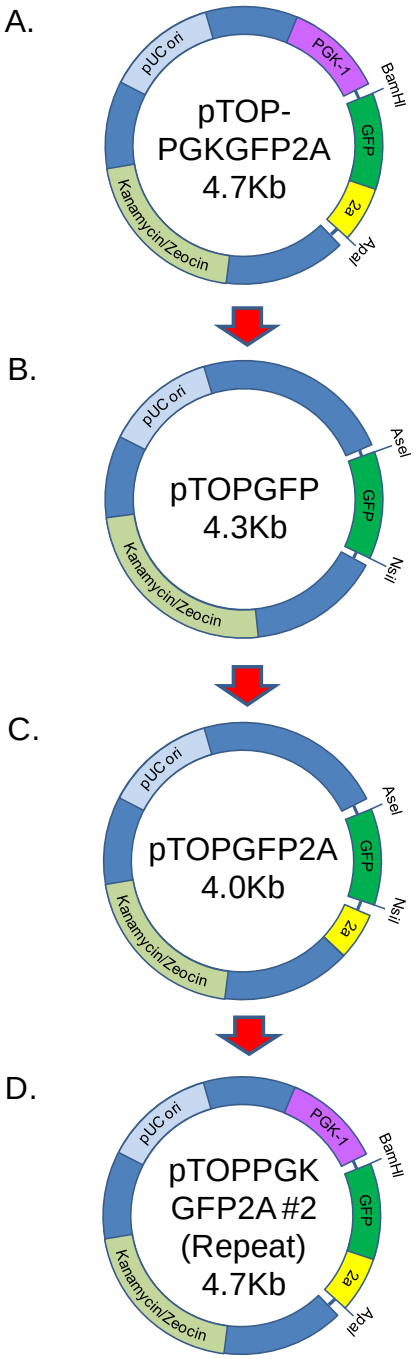


Figure 6.2 A) The GFP2A fragment was excised from JC3 and cloned downstream of the PGK promoter to form the plasmid, pTOPPGKGFP2A. B) Due to mutations present in the GFP gene, the gene was re-amplified and cloned into Blunt II TOPO. C) The GFP gene was cloned upstream of the 2A sequence to form the plasmid, pTOPGFP2A. D) The GFP2A was excised and cloned downstream of the PGK promoter to create the plasmid pTOPPGKGFP2A #2 (Repeat).

6.2.2. pTOPGFP

Primers (p2AGFP_For and p2AGFP-Rev) that incorporated the unique restriction enzyme sites, *Ase*I and *Nsi*I, were designed to amplify the GFP gene (Appendix A.1.3.4). The reverse primer lay upstream of the stop codon removing it from the end of the GFP gene. Using pTOPPGKGFP as a template (Section 3.7.1), the GFP gene was successfully amplified as described in Section 2.1.1 and Appendix A.1.4.4. The amplified GFP gene was gel purified (Section 2.1.6) and subsequently cloned into Blunt II TOPO (Section 2.1.2) to create pTOPGFP (Figure 6.2). Following transformation (Section 2.2.2), the plasmid DNA was isolated (Section 2.2.5). The successfully ligated plasmid was confirmed using restriction enzyme digestion with *Ase*I and *Nsi*I.

6.2.3. pTOPGFP2A

Using the restriction enzymes *Ase*I and *Nsi*I, the GFP DNA fragment was removed by enzyme digestion and gel purified in preparation for cloning (Section 2.1.9). The plasmid vector, pTOPPGKGFP2A was also digested with *Ase*I and *Nsi*I, removing the PGKGFP fragment, and gel purified. The digested and purified vector and insert were ligated, as described in Section 2.1.10, to create the plasmid pTOPGFP2A (Figure 6.2). Competent *E. coli* cells were transformed according to the protocol in Section 2.2.2, and plasmid DNA was isolated (Section 2.2.5). The plasmid, pTOPGFP2A, was confirmed using restriction enzyme digestion with *Ase*I and *Nsi*I, and *Bam*HI and *Apa*I.

6.2.4. pTOPPGKGFP2A #2 (Repeat)

Previously in Section 6.2.1, the GFP2A DNA fragment was cloned downstream of the PGK promoter, and this was repeated with the replacement GFP2A DNA fragment. The plasmid, pTOPGFP2A (Section 6.2.3), was sequentially digested with *Bam*HI and *Apa*I in preparation for cloning (Section 2.1.6). The digested GFP2A fragment was subsequently gel purified (Section 2.1.9). The plasmid vector,

pTOPPGK-, was digested and purified previously. The purified GFP2A fragment was ligated into pTOPPGK-, as described in Section 2.1.10. Following transformation (Section 2.2.2), plasmid DNA was isolated (Section 2.2.5). The successfully ligated plasmid, pTOPPGKGFP2A #2 (Repeat) (Figure 6.2), was confirmed using restriction enzyme digestion with *Nsi*I and *Sma*I.

