Promoter and Leader Sequence Effects on Immunological Potency of Recombinant Modified Vaccinia Virus Ankara Delivering Tuberculosis Antigens

Thesis

Promoter and leader sequence effects on the immunological potency of recombinant Modified Vaccinia virus Ankara delivering tuberculosis antigens.

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Contents

Abstract

Modified vaccinia virus Ankara (MVA) is a highly attenuated, replication defective strain of Vaccinia virus with the attributes of an optimal viral vaccine delivery system.

An improved vaccine for the prevention of tuberculosis (TB) is urgently required and until recently, the most advanced clinical candidate under development for use against TB was based on MVA vectoring a single mycobacterial antigen (MVA85A). Unfortunately, pre-clinical efficacy and potent immunogenicity in humans did not lead to improved protection in BCG vaccinated infants.

There are a number of design features to consider in the development of a recombinant MVA (rMVA)-based vaccine. The current study undertook to assess how these might impact upon the immunological potency of MVA vectoring TB antigens. Specifically, the influence of vaccinia promoter selection and leader sequences was assessed.

Vaccinia promoter selection influences antigen transcription and thereby expression. The relationship between antigen production, murine immunogenicity and protective efficacy was evaluated. The results support the conclusion that vaccinia promoter optimisation can increase immunological potency and efficacy.

Virally vectored antigens can be fused to sorting signals for optimised intracellular processing. The immunomodulatory effects of two leader sequences were compared. The results suggest that the sorting signal currently incorporated in virally vectored TB candidates, such as MVA85A, might be subject to further optimisation for the induction of enhanced T cell responses.

Overall, the results demonstrate that rMVA immunogenicity is intrinsically linked to the precise nature of transgene insertion. The value of an optimised vaccinia promoter might be to reduce the dose of rMVA required. Leader sequences represent an opportunity to enhance the quality of the antigen-specific response for improved protection. It is

concluded that both vaccinia promoter selection and leader sequence optimisation should be a feature of future rMVA TB vaccine development.

Abbreviations

°C	Degrees Celsius
ACK buffer	Ammonium-chloride-potassium buffer
AmpR	Ampicillin resistance gene
ANOVA	Analysis of Variance
APC	Antigen presenting cell
ATCC	American Type Culture Collection
BAC	Bacterial artificial chromosome
BCIP/NBT	5-bromo-4-chloro-3'-indolyphosphate and nitro-blue tetrazolium
BHK-21	Baby hamster kidney cell line
bp	Base pairs
CAT	Chloramphenicol acetyltransferase
ccdB	Toxin encoding gene
CEF	Chick embryo fibroblasts
CFU	Colony forming units
CPE	Cytopathic effects
CmR	Chloramphenicol resistance gene
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOT	Directly observed therapy
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ELISpot	Enzyme linked immunosorbent spot assay
EPI	Expanded Programme of Immunisation

ER	Endoplasmic reticulum
FBS	Foetal bovine serum
gDNA	Genomic DNA
GFP	Green fluorescent protein
GMP	Good Manufacturing Practice
GOI	Gene of Interest
HIV/AIDS	Human immunodeficiency virus/acquired immune deficiency syndrome
HRP	Horseradish peroxidase
ICS	Intracellular cytokine staining
IFNγ	Interferon gamma
Ig	Immunoglobulins
IL	Interleukin
JE	Japanese encephalitis
kb	Kilo-base pair
kDa	Kilo Daltons
L, R and DR	Left flank, Right flank and Direct Repeat (MVA homologous DNA)
LacZ	ß-galactosidase gene
LTBI	Latent TB infection
LB	Lysogeny broth
PAGE	Polyacrylamide gel electrophoresis
OE-PCR	Overlap extension PCR
Ori	Origin of replication
OVA	Ovalbumin
MDR	Multi-drug resistant
mH5	Modified vaccinia promoter H5
MHC	Major histocompatibility complex

MOI	Multiplicity of infection
Mtb	*Mycobacterium tuberculosis*
MVA	Modified Vaccinia virus Ankara
NIH	National Institutes of Health
NHP	Non-human primate
NSB	Non-specific binding
OADC	Oleic acid, albumin, dextrose and catalase
OD	Optical density
P7.5	Vaccinia promoter 7.5
PCR	Polymerase chain reaction
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PFU	Plaque forming units
PMA/I	Phorbol 12-myristate 13-acetate and Ionomycin
PPD	Purified protein derivative
PVDF	Polyvinylidene difluoride
SDS	Sodium dodecyl sulphate
SFU	Spot forming units
SOC	Super Optimal broth with Catabolite repression
TAE	Tris-acetate EDTA
TB	Tuberculosis
T_{CM}	T central memory cell
TCR	T cell receptor
T_E	T effector cell
T_{EM}	T effector memory cell
TMB	Tetramethylbenzidine

TNFα	Tumour necrosis factor alpha
TPA	Human tissue plasminogen activator gene sorting signal
VLP	Virus like particle
VSV	Vesicular stomatitis virus
VSV G	Vesicular stomatitis virus glycoprotein
VSVg	VSV glycoprotein C-terminal cytosolic and transmembrane domain
VV	Vaccinia virus
XDR	Extensively drug resistant
ZeoR	Zeocin™ resistance gene

Publications

Sections of Chapter 1, including Figures 1.3, 1.4 and 1.7, have been copied or adapted from the following:

Hall, Y., and Carroll, M.W. (2012). Recombinant MVA vaccines: Optimization, Preclinical, and Product Development. In Vaccinology Principles and Practice, W.J.W. Morrow, N. Sheikh, A., C.S. Schmidt, and H.D. Davies, eds. (Wiley-Blackwell), pp. 209-223.

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Chapter 1 Introduction

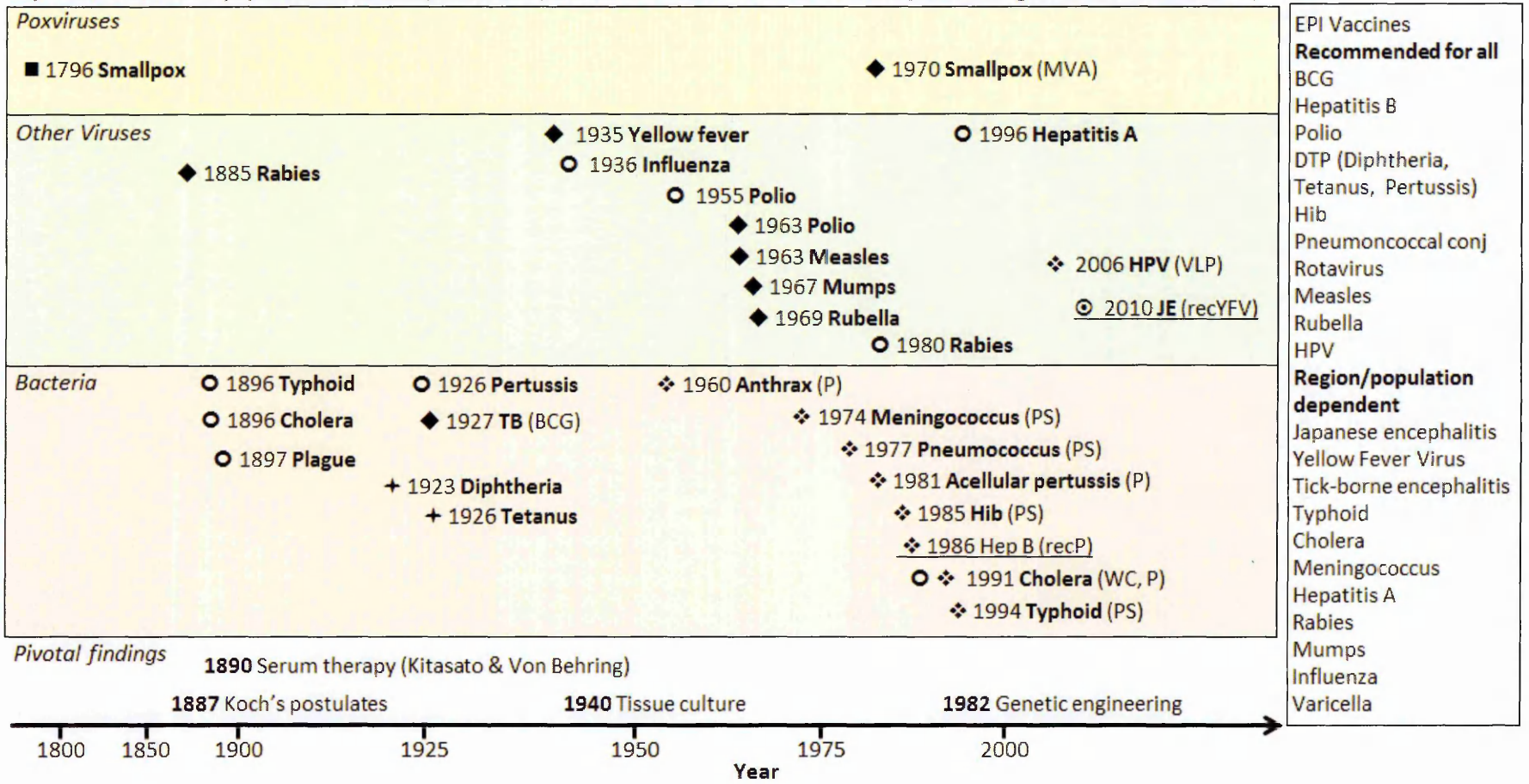
1.1 Vaccination

In 1798, Edward Jenner published his research describing inoculation with cowpox lesion matter to confer protection against smallpox (Jenner, 1798). At the end of the last century, live Vaccinia virus, a very close relative of cowpox virus was used as the vaccine in the World Health Organisation's Smallpox Eradication Programme (1966 – 1980). Jenner introduced the noun 'vaccine' to describe the cowpox material used as his inoculum, and subsequent use of the words 'vaccine', 'vaccination' and 'vaccinology' pay homage to his pioneering studies; *vacca* is Latin for cow. Jenner may not have been the first to use cowpox to achieve protection against smallpox; there is anecdotal evidence that an English farmer, named Benjamin Jesty, protected his family from smallpox via the same means 20 years earlier. The practice of variolation (inoculation with smallpox lesion matter) had also been widely implemented and can be traced back to the ancient Chinese. Jenner, however, was the first to subject the phenomenon of immunisation to scientific investigation and reporting. In the subsequent 200 years of vaccinology research there have been many significant developments.

1.1.1 Major advances in the development of novel vaccines

The history of vaccination, and findings in the related fields of bacteriology, virology and immunology that have been pivotal to vaccine development, has been reviewed (Plotkin, 2014; Plotkin and Plotkin, 2011). A brief overview is provided here and is summarised in Figure 1.1.

Figure 1.1 Summary of major developments in vaccinology research. The introduction of human vaccines based on live, naturally attenuated organisms (■), live, artificially attenuated organisms (◆), killed organisms (O), toxoids (✦) and subunits (❖) and a viral vector (⊙) are shown. Genetically engineered vaccines are underlined. (Abbreviations: P, protein; PS, polysaccharide; Hib, *Haemophilus influenzae type b;* rec, recombinant; WC, whole cell; VLP, virus-like particle; Hep, hepatitis; HPV, human papilloma virus; JE, Japanese encephalitis; YFV, Yellow Fever virus ; EPI, Expanded Programme of Immunisation).



In his publications relating to smallpox, Edward Jenner described the capacity for a naturally occurring, less virulent infection to confer protection against a related, but more deadly disease. Eighty years later, Louis Pasteur demonstrated that the same phenomenon could occur following artificial attenuation of the disease-causing agent; chickens were protected against cholera and cattle against anthrax. In 1885, the Pasteur laboratory applied induced attenuation to produce a rabies vaccine for post-exposure use in humans.