6.2.5. pTOP(PGKGFP2A)

The PGKGFP2A DNA fragment was amplified using PCR in preparation for the ligation upstream of the H10myc gene. Using the primers, pPGKGFP2A_For and pPGKGFP2A_Rev, the PGKGFP2A DNA fragment was successfully amplified (Section 2.1.1 and Appendix A.1.4.4). Due to the presence of a naturally occurring *Ase*I restriction enzyme site at the start of the DNA fragment, there was no need to incorporate this site into the primer, pPGKGFP2A_For. The unique enzyme restriction site, *Kpn*I, was incorporated into the reverse primer, pPGKGFP2A_Rev (Appendix A.1.3.4). Following amplification, the gene was cloned into Blunt II TOPO (Section 2.1.2), competent *E. coli* cells were transformed with the ligation mix (Section 2.2.2) and plasmid DNA was isolated (Section 2.2.5). The successfully ligated plasmid, pTOP(PGKGFP2A) (Figure 6.3), was confirmed using restriction enzyme digestion with *Ase*I and *Kpn*I.

6.2.6. pTOPH10myc

This plasmid was created previously (Section 3.8.2). The H10myc DNA fragment however, had been blunt cloned into TOPO as an intermediate step, and the gene orientation had not been a consideration. To ensure that the orientation was correct, the previously digested (*Bam*HI and *Xho*I) and purified H10myc DNA fragment (Section 3.8.2) was re-cloned into a TOPO vector. The plasmid vector, pTOPGFP (Section 6.2.2), was digested with *Bam*HI and *Xho*I, removing the GFP fragment. The vector was gel purified and ligated, according to the protocol in Section 2.1.10, with the H10myc insert to create the plasmid pTOPH10myc (Figure 6.3). Competent

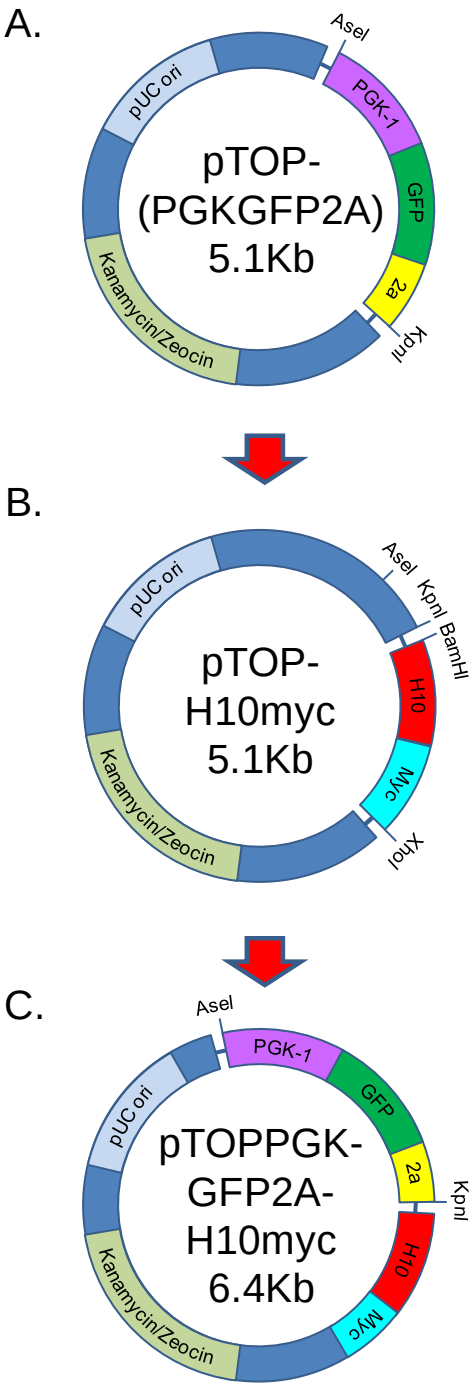


Figure 6.3 A) The PGKGFP2A fragment was PCR amplified with primers incorporating the enzyme restriction sites, *Ase*I and *Kpn*I, and cloned into Blunt II TOPO to create the plasmid, pTOP(PGKGFP2A). B) The H10myc DNA fragment was re-cloned into the TOPO vector to ensure that it was in the correct orientation. C) The PGKGFP2A DNA fragment was excised using the enzymes, *Ase*I and *Kpn*I, and cloned into pTOPH10myc, to form the plasmid, pTOPPGKGFP2AH10myc.

E. coli cells were transformed with the ligation reaction (Section 2.2.2) and plasmid DNA was isolated (Section 2.2.5). The plasmid, pTOPH10myc, was confirmed using restriction enzyme digestion with *Bam*HI and *Xho*I, and *Hin*dIII.

6.2.7. pTOPPGKGFP2AH10myc

In preparation for cloning, the PGKGFP2A DNA fragment was removed from the plasmid, pTOP(PGKGFP2A), using *Ase*I and *Kpn*I. The plasmid vector, pTOPH10myc (Section 6.2.6), was digested with the same enzymes. Subsequently, the insert and the vector were gel purified, and ligated (Section 2.1.10), to create the plasmid pTOPPGKGFP2AH10myc (Figure 6.3). Following transformation (Section 2.2.2), plasmid DNA was isolated as described in Section 2.2.5. The successfully created plasmid, pTOPPGKGFP2AH10myc, was confirmed using restriction enzyme digestion with *Ase*I and *Kpn*I, and *Hin*dIII.

6.3. Conclusion and Future work

Unfortunately, due to time limitations, it was not possible to perform expression studies using pTOPPGKGFP2AH10myc, or to incorporate the GFP and H10 genes, linked by the 2A polypeptide, into the MDV CVI988 genome. To hypothesise, the ideal result would have been the development of CPE that simultaneously expressed GFP and H10myc, the latter protein detectable using an Alexa Fluor 594 (red) antibody. In addition, with further work to correct and improve the western blot techniques, it would be possible to detect the presence of the discrete GFP and H10myc proteins. Finally, and looking ahead, work to link different haemagglutinin genes would complete the study to create an effective recombinant vaccine for Marek's Disease Virus and influenza that would protect against multiple influenza subtypes.

Appendix: PCR Programs

A.1.1 Recipes

A.1.2 Reaction Conditions

A.1.3 Primers

A.1.4 Specific Reaction Conditions

A.1.1 Recipes

1. Phusion High Fidelity DNA Polymerase (Finnzyme, Finland)

5x Buffer	10 µl
dNTP (10 mM)	1 µl
Primer 1 (10 pmol/µl)	5 µl
Primer 2 (10 pmol/µl)	5 µl
Template DNA/cDNA	1 µl
Pfu Polymerase	0.5 µl
dH_2O	27.5 µl

   - Template DNA diluted to between 1-50 ng/µl
   - cDNA not diluted

2. Taq DNA polymerase (Invitrogen)

10x Buffer	5 µl
$MgCl_2$ (50 mM)	3 µl
dNTP (10 mM)	1 µl
Primer 1 (10 pmol/µl)	1 µl
Primer 2 (10 pmol/µl)	1 µl
Taq DNA Polymerase	2.5 µl
dH_2O	36.5 µl

   - Taq DNA Polymerase diluted 1/5 in dH_2O
   - Reaction mixture aliquoted into 10 µl quantities
   - Template DNA introduced in bacterial colony

A.1.2 Reaction Conditions

A. Mareks FSA and FSB

Pre heat	-	98°C		
Initial Denaturing	-	98°C	1 min	
Denaturing	-	98°C	30 sec	x 35 cycles
Annealing	-	* °C	40 sec	
Extension	-	72°C	* min	
Final Extension	-	72°C	7 min	
		4°C	Hold	

B. H10 Amplification

Pre heat	-	98°C		
Initial Denaturing	-	98°C	1 min	
Denaturing	-	98°C	20 sec	x 35 cycles
Annealing	-	* °C	30 sec	
Extension	-	72°C	* min	
Final Extension	-	72°C	7 min	
		4°C	Hold	

C. Taq DNA polymerase (Invitrogen)

Pre heat	-	95°C		
Initial Denaturing	-	95°C	3 min	
Denaturing	-	94°C	45 sec	x 35 cycles
Annealing	-	* °C	45 sec	
Extension	-	72°C	* min	
Final Extension	-	72°C	7 min	
		4°C	Hold	

Note - Annealing temperatures and extension times (*) varied according to the requirements of specific reactions. See Specific Reaction Details (Section A.1.4)