Fundamental to the development of vaccines has been the discovery of their causative agents. In 1877, Robert Koch published discussions on the causative agents of disease, giving rise to Koch's postulates: a set of 4 criteria through which to confirm the relationship between a microbe and an infectious disease. Between 1876 and 1884, Robert Koch identified the bacteria responsible for anthrax, tuberculosis (TB) and cholera. The discovery of these and other causative agents of disease was an important precursor to the development of effective vaccines; after the artificial attenuation work of Pasteur came the finding that whole-cell preparations of dead bacteria, killed by heat or chemical treatment, could be safely administered to confer protection against their live counterpart. In the late 19th century, this process gave rise to bacterial vaccines against typhoid, cholera and plague.

Towards the turn of the 20th century, it was discovered that the bacterium responsible for diphtheria infection produced a toxin and that anti-toxin, i.e. the serum of a toxin-inoculated animal, was protective upon transfer to another animal. In 1907, Emil von Behring demonstrated improved efficacy with toxin - anti-toxin (TAT) mixtures and this was implemented for human use. In 1923, TAT was superseded by the use of formalin-inactivated toxin, or 'toxoid', the immunogenicity of which was later shown to be enhanced by the addition of aluminium salts (Glenny *et al.*, 1926). This was the first example of an adjuvanted vaccine, i.e. a vaccine to which a substance had been added to potentiate the immune response evoked to it.

The artificial attenuation work of Pasteur had been achieved using exposure to adverse conditions such as oxygen and/or heat. The first example of attenuation achieved via serial passage was provided by scientists Albert Calmette and Camille Guérin, who grew *Mycobacterium bovis* on artificial medium. After 230 passages, performed over a period of 14 years, the bacillus of Calmette and Guérin (BCG) was less virulent and provided protection against *M. tuberculosis* in animal models. The *M. bovis* BCG vaccine strain was first used in humans in 1927 and later, evidence for its protective efficacy in children was described (Calmette, 1931).

The first viral vaccine to be attenuated by serial passage was a vaccine for Yellow Fever virus (Theiler and Smith, 1937). Viral vaccine development was later accelerated by the advent of methodology for large-scale propagation of viruses *in vitro* (Enders *et al.*, 1949); the development of these improved tissue culture techniques led to the production of live attenuated vaccines against poliovirus, measles, rubella and mumps. A further development related to the culture of viruses *in vitro* was the ability to induce attenuation of RNA viruses via reassortment. This process was applied in the generation of vaccines against influenza and rotavirus, and more recently a new rotavirus vaccine (Clark *et al.*, 1996).

An alternative to the use of whole cell preparations has been the development of vaccines based on individual components of the disease-causing organism. The first examples of 'subunit' vaccines were based on inactivated toxin (diphtheria and tetanus toxoid). More recent examples based on isolated proteins include vaccines for use against anthrax, pertussis and hepatitis B. There are also vaccines based on polysaccharides, e.g. for meningococci and pneumococci, which have been improved by conjugation to protein antigens. Another subunit combination, developed for use against cholera, comprises a killed whole cell (WC) preparation in combination with a cholera protein. Some virus

proteins, e.g. the L1 protein of human papilloma virus (HPV), have increased immunogenicity on account of forming virus-like particles (VLP).

The most recent general development to impact on the field of vaccinology is the advancement of techniques related to genetic engineering. This has made possible the production of recombinant protein, as first demonstrated for hepatitis B surface antigen (Valenzuela *et al.*, 1982). It has also been possible to deliver a subunit antigen for one infectious disease target using an unrelated organism as a recombinant delivery system. Thus far, one example has been licensed: a Yellow Fever Virus (YFV) carrying a Japanese encephalitis (JE) antigen.

An important consideration for more recent vaccine development efforts is safety. To date, smallpox remains the only disease to have been eradicated via vaccination; eradication was declared in 1979 (Fenner *et al.*, 1988). Live Vaccinia virus was used as the vaccine and whilst clearly efficacious, it was observed to cause severe complications in some individuals, for example those with immunological disorders (Lane *et al.*, 1969). Therefore, a safer alternative was required. Vaccinia virus Ankara was attenuated via serial passage on chick embryo fibroblast (CEF) cells. After more than 500 passages, the resultant strain was replication-defective in mammalian cells and non-pathogenic in animal models and was re-named Modified Vaccinia virus Ankara (MVA) (Mayr *et al.*, 1978). Later, genomic analysis and marker rescue revealed the attenuation to be the result of multiple deletions and mutations (Meyer *et al.*, 1991; Wyatt *et al.*, 1998), all of which were confirmed when the MVA genome was sequenced (Antoine *et al.*, 1998). Towards the end of the smallpox campaign, MVA was used to vaccinate over 120,000 individuals without the complications observed for the parent strain. Whilst the contribution of MVA to the eradication campaign may be unclear, it was demonstrated to be safe in all groups. Recently, MVA has been licensed for use as a smallpox vaccine in Europe (IMVANEX®, Bavarian Nordic) and Canada (IMVAMUNE, Bavarian Nordic).

1.1.2 Future goals for vaccinology research

As described above, there are now many effective vaccines available for the prevention of important human diseases and these are used in the World Health Organisation's Expanded Programme of Immunisation (EPI) (see Figure 1.1). However, vaccines are still required for prevention of the three biggest causes of morbidity and mortality: TB, HIV/AIDS and malaria. There are also vaccines under development for the immunotherapeutic treatment of cancers.

A vaccine for TB (*M. bovis* BCG) was developed and remains in use, but is now recognised to be only partially effective beyond adolescence and so an alternative is required (discussed further, below). It now known that for the majority of the vaccines successfully implemented to date, protective immunity is achieved via the induction of humoral responses, i.e. protective antibodies (Plotkin, 2010). For the outstanding vaccine targets, for which antigenic diversity and complexity of the pathogen/stages of the infectious disease are an issue, greater levels of cellular immunity are believed to be required. As such, it has become apparent that a thorough understanding of the protective immune response is an essential aspect of vaccinology research.

Humoral immunity

That an immunological component (antibodies) existed in blood serum became evident in 1890, when von Behring and Kitasato performed serum transfer studies to confer protection against diphtheria. In 1931, Theiler demonstrated the neutralising properties of this component with regards to viral infectivity. Then in 1939, Tiselius and Kabat identified antibodies as gamma globulins.

Immunoglobulins (Ig) are produced by B cells - lymphocytes that mature in the bone marrow. They are glycoproteins comprising heavy and light chain elements the structure of which form a variable, antibody binding fragment (Fab) and a crystallisable fragment (Fc) which is constant (Schroeder and Cavacini, 2010). A schematic is shown in Figure 1.2A.

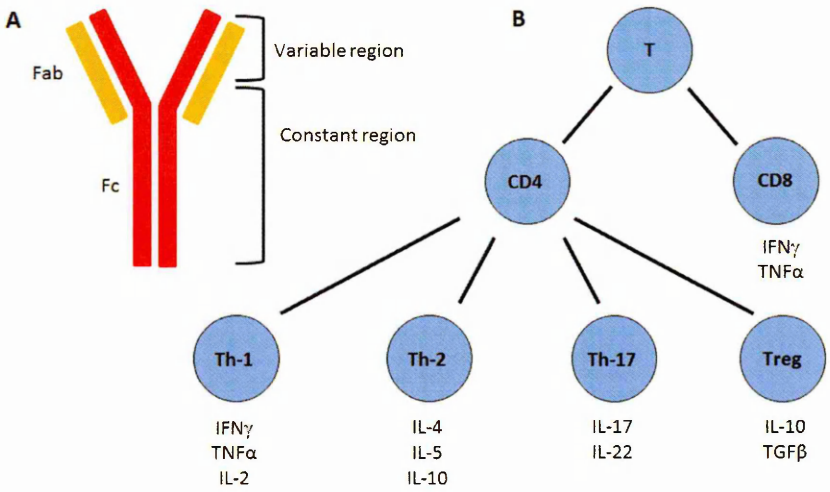


Figure 1.2 Components of the adaptive immune system. A) The structure of immunoglobulin comprising heavy (red) and light (orange) chains. B) Principal T-cell subsets and the cytokines they secrete (IFNγ; interferon gamma, TNFα; tumour necrosis factor alpha, IL; interleukin).

For the most part, Ig molecules are secreted, but they can also serve as a B cell surface receptor. Antibodies function by binding antigen at the variable region and then stimulating immunological pathways via Fc receptors (FcR); the majority of cells contributing to immune function expressed FcR. Five main classes of Ig have been categorised based on differences in the structure of the Fc fragment, two of which (IgG and IgA) have sub-classes, also referred to as isotypes (see Table 1.1). For each Fc type there is a complementary FcR capable of activating or inhibiting immunological responses. Differences in the heavy chain confer differences in biological activity, since it is the heavy chain which defines the Fc portion, and thereby the FcR that will be bound and the role of the Ig molecule within the immune system.

Arguably the most important class of antibody, in the context of vaccine induced protection, is IgG. It is the most abundant and induction of antigen-specific IgG is a correlate of protection for many vaccines (Plotkin, 2010). In addition to influencing

immune pathways, a vital role for IgG (as well as IgA) is to bind directly to toxins and viruses, thereby neutralising them by impeding subsequent interaction with the host.

Table 1.1 The 5 classes of immunoglobulin. A summary of the main characteristics and biological functions of human Ig classes. IgG and IgA are present as one of 4 and 2 isotypes, respectively (Arnold *et al.*, 2007; Schroeder and Cavacini, 2010).

Ig	Heavy chain	Function and characteristics
IgG	γ1	Neutralises toxins and viruses.
	γ2	Binds complement; a serum enzyme system leading to pathogen destruction.
	γ3	Is the most abundant serum Ig, circulating at 10-15 mg/ml. Can be transferred across the placenta.
	γ4	As above, but does not bind complement.
IgA	α1, α2	Secretory forms (IgA1 and IgA2) provide mucosal immunity. Both associated with mucosal epithelia, tears, saliva and milk. Serum IgA consists predominantly of IgA1 (~ 1 mg/ml).
IgM	μ	Associated with primary immune response. Binds complement. Serum Ig has a pentameric structure (~ 2.5 mg/ml). Also serves as a B cell receptor.
IgD	δ	Low abundance in serum (< 30 μg/ml). Is expressed on immature (differentiating) B cells, with IgM.
IgE	ε	Least abundant serum Ig (< 1 μg/ml). Associated with hypersensitivity, allergy and parasitic infections.

Cell mediated immunity

It became apparent in the 1940's that some infectious diseases were not preventable by induction of antibody responses; it was shown that the delayed type hypersensitivity (DTH) reaction associated with skin tests could be induced after transfer of cells, but not by serum. In 1968, the T-cell lymphocyte was identified as having cell-cytotoxic properties that were induced upon cellular infection.

T cells are lymphocytes that mature in the thymus. They are diverse and are characterised by T-cell antigen surface receptors (TCR). The essential role of T cells is to recognise antigen that has been processed within a host cell; T cell receptors are only able to bind antigen in the form of a peptide fragment bound to a major histocompatibility complex (MHC) molecule.

There are two classes of MHC molecule. All cells, except for red blood cells, express MHC Class I. The role of this molecule is to bind antigen encountered within the cell, i.e. following infection with an intracellular pathogen. MHC Class II molecules bind antigen that has been endocytosed by the cell. They are expressed by 'professional' antigen presenting cells (APC) such as dendritic cells (DC), macrophages and B cells.

There are 2 main types of T cell, defined by the MHC receptors that they express. T cells expressing CD8 (CD8+ T cells) bind MHC Class I molecules. Upon activation, CD8+ T cells are effective in lysing infected cells directly and so are called cytotoxic T lymphocytes (CTL). T cells expressing CD4 (CD4+ T cells) bind MHC Class II. The role of CD4+ T cells, upon activation, is to release cytokines – cell signalling molecules that stimulate further T cell function, macrophage activation and B cell antibody production. Thus, they are also known as T helper (Th) cells and can be further subdivided into Th-1, Th-2 and Th-17 subsets based on their cytokine secretory profile. Th-1 and Th-17 enhance inflammatory immune responses, while Th-2 promotes non-inflammatory responses. An essential contributor to the regulation of inflammation are another subset of CD4+ helper cells called regulatory T

cells (Treg) (Vignali *et al.*, 2008). As well as lysing antigen-bearing cells directly, cytotoxic CD8+ cells are also able to release the effector cytokines IFNγ and TNFα. A summary is shown in Figure 1.2B.