A.1.3 Primer Sequences

A.1.3.1 Chapter 3: UL41 Open Reading Frame Primers

Primer	Sequence
UL41 Flanking Sequences	
MareksFSA_For and *Age*I	ATACCGGTTCATACATCCATATACTCCC
MareksFSA_Rev and *Pac*I	CATTAATTAAAGATTTCACTACATAGGCAG
MareksFSB_For and *Kpn*I	TTGGTACCCTGTGTCAATTCTTCGTTCG
MareksFSB_Rev and *Age*I	TCACCGGTCGCTTCCAGAGTATGCAG
H10 Gene Specific Primer	
H10N1RT	AGCCAATGGAACCGTCG
H10 Amplification	
H10N1For and *Xho*I	CTCGAGTGAAGAAGTGACCAATGC
H10Rev and *Xba*I	GGTCTAGAGGCTAAATACAGATTGTGC
CMVH10 Amplification	
pCMVHA_For and *Age*I	AAACCGGTATTCTTTCCTGCGTTATCCC
pCMVHA_Rev and *Age*I	CAACCGGTCTACAAATGTGGTATGGCTG
CMVH10 Sequence Primers	
pTOPCMVHA_For1	GGCTCGTATGTTGTGTGG
pTOPCMVHA_For2	GGACTTTCCTACTTGGCAG
pTOPCMVHA_For3	CTGGCTTCACTTATGGGTC
pTOPCMVHA_For4	TAGAGAATGGATGGGAAGG
pTOPCMVHA_Rev1	TCTTCGCTATTACGCCAG
H10 Colony PCR primers	
pCMVHAID_For	ATCTATCGGTCGTGTTCAGG
pCMVHAID_Rev	GGTGTTGTTTCTTATGCTCTCC
H10 Reverse Orientation primer	
pCMVHA[REV]ID_Rev	GATGGGAAGGAATGGTGG
MDV030 cDNA PCR primers	
VP23cloneFor	TTGTTTCCGACTCACGACTG
VP23cloneRev	TACTTCCAACGGCACGATAG
vIL-8 cDNA PCR primers	
vIL-8For	CTGGAGGTTGTGGAATACGC
vIL-8Rev	CCAATAGTAGGAGGTGTGGGCTC

Primer	Sequence
H10 cDNA PCR primers	
pCMVHAID_For2	ATTCAATAACCGACATCTGGAC
pCMVHAID_Rev	As above
pTOPFSABHA_For2	ACCCTGTTTGACTCCCG
pTOPFSABH10 Sequence Primers	
pTOPFSABHA_For3	GATAGAATAGTGTCCGCTGG
pTOPFSABHA_For4	TGAAGACTTGCCATCGG
pCMVHAID_For	As above
pTOPCMVHA_For2	As above
pTOPCMVHA_For3	As above
pTOPCMVHA_For4	As above
pTOPFSABHA_For9	ATACTCCCATGATGACCCTC
pTOPFSABHA_For10	AGGAATAACTATGGGCAGC
pTOPFSABHA_For11	AAGGAGGTTTTTATGCCG
pTOPFSABHA_For12	ACAACTCATCATTACCTCCG
pTOPFSABHA_For13	GCTATTATCGCATTTGTCG
pTOPCMVHA_Rev1	As above
pTOPFSABHA_Rev2	ATGGCGTAGAGTCGTTGC
pTOPFSABHA_Rev3	CGTTTCCATTTGTTGCTG
pTOPFSABHA_Rev4	GCGTTAGTATCTTCCAGTGC
pTOPFSABHA_Rev5	ATCATCAGTCCGTCGTCG
pTOPFSABHA_Rev6	CATAACCACTTGAATGTCGC
pCMVHAID_Rev	As above
pTOPFSABHA_Rev8	CTCCATTGTGTGAGAAGGTG
pTOPFSABHA_Rev9	ATTGGTCACTTCTTCACTCG
pTOPFSABHA_Rev10	GGCTATGAACTAATGACCCC
pTOPFSABHA_Rev11	GCCAGTGAGTCATTGTCG
pTOPFSABHA_Rev12	TCCCCACAGAATACTCCC
pTOPFSABHA_Rev13	ATTCTACATTAGCCCACCC
pTOPFSABHA_Rev14	CACATTATCACACCTGCGAG
UL41 Flanking Sequence B (Rpt)	
CVI988FSB_Rev and *Age*I	CCACCGGTCCGCTAGCACTATCGTATCCC
UL41 (Deletion) Colony PCR	
UL41MutBAC_For	GCATACTCTGGAAGCGTGTC
UL41MutBAC_Rev	GCTATGTGCAAATCAACCC
H10 (Truncated) Amplification	
pTOPCMVHA10T_For and *Age*I	AAACCGGTATTCTTTCCTGC
pTOPCMVH10T_Rev and *Age*I	GAACCGGTAGAAGAGAGTTTCACTGG

Primer	Sequence
GFP Amplification	
pGFP_For and *Nhe*I	TCGCTAGCATCCACAAGTCGCCACC
pGFP_Rev and *Acc*I	AAGTCTACCTGATTATGATCTAGAGTCG
UL44 cDNA PCR primers	
pUL44_For	CTTTTATCTCCGAGCAACG
pUL44_Rev	GCTGAGGAGATTTCTATGGC
GFP Colony PCR primers	
peGFP_For	GGGCGATGCCACCTACGGCAAG
peGFP_Rev3	GATGGTGCGCTCCTGGACGTAG
H10 Amplification (Repeat)	
pH10 (Rpt)_For and *Xho*I	ATCTCGAGTGAAGAAGTGACC
pH10 (Rpt)_Rev and *Hin*dIII	AGAAGCTTAATACAGATTGTGCATCGC
-H10myc- Amplification	
pH10myc_For and *Bam*HI	AAGGATCCAAAGAGGTTGTGCATGAAGG
pH10myc_Rev and *Xho*I	AACTCGAGGGGCAAACAACAGATGGC
NA (H5N1) Amplification	
pNA_For (fixed) and *Nhe*I	CCGCTAGCATGAATCCAAATCAGAAG
pNA_Rev and *Xho*I	AACTCGAGAACTACTTGTCAATGGTG
HA (H5N1) Amplification	
pDONRH5_For and *Xho*I	TTCTCGAGTTTGACTGATAGTGACCTG
pDONRH5_Rev and *Hin*dIII	TAAAGCTTCGGGTCAATGCAAATTCTGC
H5myc Amplification	
pH5myc(rpt)_For and *Bam*HI	TAGGATCCTAAAGCAGGCTCCGCCATG
pH10myc_Rev and *Xho*I	As above

A.1.3.2 Chapter 4: US10 Open Reading Frame Primers

Primer	Sequence
US10 Flanking Sequences	
pUS10FSA_For and *Bam*HI	ATGGATCCTATCTGACAAATCTTCGGG
pUS10FSA(4)_Rev and *Pac*I	ACTTAATTAAACAGACCTACTTGCTACC
pUS10FSB_For and *Kpn*I	TTGGTACCTGCGTATTTTCCCCGTGC
pUS10FSB_Rev and *Bam*HI	AAGGATCCACTGCGTACAGATTTAGGC
US10pTOPFSAB Sequence Primers	
pTOPCMVHA_For1	Section A1.3.1
US10pTOPFSAB_F2	GCATACATCCTACTTATCGCAC
US10pTOPFSAB_F3	AGTGAAATCTTTAGGGAGGG
US10pTOPFSAB_F4	AAAACAAGCCGAATGGG
US10pTOPFSAB_F5	TTTGAATACTGGAGACGAGC
US10pTOPFSAB_F6	ACCTCCAACATCCATTCG
US10pTOPFSAB_F7	CAGATAACCATTTGACACCAC
pTOPCMVHA_Rev1	Section A1.3.1
US10pTOPFSAB_R2	AATGTATGGAAGCCCGAG
US10pTOPFSAB_R3	TATGCCGTGGTTCTTGC
US10pTOPFSAB_R4	TAAGTAGGATTCCCCGTCTC
US10pTOPFSAB_R5	AAAAATGCTATCGGAGGC
US10pTOPFSAB_R6	ATGGAGCCGAAAGCAG
US10pTOPFSAB_R7	CATCTTTGCCATCTCTGC
GFP Amplification/Colony PCR primers	
p2aGFP_For and *Ase*I	CCATTAATTCCTCTTCCTCATCTCC
p2aGFP_Rev and *Nsi*I	CTATGCATCTTGTACAGCTCGTCC
H10myc Colony PCR	
pH10myc_For	See Appendix A.1.3.1
pH10myc_Rev	See Appendix A.1.3.1