Each T cell subset can be further categorised according to its state of activation (Broere *et al.*, 2011). 'Naïve' T cells, having matured in the thymus, reside in the spleen and lymph nodes. Upon infection, antigen-loaded APC migrate to the lymphoid tissues where TCR and MHC-bound antigen interact. This leads to T cell activation and in this state T cells become effector cells (T_E). Critical to the principle of vaccine-induced T cell immunity is the ability of some effector T cells to remain beyond antigen elimination, as these long-lasting populations contribute to immunological memory.

Effector memory cells (T_{EM}) remain at the periphery, including mucosal sites, where they are able to encounter secondary infection and develop immediate effector function. Central memory cells (T_{CM}) reside in the secondary lymphoid tissue (spleen and lymph nodes) where they can mount a recall response, which although delayed in terms of mounting effector functions has greater proliferative potential (Sallusto *et al.*, 2010).

Induction of cell mediated immunity

For the outstanding vaccine targets, humoral responses may remain important for prevention of initial infection, but induction of cell-mediated immunity is now believed to be essential for effective infection control (Plotkin, 2010). Further, protection may depend upon the activation of particular T cell subsets, specifically, the induction of T cells offering protective effector function within which are subsets that will be programmed to have both effector memory and central memory function.

For any given vaccine target, antigen discovery is important; antigen-specific responses must confer protection. Sophisticated transcriptomic, proteomic and reverse vaccinology (*in silico*) approaches are being applied to identify novel vaccine antigens. However,

beyond antigen selection, it is vaccine formulation and/or the delivery system employed that will determine the type of immune response that is promoted, thereby impacting on the potential success of the vaccine.

Adjuvant research has identified a number of compounds that can evoke humoral and cellular immunity; however, the ability to promote robust cellular immunity without adverse effects is a challenge (Reed *et al.*, 2013). An alternative to adjuvanting protein subunits is to apply genetic engineering to the challenge of antigen delivery. There are vaccines under clinical development based on plasmid DNA and many recombinant organisms. In these vaccines the genetic code for the vaccine antigen is delivered for expression in the host, i.e. the vaccine recipient. This gives rise to intracellular manifestation of antigen, as would occur in intracellular pathogen infection. Thus, plasmid DNA and recombinant viral and bacterial vaccines are able to induce effective CD8+ CTL responses.

Plasmid DNA vaccines are simple to produce and induce both CD4+ and CD8+ T cell immunity (Maecker *et al.*, 1998). The first demonstration of protective DNA vaccination was performed in mice immunised with an influenza antigen (Ulmer *et al.*, 1993). Plasmid DNA vaccines have proved to be an excellent tool in pre-clinical research, and there are some examples licensed for veterinary use (Draper and Heeney, 2010) . However, plasmid DNA alone has been found to be less immunogenic in larger animals and strategies are required for enhancing their efficacy (Saade and Petrovsky, 2012).

Responses to DNA-vectored antigen can be enhanced by co-administration with adjuvants, or by advanced delivery techniques, e.g. gene gun technology. It is also possible to increase T cell responses via heterologous prime-boosting. This was first demonstrated following a DNA prime and MVA boost protocol in mice, which provided protection against malaria (Schneider *et al.*, 1998). High levels of antigen-specific CD8+ T cells were observed and induction of similarly enhanced T cell responses were subsequently demonstrated in

humans (McConkey *et al.*, 2003). Currently, a vaccine candidate based on DNA-MVA immunisation is under clinical development for HIV (Munseri *et al.*, 2015).

The benefit of recombinant bacterial or viral vectors is that, relative to plasmid DNA or protein subunits, they are antigenically diverse and highly immunogenic, without the need for additional adjuvants or specialised delivery methods. There are vaccines based on recombinant bacteria and viruses in clinical trials, but as yet, the only licensed vaccine is a recombinant YFV – JE chimera. That bacterial vaccine vectors might not be as advanced as viral vectors may stem from their complexity and the need to render them safe for use in human subjects and concomitant release to the environment (da Silva *et al.*, 2014). A greater number of viral vectored vaccines, meanwhile, are under development.

1.1.3 Viral Vector vaccines

A summary of the principal viral vaccine vectors currently under investigation is summarized in Table 1.2. As shown, a number of viral vectors are based on poxviruses.

The first viral vectored vaccines were based on live Vaccinia virus. Following the eradication of smallpox, and supported by recent advances in genetic engineering, Vaccinia virus was investigated as a tool for recombinant antigen expression. In the first instance, it was shown to be possible to introduce and express foreign genes in recombinant Vaccinia virus (rVV) (Mackett *et al.*, 1982; Panicali and Paoletti, 1982). Soon after it was demonstrated that rVV could evoke protective, antigen-specific immune responses in an animal model of hepatitis B (Moss *et al.*, 1984). Later, MVA was turned to as an alternative viral vector with an improved safety profile and was also demonstrated to be effective pre-clinically (Sutter *et al.*, 1994).

Table 1.2 Viruses used as vaccine vectors. The principal viral vector platforms under investigation for the development of novel vaccines.

Virus Family	Viral Vector(s)	Advantages and disadvantages[†]
DNA viruses		
Poxviridae	Vaccinia virus, MVA, NYVAC, ALVAC and FPV	Genome 130 – 280 kb. Large capacity for foreign DNA (>25 kb). Safe history of use in humans/veterinary applications. Established platforms for product development. [†]Anti-vector immunity may arise.
Adenoviridae	Adenovirus	Genome 36 – 38 kb. Capacity for 7-8 kb foreign DNA. Safe history of use in humans/veterinary applications. Established platforms for product development. [†]Possible interference from pre-existing immunity.
RNA viruses		
Rhabdoviridae	VSV	Genome 13 – 16 kb [†]Limited capacity for foreign inserts.

Abbreviations: MVA, modified Vaccinia virus Ankara; NYVAC, Vaccinia virus attenuated via the deletion of 18 open reading frames; ALVAC, attenuated canarypox virus; FPV, fowlpox virus; VSV, Vesicular stomatitis virus.

Vaccinia virus (VV) is a member of the *orthopoxvirus* genus within the *Poxviridae* family. Other poxviruses in use as viral vectors include attenuated canarypox virus (ALVAC), fowlpox virus (FPV) and a strain of VV attenuated by genetic manipulation (NYVAC). While none of these vectors have been licensed for human use, Phase I – III clinical studies for a range of disease targets and cancer are ongoing, and there are examples of licensed veterinary vaccines based on ALVAC, FPV and VV (Draper and Heeney, 2010). Thus, poxviruses are an effective vaccine delivery platform and are amenable to large-scale production.

After poxviruses, the most commonly employed viral vector platform for the development of novel vaccines is adenovirus (Ad). Adenovirus naturally infects humans resulting in a range of clinical outcomes. The infection can be asymptomatic, cause common cold symptoms or, in some cases, severe respiratory disease. In common with poxviruses, Adenovirus exhibits a broad tropism, i.e. can infect a range of cell types. Like poxviruses, there are examples of rAd under Phase I – III clinical development for bacterial, viral and parasitic diseases, as well as for cancer immunotherapy (Rollier *et al.*, 2011). The capacity of recombinant poxviruses to carry foreign DNA may be greater, but the pay load for rAd may still amount to several antigens. In Ad vector methodology, genes are removed from the virus in order to render it replication deficient. A 'gutless' Ad vector may accommodate up to 8 kb of foreign DNA. To date, the most commonly used Ad virus serotype has been human Ad virus serotype 5 (AdHu5). Pre-existing immunity may be a disadvantage for this vector, but more recently this has been circumvented by using chimpanzee serotypes.

There are fewer examples of other viruses that have been used as viral vaccine vectors. A relatively recent example to come to prominence is based on Vesicular stomatitis virus (VSV). VSV is a member of the *Rhabdoviridae* family of viruses. It infects animals of agricultural importance (horses, cattle, pigs) and can cause a flu-like illness in humans. The virulence of VSV is associated with its glycoprotein (G). Cleverly, VSV G can be replaced

with an alternative antigen with the effect that the virus is attenuated, induces little neutralising immunity against itself while promoting strong humoral and cell-mediated responses against a recombinant antigen (Roberts *et al.*, 1999). Most recently, VSV viral vector technology has been implemented in a Phase III trial against Ebola virus disease (Camacho *et al.*, 2015).

There are other viral vaccine vectors under investigation for which there is currently less clinical data through which to establish safety and efficacy. For each of these, there may also be other disadvantages such as potential to integrate into the human genome, reduced tropism and pre-existing immunity. They include Adeno-associated virus, Measles virus, Sendai virus and Baculovirus and have been subject to review (Brave *et al.*, 2007; Liu, 2010; Tripp and Tompkins, 2014; Ura *et al.*, 2014).

With many examples of MVA and Adenovirus having been tested in clinical studies, there is now detailed evidence for the type of T cell immunity they induce. Both are able to induce CD8+ responses that include effector memory and central memory functions (Rollier *et al.*, 2011). Thus, the responses are encouraging from the perspective of novel vaccine development.

1.1.4 Prospects and progress for MVA based vaccines

MVA is a highly attenuated, replication defective strain of vaccinia virus with attributes of an optimal viral delivery system including; large capacity for foreign DNA, proven clinical safety and ability to induce potent and long-lasting humoral and cell-mediated immune responses which can be further complemented in heterologous prime-boost regimens. Efficacy of rMVA based vaccines has been demonstrated in numerous pre-clinical and clinical studies against a range of targets including HIV, malaria, tuberculosis and an array of tumour associated antigens (Gomez *et al.*, 2008; Harrop and Carroll, 2006). In addition to its proven clinical safety and ability to elicit protective immunological responses, the advantages of rMVA as a viral vaccine vector now extend to include established

manufacturing processes (compliant with Good Manufacturing Practice; GMP), low costs of goods, ease of storage and clear requirements for regulatory submission (Hall and Carroll, 2012). That rMVA based vaccines are a reliable prospect is evident from the number of candidates in clinical development, with a number of institutions committed to rMVA based research (see Table 1.3).

Pre-existing and anti-vector immunity

As stated above (Table 1.2), anti-vector immunity may be the biggest disadvantage presented by the use of MVA as a viral vaccine vector. Pre-existing immunity has the potential to limit the efficacy of a viral vector and, following the eradication of smallpox, there was a widespread population with humoral immunity to vaccinia virus. However, with the cessation of global vaccination, this population has halved (Bray, 2003) and is decreasing. Even so, the effects of pre-existing vector immunity still require consideration in the context of multiple-immunisation regimes and in light of the number of rMVA vaccines under clinical study.

Pre-clinically, MVA has been shown to evoke less potent vector-specific immune responses than replication competent vaccinia, without compromising the strength of immune response elicited against a foreign antigen (Ramirez *et al.*, 2000a). Recombinant MVA has also been shown to be effective under conditions of pre-existing immunity to the vector (Ramirez *et al.*, 2000b; Redchenko *et al.*, 2004). There are now clinical examples for immunogenic vaccine antigen delivery by rMVA against a background of MVA-specific antibody, pre-existing, or induced through repeated rMVA immunisation (Harrop *et al.*, 2007). rMVA's ability to induce transgene responses in the presence of pre-exiting MVA immunity in homologous prime boost regimens may be due in part to the rapid expression kinetics of Vaccinia virus promoters and a lack of requirement for cell to cell spread of the vector. For each novel rMVA product, however, the impact of pre-existing immunity on rMVA efficacy should be evaluated (Kannanganat *et al.*, 2010).

Table 1.3 Recombinant MVA in clinical development. Examples of clinical candidates under development by institutes active in the field of rMVA research.

Target	Vaccine(s)	Antigens	Clinical Phase	Ref
University of Oxford (UK)				
Tuberculosis	MVA85A	Ag85A	II	(Sheehan *et al.*, 2015)
Seasonal Influenza	MVA-NP+M1	NP+M1	II	(Berthoud *et al.*, 2011)
Malaria	MVA ME-TRAP	ME-TRAP	IIb	(Sebastian and Gilbert, 2015)
	MVA MSP1, MVA AMA1, MVA CS	MSP1, AMA1, CS	IIa	
	MVA RH5, MVA PvDBP	RH5, PvDBP	Ia	
HIV infection (Clades A, B, C and D)	MVA.HIV$_{CONSV}$	14 conserved regions of the HIV-1 proteome	Ib	(Letourneau *et al.*, 2007)
Bavarian Nordic (Denmark)				
Respiratory Syncytial Virus	MVA-BN RSV	RSV F, GP (x2), N, M2	I	NCT 02419391
Ebola/Marburg Virus Disease	MVA-BN Filo	Zaire Ebola virus GP, Sudan Ebola virus GP, Marburg virus GP, and Tai-Forest Ebola virus NP	III	(Tapia *et al.*, 2015)
Transgene (France)				
Cancer; lung	TG4010	MUC1 and IL-2	IIb	(Quoix *et al.*, 2011)
HPV - induced disease	TG4001	HPV16 E6, E7 and IL-2	IIb	(Brun *et al.*, 2011)
Oxford Biomedica (UK)				
Cancer; colorectal, mesothelioma, ovarian.	TroVax®	5T4	II	(Harrop *et al.*, 2012)
National Institute of Health (USA)				
Ebola Virus Disease	MVA Ebola Z	Zaire Ebola virus GP	Ib	(Zhou and Sullivan, 2015)
HIV	MVA-CMDR	CRF01_AE gag, env and pol	I	(Earl *et al.*, 2009)

Abbreviations: Ag85A, mycobacterial mycolyl transferase 85A; AMA-1, apical membrane antigen 1; CMDR, Chiang Mai Double Recombinant; GP, glycoprotein; HPV, human papilloma virus; IL-2, interleukin 2; M1, matrix 1 protein; ME-TRAP, multiple epitope string fused to thrombospondin-related adhesion protein; MSP-1, merozoite surface protein 1; MUC1, mucin 1; NP, nucleoprotein; PvDBP, *Plasmodium vivax* Duffy-binding protein; RH5, reticulocyte binding protein-like homologue 5; RSV F, G, N, M2, respiratory syncytial virus fusion protein, nucleoprotein, and matrix protein 2; NCT02419391; clinical trial ID for a Phase I Trial to Evaluate Safety, Tolerability and Immunogenicity in Healthy Adult Subjects.

Heterologous prime-boost regimens

The potential for heterologous prime-boosting to enhance rMVA efficacy, particularly for the improvement of immunisation strategies which require cell mediated immunity, is now well established. Recombinant MVA administered in prime-boost regimens involving plasmid DNA or alternative viral vectors has been demonstrated to drive strong T cell responses (McConkey *et al.*, 2003) and may be more effective when rMVA boosts non-MVA prime (Vuola *et al.*, 2005), although this may differ between vaccine targets. Heterologous prime-boost regimens are being pursued in a clinical setting for vaccines targeted against HIV (Iyer and Amara, 2014), malaria (Hill *et al.*, 2010) and tuberculosis (Ottenhoff and Kaufmann, 2012).

Immunisation route

Route of administration is sometimes thought to be another feature of the immunisation protocol that can influence rMVA efficacy. Intramuscular (i.m) and intradermal (i.d) delivery are commonly used for rMVA delivery with the latter leading to higher immune responses in some pre-clinical studies, which is evidently a result of the increased number of professional antigen-presenting cell encountered at this site (Abadie *et al.*, 2009). However, any benefit conferred by i.d immunisation is outweighed by its reduced practicality in the clinic and tendency for localised reaction. In any event, clinical comparison of rMVA i.d and i.m administration demonstrates no significant difference in immunogenicity (Berthoud *et al.*, 2011; Harrop *et al.*, 2006). Mucosal delivery may be advantageous for diseases in which the pathogen is first encountered at the mucosa and this is under investigation for rMVA targeting HIV (Maeto *et al.*, 2014) and tuberculosis (Satti *et al.*, 2014).

Summary

In conclusion, while vector immunity must remain a consideration for MVA-based vaccines, there are many more favourable characteristics and the opportunity to optimise its use.

There are now a number of vaccines under clinical study and there have been developments in manufacturing platforms and bespoke cell lines for manufacture.

1.2 Rational design of recombinant MVA

The principal feature of a vaccine based on a recombinant MVA virus is the antigen, or antigens, it has been engineered to deliver. For a given target disease, these antigens must be capable of evoking an immune response that imparts long-lasting, protective immunity, otherwise the vaccine will not be effective. As described below, there are aspects of rMVA construction that may impact upon the ability of the vectored antigen(s) to achieve this. The development of optimised rMVA is, therefore, very important. Our current understanding of rMVA design and optimisation has been honed over three decades of recombinant Vaccinia virus research. Underpinning the research are background knowledge in poxvirus biology and advances in genetic engineering.

1.2.1 MVA life cycle and gene expression

MVA is a member of the *orthopoxvirus* genus within the *Poxviridae* family. The virus contains a single copy of double stranded DNA genome, approximately 178 kb in length (Antoine *et al.*, 1998). Unlike other DNA viruses, vaccinia replication occurs exclusively in the host cell cytoplasm and uses virus encoded polymerases and transcription factors. In common with most other viruses, replication is controlled via tight temporal regulation of gene expression falling into specific categories - early, intermediate and late (Broyles, 2003), with an additional, immediate-early class of genes more recently described (Assarsson *et al.*, 2008; Yang *et al.*, 2010). The infectious virion has packaged within it enzymes and transcription factors required for early gene expression so that viral messenger RNA (mRNA) transcripts are detectable within 20 minutes post-infection (Baldick and Moss, 1993). Under the tight regulation of vaccinia transcriptional promoters, the first genes to be expressed code for immune modulators and for enzymes and transcriptional factors required for intermediate expression. After viral DNA replication,

intermediate gene transcription is activated and viral late gene transcription factors are expressed to enable subsequent late gene expression to occur. The proteins and enzymes required for early transcription are expressed at late times so that they are encapsulated into progeny virus ready for the next round of replication. The majority of virus particles are then released during cell lysis. The tightly regulated cascade of viral early, intermediate and late transcription is shown in Figure 1.3. Also shown is the point at which replication is defective for MVA infecting non-permissive cell types.

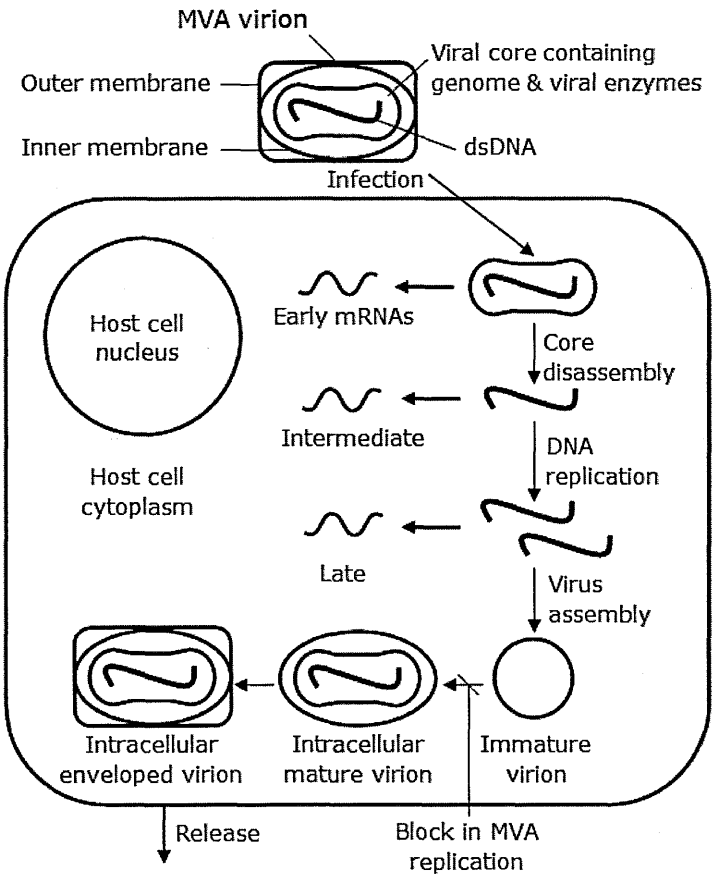


Figure 1.3. MVA life cycle. The infectious MVA virion enters the cell and undergoes replication prior to the release of viral progeny. In non-permissive cells, there is a block in MVA replication.

As MVA replication is blocked in the final step of virus particle maturation, authentic gene expression is still able to occur at all stages, a feature confirmed for high level expression of recombinant genes (Sutter and Moss, 1992) capable of evoking an effective immune response (Sutter *et al.*, 1994). That MVA replicates in the host-cell cytoplasm with relative

autonomy from the host cell nucleus represents another favourable characteristic of rMVA-based vaccines from the perspective of GM safety i.e. the lack of opportunity for insertion of viral DNA into the host genome.

Host range restriction

During the course of its attenuation in primary chick embryo fibroblasts (CEF), approximately 30 kb of the parent vaccinia genome was deleted rendering MVA replication defective in human and most other mammalian cells. Whilst the genetic basis for host range restriction has not been fully elucidated, it is known to involve multiple gene defects (Meyer *et al.*, 1991; Wyatt *et al.*, 1998). Initially, propagation of MVA was thought to require culture in CEF, but later other non-human cell lines permissive to MVA growth were identified, and one in particular, baby hamster kidney cells (BHK-21) were found to support efficient replication (Carroll and Moss, 1997; Drexler *et al.*, 1998; Okeke *et al.*, 2006) and production of rMVA (Carroll and Moss, 1997). The most common cell cultures now in use for *in vitro* manipulation of MVA are primary CEF and BHK-21. Additional designer avian cell lines have been developed for optimised scalability and robustness for product-scale amplification of MVA (Brown and Mehtali, 2010; Jordan *et al.*, 2011; Jordan *et al.*, 2009).

1.2.2 The first recombinant Vaccinia viruses

The first examples of recombinant Vaccinia virus used homologous recombination as the mechanism for gene transfer (Mackett *et al.*, 1982; Panicali and Paoletti, 1982) and this is still the most widely used technique for generation of rMVA. Methods for constructing rMVA are discussed in more detail in Section 1.3. Briefly, MVA permissive cells are infected with wild type virus and then transfected with a transfer plasmid vectoring exogenous DNA. Homologous recombination into a specific site of the MVA genome is mediated by homologous sequences flanking the transgene (Earl *et al.*, 2001b). In addition to the gene of interest, the transfer plasmid contains a vaccinia promoter for control of its expression and, dependent upon the precise nature of the protocol, a selection marker to aid isolation

of recombinant virus. An example based on transient expression of green fluorescent protein (GFP) (Wyatt *et al.*, 2008b) is shown in Figure 1.4. During MVA replication, homologous recombination between genomic and transfer plasmid DNA occurs at a frequency of approximately 1:1000 (Kremer *et al.*, 2012). Thus, when genomic DNA is repackaged to yield MVA progeny, a proportion of the population, reflective of this frequency, is recombinant. Purified recombinant virus must subsequently be prepared through a process enabling selective enrichment.