A.1.3.3 Chapter 5: UL50 Open Reading Frame Primers

Primer	Sequence
UL50 Flanking Sequences	
pUL50FSA_For	CAGTAGGTAAATGTCGTCGCC
pUL50FSA_Rev and *Pac*I	TATTAATTAAGTAACACGGGTATGAGCC
pUL50FSB_For and *Kpn*I	TAGGTACCAGCGTATCGTATGTATCGTC
pUL50FSB_Rev and *Age*I	AAACCGGTCCAGGGAACTGTTAGTGC
UL50pTOPFSAB Sequence Primers	
pTOPCMVHA_For1	Section A1.3.1
pUL50TOPFSAB_For2	TCCATCAACAATGCTTCG
pUL50TOPFSAB_For3	TCCCTTGCTTCTAATTCTCC
pUL50TOPFSAB_For4	GCAGTAGTAGGGCTGTTCC
pUL50TOPFSAB_For5	TTGTGTTGGATGGTCGG
pUL50TOPFSAB_For6	TCAAAATAGCAACGCCG
pUL50TOPFSAB_For7	ATCGCAGTTTCGGTAGC
pUL50TOPFSAB_For8	CAGATACCCTCTACTAACTCCG
pUL50TOPFSAB_For9	ACGAACAAAAGGTCACAGC
pUL50TOPFSAB_For10	GTGAATGACCACATAGCCC
pUL50TOPFSAB_For11	CTTATGACCGAATGTTGGG
pTOPCMVHA_Rev1	Section A1.3.1
pUL50TOPFSAB_Rev2	CTTTCCCAACATTCGGTC
pUL50TOPFSAB_Rev3	GTAAATGTCTTCATCGGGC
pUL50TOPFSAB_Rev4	CAGTCAGACCTCGTTTACCC
pUL50TOPFSAB_Rev5	GACTACAGAAGACTCAAAGCAG
pUL50TOPFSAB_Rev6	CAATGGACGAGTCAACCG
pUL50TOPFSAB_Rev7	TCATCTGGGTTTTCTGTGC
pUL50TOPFSAB_Rev8	CAGCGTATGTTTTGCGG
pUL50TOPFSAB_Rev9	TGATGAAACGCTTCTCTCTG
pUL50TOPFSAB_Rev10	AGCATCTCAAATGGGACG
pUL50TOPFSAB_Rev11	TATGATGGTGAGTTGGCAG
UL50 (Deletion) Colony PCR	
UL50MutBAC_For	CCAGACCCAGATAAAACAGC
UL50MutBAC_Rev	CAATGGACGAGTCAACCG
UL50 Amplification	
pUL50_For (Rpt)	TAACAGACGGAGCAACCAG
pUL50_Rev (Rpt)	ACGCATCCACATAATGAGAG

Primer	Sequence
UL50pTOPFSABUL50 Sequence Primers	
pUL50TOPFSAB_For3	As above
pUL50TOPFSAB_For4	As above
pUL50TOPFSAB_For5	As above
pUL50TOPFSAB_For6	As above
pTOPFSABUL50_F1	GTCAGATTAGGAAGCGGG
pTOPFSABUL50_F2	GTAGGTAAATGTCGTCGCC

A.1.3.4 Chapter 6: 2a Polyprotein Primers

Primer	Sequence
GFP Sequencing primers	
peGFP_For	Section A1.3.1
peGFP_Rev3	Section A1.3.1
GFP Amplification Primers	
p2aGFP_For and *Ase*I	CCATTAATTCCTCTTCCTCATCTCC
p2aGFP_Rev and *Nsi*I	CTATGCATCTTGTACAGCTCGTCC
PGKGFP2a Amplification Primers	
pPGKGFP2a_For	TGGCACGACAGGTTTCC
pPGKGFP2a_Rev and *Kpn*I	TAGGTACCATACGACTCACTATAGGGC

A.1.4 Specific Reaction Conditions

A.1.4.1 Chapter 3: UL41 Open Reading Frame

MDV UL41 FSA
Recipe A
Product size – 3167 bp
Primer 1 – Mareks FSA_For and *Age*I
Primer 2 – Mareks FSA_Rev and *Pac*I
Template DNA - CVI988 BAC DNA, Diluted
Reaction Conditions A
Annealing Temperature – 52^{o}C
Extension Time – 4 min

MDV UL41 FSB
Recipe A
Product size – 2962 bp
Primer 1 – Mareks FSB_For and *Kpn*I
Primer 2 – Mareks FSB_Rev and *Age*I
Template DNA - CVI988 BAC DNA, Diluted
Reaction Conditions A
Annealing Temperature – 52^{o}C
Extension Time – 4 min

H10
Recipe A
Product size – 1582 bp
Primer 1 – H10N1For and *Xho*I
Primer 2 – H10Rev and *Xba*I
Template DNA – H10 cDNA
Reaction Conditions B
Annealing Temperature – 49^{o}C
Extension Time – 2 min 30

CMVH10
Recipe A
Product size – 2219 bp
Primer 1 – pCMVHA_For and *Age*I
Primer 2 – pCMVHA_Rev and *Age*I
Template DNA – Diluted pCMVH10 maxiprepped DNA
Reaction Conditions B
Annealing Temperature – 55^{o}C
Extension Time – 2 min 30

CMVH10 Colony PCR
Recipe B
Product size – 2320 bp
Primer 1 – pCMVHAID_For
Primer 2 – pCMVHAID_Rev
Template DNA – Bacterial Colony
Reaction Conditions C
Annealing Temperature – 52^{o}C
Extension Time – 2 min 30