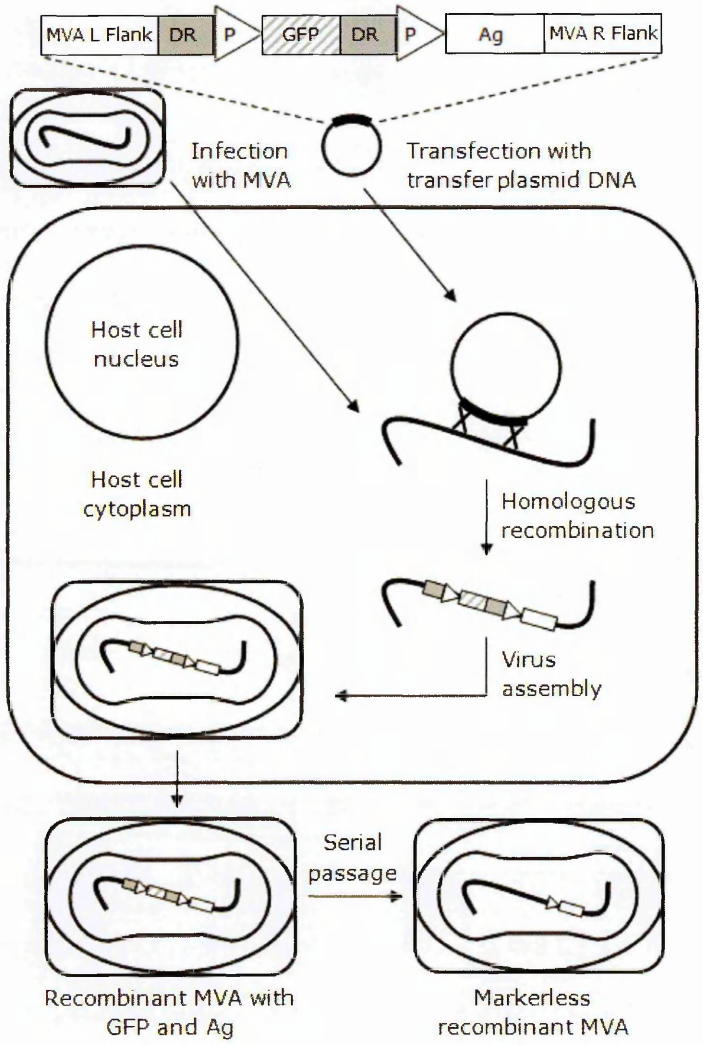


Figure 1.4 Generation of rMVA. Permissive cells are infected with MVA and then transfected with transfer plasmid DNA carrying a recombinant cassette. In addition to the antigen of interest (Ag), the cassette contains a selection marker (GFP), vaccinia promoters for transgene expression (P) and left (L) and right (R) MVA homologous flanks through with recombination into the MVA genome is mediated. Direct repeat (DR) sequences flanking GFP lead to transient expression of the marker.

1.2.3 Selection markers

Since the first rVACV were generated in the early 1980s, there have been many technical advances relating to construction and isolation of recombinants. Early methods relied on insertion of the foreign gene into the thymidine kinase (TK) coding sequence via homologous recombination and enrichment on the basis of a TK-phenotype selected by using bromodeoxyuridine (BrdU) (Mackett *et al.*, 1982, 1984; Panicali and Paoletti, 1982). Later, this method was combined with simultaneous insertion of the *Escherichia coli* Lac Z gene, coding for β-galactosidase, so that recombinants could be further distinguished from TK-mutants arising under the cytotoxic influence of BrdU; lac Z recombinant plaques turn blue when grown in the presence of X-gal (Chakrabarti *et al.*, 1985). Other genes added to transfer vectors to improve versatility in recombinant isolation include antibiotic resistance markers such as the *E. coli* neomycin gene (Franke *et al.*, 1985) and xanthine-guanine phosphoribosyl transferase (*gpt*) gene (Falkner and Moss, 1988) and additional colour markers, green fluorescent protein (GFP) (Wu *et al.*, 1995a) and *E. coli* β-glucuronidase A gene (*gus*) (Carroll and Moss, 1995).

Where markers are not cross-reactive, it is possible to combine them for improved selection, as in the case of a *gpt-gus* fusion expressed under a single vaccinia promoter (Cao and Upton, 1997). Alternatively, they can be used simultaneously in the construction of recombinants containing multiple inserts. Another strategy for the isolation of recombinant vaccinia, developed using VACV, involves use of a modified parent strain with a deficiency restored by a gene included in the transfer vector (Blasco and Moss, 1995; Perkus *et al.*, 1989).

When making rMVA products destined for clinical use, inclusion of a selection marker is not desirable. Several systems have been designed to allow for transient expression of marker genes, many employing a second recombination event for their removal (Falkner and Moss, 1990; Scheiflinger *et al.*, 1998; Wyatt *et al.*, 2008b). Where recombinant protein is

expressed on the surface of infected cells, selection markers may not be required at all if recombinant plaques can be immunostained and recombinant plaques visualised for isolation and further purification (Earl *et al.*, 2001b). A popular approach in the development of rMVA based vaccines today is the use of transiently expressed fluorescent markers. A transfer plasmid vectoring transient GFP has been described (Wyatt *et al.*, 2008b) in which recombinant virus is plaque purified and then subjected to serial passage so that the GFP marker, located in between two direct repeat sequences, is eventually lost through a second recombination event.

1.2.4 Vaccinia promoter selection

As described above (Section 1.2.1), Vaccinia virus replication occurs in the host cell cytoplasm, using vaccinia-encoded proteins for genome replication and transcription. The role of the vaccinia promoter sequence is to bind one or more transcription factors in order to recruit RNA polymerase and initiate transcription. The expression of a foreign gene by a recombinant vaccinia virus (rVV) is, therefore, dependent upon insertion downstream of a vaccinia promoter.

An alternative promoter can be used, but the corresponding transcriptional machinery must be provided during viral replication. Such a system has been developed based on the bacteriophage T7 system. Briefly, cells are infected with a recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase and then co-transfected with a vector carrying the gene of interest flanked by T7 promoter and terminator sequences, thereby giving rise to T7-mediated gene expression (Fuerst *et al.*, 1986). The same system has been implemented using attenuated vaccinia (Wyatt *et al.*, 1995). The T7 system has been described as an optimised *in vitro* expression tool, but has yet to be widely reported as a means for driving vaccine antigen expression *in vivo*.

Vaccinia promoters, by virtue of the transcription initiation factors they bind (reviewed by (Broyles, 2003)), control the temporal regulation of gene expression and so can be classed

as early, intermediate or late (described in Section 1.2.1). They also affect the strength of gene expression. As described below, this is an important consideration in the design of a recombinant vaccine.

Vaccinia promoter structure

Vaccinia promoter studies employing a transgene encoding an enzyme, e.g. chloramphenicol acetyltransferase (CAT), have enabled promoters to be fully characterised. Following insertion, gene expression can be accurately measured via an assay for enzymatic activity (Mackett *et al.*, 1984). The earliest promoter studies served to confirm the location of transcriptional regulatory regions within putative promoter sequences, and define their temporal category with regards to early or late expression (Weir and Moss, 1984). Among the first regulatory regions to be characterised was a 275 bp promoter sequence for a constitutively expressed protein of 7.5 kDa. This promoter (P7.5) was shown to contain tandem, independently-operating early and late promoter regions (Cochran *et al.*, 1985).

Later, in depth mutagenesis studies identified in detail the promoter elements critical to expression activity for both early (Davison and Moss, 1989a), and late promoters (Davison and Moss, 1989b). Dissection of promoter sequences by individual base pair substitutions has revealed that early promotors comprise an A/T rich critical region of approximately 16 bp, followed by a less critical region of 11-14 bp, beyond which is the initiation region (Figure 1.5A). Late promoters are also made up of 3 regions: a 20 bp A/T rich sequence, a 6 bp spacer region and a highly conserved TAAAT sequence, within which transcription is initiated (Figure 1.5B). In the course of determining the significance of individual base pairs within the promoter, mutations capable of increasing transcriptional activity were identified, thus enabling the development of optimised promoters including the synthetic promoter (Psyn), which comprises optimised, overlapping early and late elements (Chakrabarti *et al.*, 1997).

Historically, vaccinia virus genes have been categorised as either 'early' or 'late,' according to the timing of their expression relative to viral DNA replication. An intermediate category of genes has since been described in which gene expression occurs at the onset of DNA replication and via a promoter that does not utilise the complete TAAAT initiator (Vos and Stunnenberg, 1988). Intermediate promoters have been characterised via mutational studies (Baldick *et al.*, 1992) and shown to comprise a 14 bp A/T rich core element, a 10 -11 bp spacer and then a critical (initiator) region with the sequence TAAA (Figure 1.5C).

More recently, early vaccinia genes have been further categorised to include an immediate-early subset (Assarsson *et al.*, 2008; Yang *et al.*, 2010), for which there has been an initial report of a potential consensus promoter sequence (Di Pilato *et al.*, 2013).

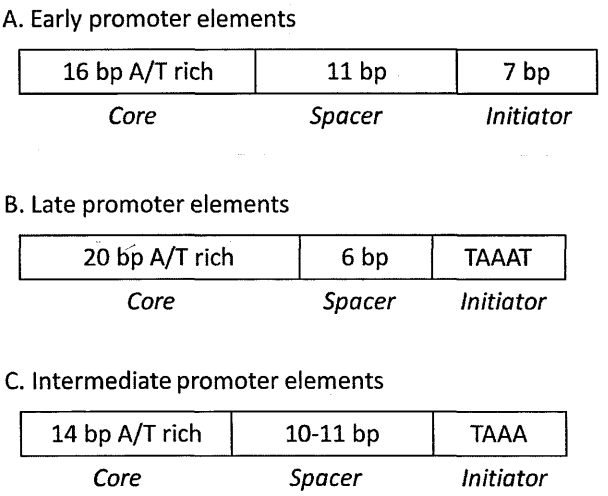


Figure 1.5 Conserved elements of vaccinia promoter sequences. All three temporal classes of promoter comprise three regions - the core, spacer and initiation region. The core sequence is rich in adenine/thymine (A/T) bases. The initiation region is the site at which transcription starts. The core region binds transcription factors and so has sequence requirements, alterations to which will affect promoter activity.

Vaccinia promoter selection for optimal immunogenicity

Intuitively, rVV-based vaccines must strive for high levels of transgene expression to increase the immunological potency of the vectored antigen. The first examples of rVV

preceded elucidation of vaccinia promoter sequences and so regulatory regions were selected on the basis of their association with proteins that were abundant at both the early and late stages of the virus life cycle, e.g. a constitutively expressed protein of 7.5 kDa. These viruses were capable of inducing antigen-specific antibodies in animal models and induced protective immunity. The first examples demonstrated immunogenicity of influenza virus haemagglutinin (Panicali *et al.*, 1983) and immunogenicity and protective pre-clinical efficacy for hepatitis B antigen (Moss *et al.*, 1984; Smith *et al.*, 1983).

Following the characterisation of vaccinia promoters, optimisation of expression might have focused on late promoter elements which can give rise to higher protein levels; late promoters are active at a time when DNA template and transcription factors are relatively abundant. However, rVV studies have shown that promoter kinetics, and in particular late-stage expression, can be detrimental to transgene immunogenicity. Coupar *et al.* investigated the immunogenicity of rVV vectoring influenza haemagglutinin (HA) under the control of three different promoters: PF (early), P7.5 (early/late) and P11 (late). All induced antibody responses in mice, but the P11 construct produced limited T cell responses despite driving the highest levels of expression (Coupar *et al.*, 1986).

Vaccinia virus late-stage gene expression is now understood to be blocked in professional antigen presenting cells (APC). APC are vital for the induction of cell-mediated immunity as they process and present antigens to T cells. That APC are able to support the early stages of the VV life cycle, but block late-stage replication has been demonstrated for rVV in macrophages (Broder *et al.*, 1994) and in dendritic cells (DC) (Bronte *et al.*, 1997). It has also been shown that entry into DC may be less efficient for Vaccinia virus (Drillien *et al.*, 2000). As discussed above (see Section 1.1.2), a potential application for viral vaccine vector technology is to induce both humoral and cell-mediated immune responses, to counter those infectious diseases for which antibody-responses alone have proved insufficient. Late-stage expression may have a role in generating recombinant antigen in

non-APC cell types, but early-stage expression is vital for the quality of the immune response required.

As already stated, in the course of developing rVV based viral vectors, MVA was selected as a potential alternative with an improved safety profile. MVA is replication deficient, but in non-permissive cells gene expression, including the expression of recombinant genes, is able to occur (Sutter and Moss, 1992). That MVA is replication-deficient arguably increases the need to maximise transgene expression. The first rMVA-based vaccine sought to achieve this by selecting the optimised, synthetic early/late promoter Psyn; a rMVA virus was constructed to express influenza virus antigens and was successful in inducing protective immunity in mice (Sutter *et al.*, 1994). However, when Psyn was selected to drive expression of *parainfluenza virus 3* (PIV3) antigens, the resultant rMVA replicated poorly *in vitro*, with the high level of gene expression apparently detrimental to viral fitness (Wyatt *et al.*, 1996). Powerful promoters, therefore, can result in cytotoxic effects where, dependent upon the gene being expressed, the recombinant may be unstable. With a view to using a promoter optimised for expression and stability, Wyatt *et al.* developed a novel promoter of intermediate strength to Psyn and the weaker P7.5. Informed by earlier mutagenesis studies, the naturally occurring early/late promoter for vaccinia gene H5 was altered via two nucleotide substitutions to create modified H5 (mH5). An important observation for the activity of this promoter, as characterised via expression of ß-galactosidase, was that, while it was of intermediate strength to Psyn and P7.5, it delivered three-fold higher early expression relative to Psyn. As described above, this may be an advantageous feature for transgene expression in APC and induction of cellular immunity. Thus, promoter mH5 is favourable for expression, stability and immunogenicity. Indeed, the mH5 promoter has been used as a comparator in more recent vaccinia promoter optimisation studies (Becker *et al.*, 2014; Orubu *et al.*, 2012).

It is evident that vaccinia promoter selection is important to the efficacy of rMVA-based vaccines from ongoing efforts to optimise vaccinia promoter usage. As early-stage expression is advantageous for the promotion of T cell mediated immune responses, recent rMVA studies have investigated promoters driving immediate-early expression. As yet, no particular promoter features, in terms of the DNA sequence, have been associated with immediate-early expression. Preliminary attempts at driving immediate–early expression have shown that it may correlate with enhanced CD8+ T cell responses (Baur *et al.*, 2010; Di Pilato *et al.*, 2013).

1.2.5 Immunomodulatory molecules

The effectiveness of MVA based vaccines may also be improved through insertion of immune co-factors (ICF), especially where it is important to steer the immune system towards a particular type of T-helper response. A key advantage of MVA as a viral vector for vaccine antigen delivery, is its capacity for recombinant DNA; theoretically 30 kb. The first ICF to be co-expressed in rVACV was IL-2 which was shown to have an attenuating effect in nude mice (Flexner *et al.*, 1987; Ramshaw *et al.*, 1987). Subsequently, rVACV co-expressing a range of immune enhancers have been constructed with a view to creating cytokine microenvironments in which favourable antigen-specific responses are activated. Many cytokines, e.g. IL-2 and IL-12, have anti-viral and anti-tumour activity, however, their toxic side effects can be severe when delivered systemically. Reports show that co-expression of a model tumour-associated antigen (TAA) and IL-12 by a rVACV can obviate the requirement for systemic delivery of toxic levels of IL-12 (Carroll *et al.*, 1998). Co-expression of immune co-factors can offer a further dimension to safe and effective enhancement of rMVA based vaccines as has now been demonstrated in clinical studies (Dreicer *et al.*, 2009; Liu *et al.*, 2004; Ramlau *et al.*, 2008), but for each construct, safety was required to be demonstrated.

1.2.6 Antigen fusion strategies

Viral vaccine vectors and plasmid DNA vaccines carry antigens in the form of nucleic acid. To achieve effective delivery, the antigen must be expressed by the host cell, i.e. within the vaccine recipient. In order to achieve optimal secretion and presentation to immune cells, sorting signals or 'leader sequence' peptides can be expressed fused with the recombinant antigen, to direct them towards favourable intracellular trafficking pathways. Leader sequences that have been employed for this purpose include naturally occurring sorting signals taken from viral and bacterial genes. Eukaryotic genes have also provided a number of sequences and are more diverse, a product of their more complex protein trafficking which involves many intracellular compartments.

The first studies to investigate 'leader sequences' were principally aimed at investigating antigen processing and, in particular, the presentation of antigen peptides by major histocompatibility complex (MHC) class I and class II molecules (Bacik *et al.*, 1994). In these studies, rVV provided a means of delivering leader sequence-antigen chimeras to investigate the role of the sorting signals themselves. However, the practical implications for virally vectored vaccine antigens were noted and explored. It was subsequently demonstrated that fusion of an endoplasmic reticulum targeting signal, derived from an adenovirus protein (E3/19k), could enhance CD8+ T cell responses to an influenza virus antigen (Restifo *et al.*, 1995). Other examples of antigen fusions in rVV viral vaccine studies followed and, later, DNA provided a more efficient means of investigating antigen fusions. Established examples, the vectors they have been studied in, and their impact on transgene immunogenicity are listed in Table 1.4 and are summarised below.

Leader sequences reported to promote intracellular MHC II binding and, therefore, CD4+ responses, include the MHC II-binding Invariant chain (Ii), the cytoplasmic tail sequence of CD1 antigen presentation molecule (CD1) and the lysosomal-associated membrane protein signal sequence (LAMP-1). These leaders direct proteins to intersect with the MHC II

pathway in the endoplasmic reticulum (ER), endosomes and late endosomes/lysosomes, respectively (Koch *et al.*, 2000; Niazi *et al.*, 2007).

Table 1.4. Examples of leader usage in vaccine development studies. Established examples are listed in chronological order of their reporting.

Leader	Vector	Leader contribution	Ref
LAMP-1	DNA rVV	▪ Rerouting to MHC II pathway, enhanced CD4+ presentation ▪ HPV antigen retargeted to endosomal/lysosomal compartments. ▪ Improved immunogenicity	(Wu *et al.*, 1995b)
TPA	DNA	▪ Increased TB antigen expression in vitro. ▪ Increased Ab, Th-1 and Th-2 responses ▪ Improved protective efficacy.	(Li *et al.*, 1999)
Ii	DNA	▪ Rerouting to MHC II pathway via endosomal targeting	(Koch *et al.*, 2000)
LAMP-1	DNA	▪ Rerouting to MHC II pathway ▪ Improved HIV antigen expression ▪ Increased immunogenicity	(Marques *et al.*, 2003)
TPA	DNA	▪ DNA vaccine design study showing higher extracellular protein levels with TPA	(Wang *et al.*, 2006)
BVP22 Ii	DNA	▪ Both leaders used fused together ▪ Enhanced priming and expansion to B and T cell epitopes	(Mwangi *et al.*, 2007)
CD1	DNA	▪ Endosomal targeting - trafficking to MHC Class II pathway	(Niazi *et al.*, 2007)

Abbreviations: LAMP-1, lysosomal associated membrane protein – 1; rVV, recombinant vaccinia virus; HPV, human papillomavirus; Ab, antibody; Ii, MHC Class II invariant chain, BVP22, bovine herpesvirus 1 protein; CD1, cytoplasmic tail sequence of CD1 antigen presentation molecule; TPA, human tissue plasminogen activator gene sorting signal.

The signal sequence of the human tissue plasminogen activator gene (TPA) targets protein to the ER for secretion. Use of TPA as a vaccine antigen fusion has been rationalised by studies demonstrating that it improves expression levels, immunogenicity and protective efficacy. It is commonly used in TB vaccines and is discussed further in Section 1.4.3.

Ubiquitin (Ub) and *M. tuberculosis* heat shock protein 70 (HSP70) have been shown to promote presentation to the MHC I pathway and, therefore, promote CD8+ responses (Chen *et al.*, 2000; Leifert *et al.*, 2004). They achieve this by targeting antigen towards degradation pathways giving rise to peptides that are transported to the ER. There the peptides co-localise with MHC I molecules to which they can bind and be taken to the cell surface for presentation to CD8+ T cells.

Targeting antigen to the cell surface has proved effective as a means of enhancing antibody responses. This has been demonstrated using the transmembrane and cytoplasmic domain of Vesicular Stomatitis Virus glycoprotein (VSVg) fused to the C terminus of beta subunit of human chorionic gonadotropin (Srinivasan *et al.*, 1995). Antibody responses, as well as CD8+ responses, have also been promoted by targeting antigen to the endosomal and lysosomal compartments using LAMP-1 (Wu *et al.*, 1995b).

Another type of 'leader' sequence reported to influence the immunogenicity of recombinant vaccines is the BVP22 gene (bovine herpes virus 1 tegument protein). BVP22 is thought to promote intercellular trafficking, although the precise mechanism through which it achieves this is still under debate (Leifert *et al.*, 2004; Mwangi *et al.*, 2005).

A benefit of the antigen fusion approach is that findings are relevant to, and so can be exploited by, all vaccines based on *in vivo* expression. This includes viral vectors such as MVA and Ad as well as plasmid DNA vaccines.

1.2.7 Insertion site

Following the design of the recombinant antigen, the site of insertion into the MVA genome must be selected. In addition to the TK region described above, other sites targeted for exogenous DNA insertion are the haemagglutinin (HA) gene (Antoine *et al.*, 1996), the more commonly used deletion (Del) sites formed during the course of MVA's attenuation (Sutter and Staib, 2003) and, most recently, intergenic regions (IGR) (Timm *et al.*, 2006). Insertion

into IGRs adjacent to essential MVA genes has been used as a strategy for generating stable rMVA where exogenous DNA has otherwise been observed to promote instability. In the event of a deletion or truncation, the essential genes are affected and the strain rendered unable to replicate; thus only full length recombinants are propagated (Manuel *et al.*, 2010; Wyatt *et al.*, 2009). A diagram of the MVA genome highlighting the positions of the Deletion sites commonly targeted for antigen insertion is shown in Figure 1.6.

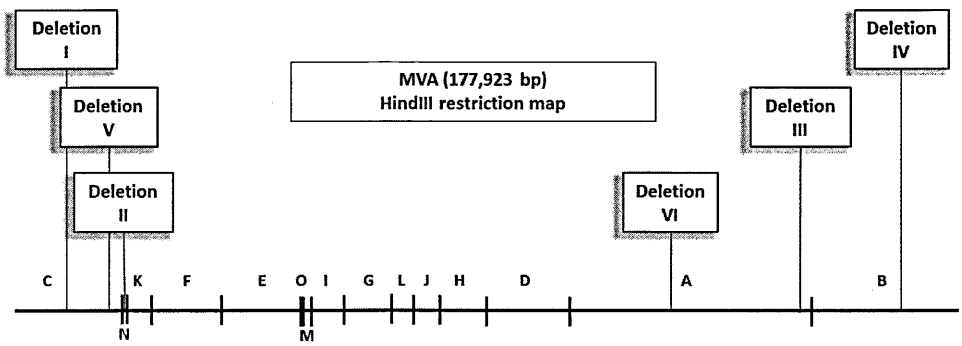


Figure 1.6 HindIII restriction map of the MVA genome. Fragments forming after HindIII restriction enzyme digest of the linear genome are labelled A – O in order of descending size. The positions of the six major genomic deletion sites arising during MVA's attenuation are indicated. The figure is adapted from a SeqBuilder (DNASTAR) file of the MVA DNA sequence (Accession number U94848).

1.3 Construction of recombinant MVA

Once the genetic configuration of a novel rMVA has been decided upon, the required sequence must be generated and inserted into the MVA genome. The first examples of recombinant vaccinia virus used homologous recombination as the mechanism for gene transfer (Mackett *et al.*, 1982; Panicali and Paoletti, 1982) and this technique remains widely used (Kremer *et al.*, 2012). However, bacterial artificial chromosome technology is another approach that is now in use and which has favourable attributes.