CVI988 cDNA PCR – MDV30
Recipe B
Product size – 530 bp
Primer 1 –VP23cloneFor
Primer 2 –VP23cloneRev
Template DNA – CVI988/H10 cDNA
Reaction Conditions C
Annealing Temperature – 54^{o}C
Extension Time – 2 min 30

CVI988 cDNA PCR –vIL-8
Recipe B
Product size – 569 bp
Primer 1 –vIL-8For
Primer 2 –vIL-8Rev
Template DNA – CVI988/H10 cDNA
Reaction Conditions C
Annealing Temperature – 54^{o}C
Extension Time – 2 min 30

CVI988/H10 cDNA PCR – H10
Recipe B
Product size – 212 bp
Primer 1 –pCMVHAID_For2
Primer 2 –pCMVHAID_Rev
Template DNA – CVI988/H10 cDNA
Reaction Conditions C
Annealing Temperature – 54^{o}C
Extension Time – 2 min 30

MDV UL41 FSB (Rpt)
Recipe A
Product size – 3144 bp
Primer 1 – Mareks FSB_For and *Kpn*I
Primer 2 – CVI988 FSB_Rev and *Age*I
Template DNA - CVI988 BAC DNA, Diluted
Reaction Conditions A
Annealing Temperature – 52^{o}C
Extension Time – 4 min

H10 Colony PCR
Recipe B
Product size – 1582 bp
Primer 1 – H10N1For
Primer 2 – H10Rev
Template DNA – Bacterial Colony
Reaction Conditions C
Annealing Temperature – 52^{o}C
Extension Time – 2 min 30

Forward Orientation H10 Colony PCR
Recipe B
Product size – 2503 bp
Primer 1 – pCMVHAID_For
Primer 2 – pCMVHAID_Rev
Template DNA – Bacterial Colony
Reaction Conditions C
Annealing Temperature – 52^{o}C
Extension Time – 2 min 30

Reverse Orientation H10 Colony PCR
Recipe B
Product size – 1125 bp
Primer 1 – pCMVHAID_For
Primer 2 – CMVHA[REV]ID_Rev
Template DNA – Bacterial Colony
Reaction Conditions C
Annealing Temperature – 52^{o}C
Extension Time – 2 min 30

UL41 (Deletion) Colony PCR
Recipe B
Product size – Gene deletion: 507 bp; No gene deletion: 1731 bp
Primer 1 – UL41MutBAC_For
Primer 2 – UL41MutBAC_Rev
Template DNA – Bacterial Colony
Reaction Conditions C
Annealing Temperature – 52^{o}C
Extension Time – 2 min 30

CMVH10 (t)
Recipe A
Product size – 2129 bp
Primer 1 – pTOPCMVHA10T_For and *Age*I
Primer 2 – pTOPCMVH10T_Rev and *Age*I
Template DNA – Diluted pTOPCMVH10 maxiprepped DNA
Reaction Conditions B
Annealing Temperature – 52^{o}C
Extension Time – 2 min 30

GFP
Recipe A
Product size – 776 bp
Primer 1 – pGFP_For and *Nhe*I
Primer 2 – pGFP_Rev and *Acc*I
Template DNA – Diluted pEGFP-N1 DNA
Reaction Conditions B
Annealing Temperature – 52^{o}C
Extension Time – 2 min 30

CVI988 cDNA PCR – UL42
Recipe B
Product size – 1102 bp
Primer 1 –pUL42_For
Primer 2 –pUL42_Rev
Template DNA – CVI988/H10 cDNA
Reaction Conditions C
Annealing Temperature – 54^{o}C
Extension Time – 2 min 30

CVI988 cDNA PCR – UL44
Recipe B
Product size – 1212 bp
Primer 1 –pUL44_For
Primer 2 –pUL44_Rev
Template DNA – CVI988/H10 cDNA
Reaction Conditions C
Annealing Temperature – 54^{o}C
Extension Time – 2 min 30

PGKGFP Colony PCR
Recipe B
Product size – 193 bp
Primer 1 – peGFP_For
Primer 2 – peGFP_Rev3
Template DNA – Bacterial Colony
Reaction Conditions C
Annealing Temperature – 59^{o}C
Extension Time – 1 min 30

H10 (Rpt)
Recipe A
Product size – 1572 bp
Primer 1 – pH10(Rpt)_For and *Xho*I
Primer 2 – pH10(Rpt)_Rev and *Hin*dIII
Template DNA – Diluted pTOPCMVH10 maxiprepped DNA
Reaction Conditions B
Annealing Temperature – 52^{o}C
Extension Time – 2 min 30

H10myc
Recipe A
Product size – 1679 bp
Primer 1 – pH10myc_For and *Bam*HI
Primer 2 – pH10myc_Rev and *Xho*I
Template DNA – Diluted pcDNA3.1H10myc DNA
Reaction Conditions B
Annealing Temperature – 52^{o}C
Extension Time – 2 min 30

NA (H5N1)
Recipe A
Product size – 1364 bp
Primer 1 – pNA_For (fixed) and *Nhe*I
Primer 2 – pNA_Rev and *Xho*I
Template DNA – pDONR207NA, diluted
Reaction Conditions B
Annealing Temperature – 48^{o}C
Extension Time – 1 min 30

HA (H5N1)
Recipe A
Product size – 1814 bp
Primer 1 –pDONRH5_For and *Xho*I
Primer 2 –pDONRH5_Rev and *Hin*dIII
Template DNA – pDONR207H5, diluted
Reaction Conditions B
Annealing Temperature – 52^{o}C
Extension Time – 2 min

A.1.4.2 Chapter 4: US10 Open Reading Frame

MDV US10 FSA
Recipe A
Product size – 1436 bp
Primer 1 – pUS10FSA_For and *Bam*HI
Primer 2 – pUS10FSA(4)_Rev and *Pac*I
Template DNA - CVI988 BAC DNA, Diluted
Reaction Conditions A
Annealing Temperature – 52^{o}C
Extension Time – 3 min

MDV US10 FSB
Recipe A
Product size – 2184 bp
Primer 1 – pUS10FSB_For and *Kpn*I
Primer 2 – pUS10FSB_Rev and *Bam*HI
Template DNA - CVI988 BAC DNA, Diluted
Reaction Conditions A
Annealing Temperature – 50^{o}C
Extension Time – 3 min

PGKGFP Colony PCR
Recipe B
Product size – 793 bp
Primer 1 – p2aGFP_For
Primer 2 – p2aGFP_Rev
Template DNA – Bacterial Colony
Reaction Conditions C
Annealing Temperature – 52^{o}C
Extension Time – 1 min 30

PGKH10myc Colony PCR
Recipe B
Product size – 1680 bp
Primer 1 – pH10myc_For
Primer 2 – pH10myc_Rev
Template DNA – Bacterial Colony
Reaction Conditions C
Annealing Temperature – 52^{o}C
Extension Time – 1 min 30

A.1.4.3 Chapter 5: UL50 Open Reading Frame

MDV UL50 FSA
Recipe A
Product size – 3086 bp
Primer 1 – pUL50FSA_For
Primer 2 – pUL50FSA_Rev and *Pac*I
Template DNA - CVI988 BAC DNA, Diluted
Reaction Conditions A
Annealing Temperature – 52^{o}C
Extension Time – 4 min

MDV UL50 FSB
Recipe A
Product size – 3053 bp
Primer 1 – pUL50FSB_For and *Kpn*I
Primer 2 – pUL50FSB_Rev and *Age*I
Template DNA - CVI988 BAC DNA, Diluted
Reaction Conditions A
Annealing Temperature – 52^{o}C
Extension Time – 4 min

UL50 (Deletion) Colony PCR
Recipe B
Product size – Gene deletion: 594 bp; No gene deletion: 1821 bp
Primer 1 – UL50MutBAC_For
Primer 2 – UL50MutBAC_Rev
Template DNA – Bacterial Colony
Reaction Conditions C
Annealing Temperature – 52^{o}C
Extension Time – 2 min 30

UL50
Recipe A
Product size – 3123 bp
Primer 1 – pUL50_For (rpt)
Primer 2 – pUL50_Rev (rpt)
Template DNA - CVI988 BAC DNA, Diluted
Reaction Conditions A
Annealing Temperature – 48^{o}C
Extension Time – 4 min

A.1.4.4 Chapter 6: 2a Polyprotein

GFP
Recipe A
Product size – 793 bp
Primer 1 – p2aGFP_For and *Ase*I
Primer 2 – p2aGFP_Rev and *Nsi*I
Template DNA – Diluted pTOPPGKGFP maxiprep DNA
Reaction Conditions B
Annealing Temperature – 52^{o}C
Extension Time – 2 min

PGKGFP2a
Recipe A
Product size – 1625 bp
Primer 1 –pPGKGFP2a_For
Primer 2 –pPGKGFP2a_Rev and *Kpn*I
Template DNA – pTOPPGKGFP2a miniprep DNA
Reaction Conditions B
Annealing Temperature – 52^{o}C
Extension Time – 2 min

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