1.3.1 Transfer plasmid methodology

As described above in Section 1.2.2, and in Figure 1.4, transfer plasmid methodology exploits the tendency for homologous DNA sequences to undergo recombination (Sigal and Alberts, 1972). Transfer plasmids contain the recombinant cassette destined for insertion in the MVA genome, flanked by sequences homologous to the intended site of insertion (Figure 1.7A). Recombination between the homologous sequences occurs when purified transfer plasmid DNA is transfected into MVA-infected cells.

The precise nature of the homologous flanks determines not only the site of insertion but also the orientation of the inserted sequence and the outcome of recombination in terms of any potential deletion of viral DNA (Figure 1.7B). The design of the intervening recombinant cassette determines the nature of the recombinant strain generated. Key features include the antigen to be inserted, the vaccinia promoter selected for control of its expression and a selection marker under the control of its own promoter; an example incorporating green fluorescent protein (GFP) is shown in Figure 1.7C. Where a markerless recombinant is required, transient expression can be achieved by inclusion of a direct repeat sequence that will lead to a further recombination event through which the marker will be removed (Figure 1.7D).

Transfer plasmids are based upon *E. coli* cloning vectors and so are readily propagated and isolated for repeat rounds of manipulation; they have an origin of replication (ori) enabling maintenance at high copy number and maintain selective pressure via an antibiotic resistance marker (Figure 1.7D). Using standard cloning methods (Green, 2012), any alteration can be made to the portion of the transfer plasmid representing the recombinant cassette, including introduction of N and/or C terminal fusions. These may be B cell epitopes to allow for immunodetection of expressed product, or leader sequences that will influence intracellular processing of the antigen upon its expression in MVA-infected cells.

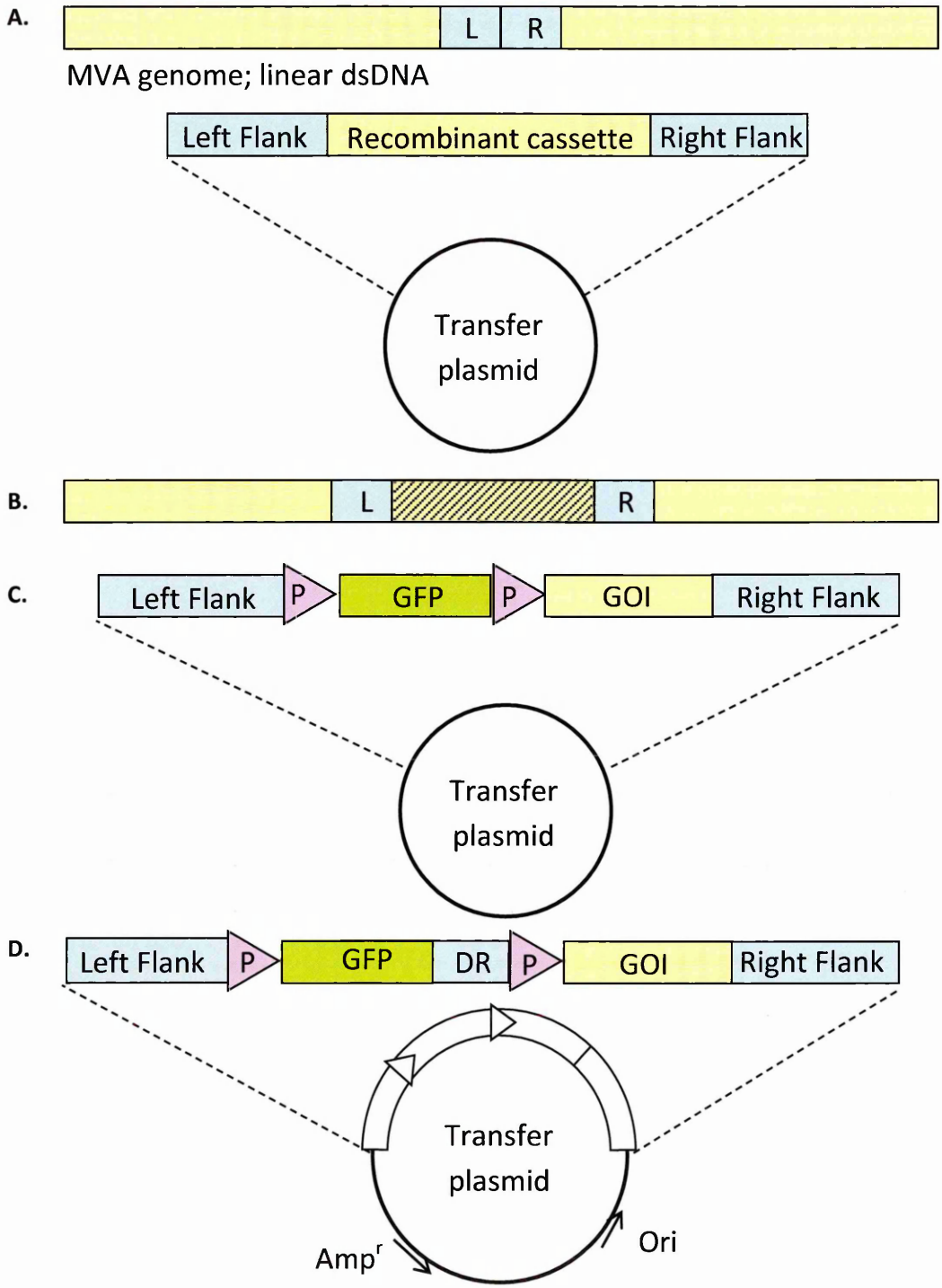


Figure 1.7 Main features of the transfer plasmid. A) In the linear, double-stranded DNA (dsDNA) of the MVA genome, sequences to the left (L) and right (R) of the insertion site are repeated in the transfer plasmid either side of the recombinant cassette. B) If L and R are not directly adjacent, the intervening sequenced (hatched area) will be deleted upon recombination. C) The cassette contains the gene of interest (GOI), a vaccinia promoter (P) to drive its expression and a selection marker (GFP) under the control of its own promoter. D) If a markerless recombinant is required, a direct repeat (DR) of a portion of the preceding flank is included. The transfer plasmid has an origin of replication (ori) for use as an *E. coli* cloning vector, and an antibiotic resistance gene, e.g. ampicillin (Amp^r).

The transfer plasmid may also incorporate Gateway® cloning (ThermoFisher Scientific, USA) for rapid insertion of the gene of interest (Hartley *et al.*, 2000). The Gateway® system employs a recombination mechanism based on lambda phage attachment sites to allow for movement of genes between plasmids without traditional cloning methods, i.e. restriction digest and ligation. According to Gateway® terminology, the transfer plasmid is converted to a Destination vector (pDEST) so that the gene of interest (GOI) can be received, in frame with existing N/C terminal fusions, from another plasmid termed an Entry vector (pENTR). A combination of antibiotic resistance markers and a copy of the ccdB lethal gene ensure that the desired plasmid is readily isolated following GOI insertion. Using the Gateway® system, transfer plasmids can be generated that are ready for any GOI to be efficiently introduced.

1.3.2 Bacterial Artificial Chromosomes

Bacterial artificial chromosomes are also *E. coli* cloning vectors, but they are based on a naturally occurring collection of functional segments known as the fertility factor, 'F'. The initial description of F was related to the apparent ability or inability of bacteria to participate in genetic exchange (Lederberg *et al.*, 1952). Since then, F has been characterised as a replicon on an episomal plasmid (Wollman and Jacob, 1958), the constitutive components of which enable maintenance at one or two copies per cell, with faithful allocation to daughter cells upon replication (Ogura and Hiraga, 1983).

Advances in the understanding of F, in particular the role of individual regulatory genes in the control of replication and copy number, has informed the development of F-based vectors for cloning of extrachromosomal DNA at low copy number (Hosoda *et al.*, 1990; O'Connor *et al.*, 1989; O'Connor *et al.*, 1986). A driver for this area of cloning has been the need to propagate large segments of DNA, which in a high copy number system would be prone to recombination events. In an application related to human genome sequencing, an F-based vector was developed for the cloning and stable maintenance of fragments up to

300 kb. It was in this work that the term bacterial artificial chromosome (BAC) was introduced (Shizuya *et al.*, 1992).

The advent of BAC vectors provided a new means with which to manipulate viral genomes. Briefly, the defining features of the BAC plasmid are introduced to the viral genome using transfer plasmid methodology. Having inserted the regulatory genes of the F replicon, along with an antibiotic resistance marker, extracted viral genomic DNA can be electroporated into *E. coli* to be maintained and propagated as a BAC vector. This affords the opportunity to further manipulate the viral genome within the bacterial cloning vector. To produce recombinant virus, the BAC DNA is isolated and transfected into eurkaryotic cells for expression and repackaging to virions.

The generation of viral recombinants using the BAC system was first demonstrated with baculovirus (Luckow *et al.*, 1993) and then herpesvirus (Messerle *et al.*, 1997) and cytomegalovirus recombinants (Borst *et al.*, 1999). Application of BAC methodology to the manipulation of Vaccinia virus necessitated two additional aspects to accommodate features of the vaccinia virus life cycle. Firstly, a means through which to circularise the linear dsDNA vaccinia genome; only circular DNA can be transferred to *E. coli* for further propagation, and secondly, use of a helper virus to yield infective virions from transfected eurkaryotic cells (Domi and Moss, 2002). The approach has since been applied to MVA (Cottingham *et al.*, 2008).

The principal advantage of BAC technology for the generation of rMVA is that it negates the need to plaque purify; upon repackaging, the resultant virus should represent a homogenous recombinant strain. The main disadvantage is the lengthy set-up time required for creating a BAC based MVA vector and the risk of contamination of the cloned genome with bacterial insertion sequences (Cottingham, 2012).

1.3.3 Preparation of recombinant MVA for clinical use

Regardless of the methodology selected for construction, rMVA can be generated and amplified under regulated (GMP) conditions for use in clinical trials. Retention of the selection marker at this stage is undesirable, but it can be removed via a recombination event using either methodology (Cottingham and Gilbert, 2010; Wyatt *et al.*, 2008b). Evidence that rMVA are amenable to downstream development and large scale production is provided by the range of diseases they are being developed for use against, of which TB is one.

1.4 Recombinant MVA for the prevention of tuberculosis

Tuberculosis (TB) ranks alongside HIV/AIDS as the leading cause of death attributable to an infectious disease. In 2014, 9.6 million people fell ill with TB and 1.5 million people died, of which 0.4 million were co-infected with HIV (WHO, 2015). In addition, it is estimated that a third of the global population may be latently infected with TB and at risk of reactivation of disease.

A number of first and second-line drugs are available for the treatment of TB, but treatment schedules are challenging; they are dependent upon early diagnosis and lengthy treatment regimens which require directly observed therapy (DOT) and follow up support (Zumla *et al.*, 2013). Drug resistance screening is also vital as there are an increasing number of multi-drug resistant (MDR) and extensively-drug resistant (XDR) strains of *M. tuberculosis* emerging.

As described earlier, a vaccine for TB was developed in the 1920's and remains in use. BCG is based on a live, attenuated form of *M. bovis* and is administered to infants as part of the Expanded Programme of Immunisation (EPI). BCG, however, has variable efficacy in different populations, ranging from 0 – 80% (Fine, 1995); it is considered ineffective at preventing pulmonary TB in adults, but provides some protection against the most serious

forms of TB in children. Another disadvantage of the BCG vaccine is that it is live and replication competent and so is contraindicated for HIV positive patients. Thus, in areas of high HIV incidence, where TB is also commonly prevalent, BCG is unsuitable for use in a proportion of those requiring protection.

While the combination of improved diagnosis and treatment has done much to control TB, eradication of the disease is generally accepted to be dependent upon licensure of a vaccine superior to BCG (Raviglione *et al.*, 1995). As with other diseases, the natural immune response to TB disease has been studied with a view to developing a vaccine that will emulate it.

1.4.1 Tuberculosis disease and immunity

The aetiological agent of TB is *Mycobacterium tuberculosis* (Mtb). The bacterium is spread by the aerosol route when an individual with active disease is coughing. Mtb is an intracellular pathogen which in the lung invades professional phagocytic cells such as macrophages and dendritic cells. A consequence of the immune response to Mtb infection is the formation of granulomatous lesions, with the loss of healthy lung tissue.

In the first stages of infection, innate immunity promotes phagocytosis of Mtb as well as the recruitment of further phagocytes, which in turn are also infected (Ernst, 2012). This is the beginning of granuloma formation. After a short delay, an adaptive immune response commences with the induction of IL-12 release. Phagocytosis of mycobacteria is a strong inducer of IL-12 and leads to initiation of a Th-1 type response, characterised by IFNγ release (Ladel *et al.*, 1997). In the adaptive immune response, professional APC migrate to the draining lymph nodes where they present MHC-bound antigen to T cells. Antigen-specific T cells subsequently expand and home to the site of infection to promote downstream antimicrobial pathways, e.g. phagolysosome fusion. At this stage, the adaptive immune response further contributes to granuloma formation. Whilst

immunopathology might ultimately be of detriment to the host, it is essential if dissemination of Mtb is to be prevented.

As Mtb is an intracellular pathogen, presentation by MHC Class II molecules and recognition by CD4+ T cells is the dominant feature of the immune response. In particular, Th-1 immunity is induced and Th-1-type cytokines, IFNγ and TNFα, are essential for the control of bacterial growth (Flynn, 2004). Mtb infection has also been shown to induce CD8+ T cell responses and it has been proposed that the relatively weaker ability of BCG to do the same might be a reason for its ineffectiveness (Ryan *et al.*, 2009). Another subset of CD4+ helper cells with an essential role in controlling early Mtb infection are Th-17 cells. They are stimulated by IL-23 and release IL-17, a proinflammatory cytokine which serves to further mediate inflammation (Khader and Cooper, 2008).

An effective adaptive immune response is sufficient to arrest the growth of Mtb, but is not sterilising. At this stage an individual is asymptomatic and considered to have latent TB infection (LTBI). This occurs in the majority of cases, but approximately 10% of those infected will go on to develop disease at a later date. Reactivation can be triggered by immunosuppression due to age, malnutrition, HIV infection or other factors.

1.4.2 Strategies for an improved TB vaccine

While the immune response to Mtb, and to BCG vaccination, has been studied in order to characterise protective immunity, a correlate of protection has not been found (Ottenhoff *et al.*, 2012). Th-1 immunity and IFNγ release are considered central to the control of Mtb infection and so induction of this type of immune response has been the key readout in TB vaccine development to date, and will likely remain so. However, a more balanced CD4+/CD8+ T cell response is being viewed with increasing importance, as is the induction of antibody responses for blocking initial infection (Andersen and Woodworth, 2014).

As the current TB vaccine, BCG, confers sufficient benefit to remain in use, there are two approaches under study for the development of a novel TB vaccine. Most of the novel TB vaccines under development aim to improve upon the immune response induced by BCG by serving as a booster inoculation. Others strive to replace or supplement BCG as a priming agent, for which booster vaccines may still be required. There are 13 vaccines currently under clinical study and they comprise live attenuated whole cell vaccines (including Nontuberculous mycobacteria (NTM) strains), recombinant protein in adjuvant and viral vectored vaccines (Figure 1.8). Of the vaccines delivering sub-units, the number of antigens they deliver ranges from 1 – 4.

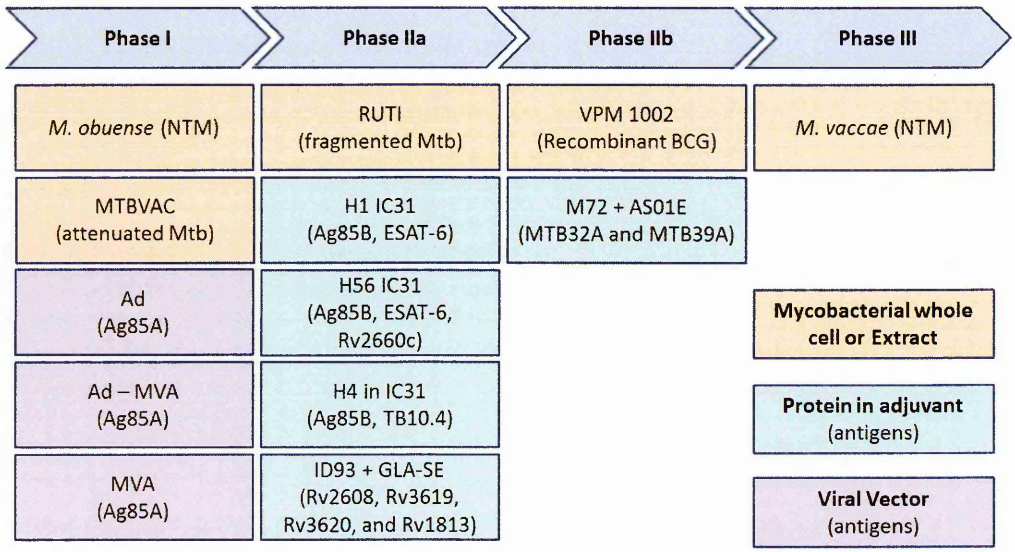


Figure 1.8 The Global Clinical Pipeline for TB Vaccine Candidates. Adapted from 'The Global Plan to End TB 2016 – 2020', (StopTBPartnership, 2015). Adjuvants: IC31, antibacterial peptide (KLKL(5)KLK) plus synthetic oligodeoxynucleotide (ODN1a) (Olafsdottir *et al.*, 2009); AS01E, Monophosphoryl Lipid A (MPL) and QS-21 (*Quillaja saponaria* purified extract) liposome formulation (Penn-Nicholson *et al.*, 2015); GLA-SE, glucopyranosyl lipid adjuvant -stable emulsion (Baldwin *et al.*, 2013)

TB vaccine antigens

Mtb antigens can be referred to by their gene number (denoted Rv) or, if well characterized, by the name commonly applied to the gene product. In 1993, the WHO

declared TB a global emergency driving an increase in TB vaccine research. Early TB vaccine candidates were focused on antigens that would be abundant and expressed in the initial stages of TB infection. These included secreted proteins of the Ag85 complex: Ag85A, Ag85B and Ag85C (mycolyl transferases essential for maintaining Mtb cell wall integrity), and early secretory antigenic target of 6 kDa (ESAT-6; Rv3875). Other antigens were identified on the basis of their immunodominance, e.g. TB10.4 (Rv0288), Mtb32 (Rv0125) and Mtb39 (Rv1196). Immunogenicity and efficacy studies in animal models confirmed the validity of these vaccine candidates (Skeiky and Sadoff, 2006).

Overall, the goal for novel TB vaccines is to prevent primary infection, latent TB infection and reactivation of latent infection. As this will require protective efficacy against multiple stages of diseases, it is generally agreed that single subunit approaches will be improved upon by combining multiple antigens. Hence, efforts to identify further TB vaccine antigens are ongoing, particularly those that will target later stages of disease. A strategy for identifying such antigens has been to perform *in vitro* transcriptomic studies in which conditions of latent TB are replicated (Bacon and Marsh, 2007). Latency antigen Rv2660c (Figure 1.8) was discovered through a combination of *in vitro* and *in vivo* transcriptional analyses and was incorporated into a formulation containing earlier stage antigens to create a multi-stage vaccine (Aagaard *et al.*, 2011). In the multi-subunit vaccine ID93 (Aeras/IDRI, USA), antigen Rv1813 is upregulated in latency (Baldwin *et al.*, 2013). Another late-stage vaccine candidate demonstrating protective efficacy pre-clinically is the acyl transferase Rv0111 (Vipond *et al.*, 2006a; Vipond *et al.*, 2006b). Formulated as plasmid DNA vaccine, Rv0111 is protective is guinea pig models of aerosol infection and has been shown to contain a number of T cell epitopes as determined by murine immunogenicity studies and *in vitro* assays for human T cell epitope mapping.

TB vaccine delivery systems

With regards to the optimal delivery system for subunit candidates, plasmid DNA is a valuable tool for pre-clinical research, but in larger animals is reliant on additional strategies for improving its effectiveness, e.g. use in heterologous prime-boost regimens or gene-gun approaches. DNA-viral vector boosting strategies have been investigated for use against TB, but there are currently no clinical TB vaccine candidates based on plasmid DNA.

The feasibility of a protein in adjuvant approach is made more difficult by the absence of licensed adjuvants for potentiation of cell mediated immune responses. All of the clinical candidates based on recombinant protein are combined with proprietary adjuvants, which in themselves represent a novel area of research. The examples currently under clinical study have been shown to evoke cell mediated responses relevant to protection against Mtb infection and are listed under Figure 1.8.

Modified BCG can serve as a bacterial vector for the delivery of TB vaccine antigens. The *M. bovis* BCG strain was attenuated by serial passage, during which 38 open reading frames were deleted from the genome (Behr *et al.*, 1999). BCG can be modified to express antigens that have been deleted, or to overexpress antigens that have been retained. Currently, the only recombinant BCG under clinical development is modified to enhance endosomal escape (Kaufmann *et al.*, 2014). A key objective of this approach is that the safety and protective efficacy of the parent BCG is improved; a BCG strain overexpressing Ag85A, Ag85B and Rv3407 was recently assessed in Phase I clinical trials, but development ceased after adverse events (reactivation of shingles).

For delivery of vaccine antigens that are expressed by BCG, viral vectors exhibit many favourable characteristics for use as a boosting agent. This includes the capacity to deliver large bacterial proteins and the ability to induce potent humoral and cell mediated immune responses. Of the many viral vectors available for vaccine antigen delivery (reviewed in

Section 1.1.3), Ad and MVA are to the date the most widely evaluated (Rollier *et al.*, 2011), and their ability to boost responses to BCG has been confirmed.

1.4.3 Optimisation of MVA for delivery of TB antigens

The first studies to evaluate Vaccinia virus for the delivery of TB antigens compared replication competent and attenuated viruses Antigen-specific antibody, CD4+ and CD8+ responses were induced by both rVV and rMVA carrying MPT64, and were greater following a prime with plasmid DNA vectoring the same antigen (Feng *et al.*, 2001). A heterologous prime-boost of DNA-MVA vectoring TB antigens ESAT-6 and MPT63 also induced CD4+ and CD8+ responses in mice and was shown to provide protection against Mtb challenge (McShane *et al.*, 2001).

Immunisation protocol and rMVA formulation

Used as a heterologous boost following immunisation with BCG, MVA vectoring the TB antigen Ag85A (MVA85A) boosts CD4+ and CD8+ responses in mice (Goonetilleke *et al.*, 2003) and humans (McShane *et al.*, 2004). Until recently, MVA85A was the most clinically advanced TB vaccine. It has been proven safe and immunogenic in several groups, including infants, and can induce Th-1 and Th-17 responses which are regarded as important for protection against TB. In 2011, MVA85A became the first TB vaccine to enter an efficacy trial since BCG studies performed over 40 years prior. In the study, infants that had been immunised with BCG at birth were boosted with MVA85A or given a placebo. While the vaccine was demonstrated to be safe, no improvement upon the placebo was seen (Tameris *et al.*, 2013).

As described above (Section 1.1.4), optimisation of MVA can include investigation of heterologous prime-boost regimens. Any MVA vaccine for TB will be used in a heterologous prime-boost schedule with BCG serving as the priming agent, but this can be enhanced by further heterologous boosting, as with Ad-MVA which is being investigated in

humans (Figure 1.8, Phase I). Alternative routes of immunisation can also be explored and recently the delivery of MVA85 by the aerosol route has been assessed (Satti *et al.*, 2014).

Heterologous prime-boost regimens and route of delivery are a means of optimising rMVA beyond its construction. Prior to this it should be optimised at the genetic level for optimal expression and delivery of the recombinant antigen(s).

Optimisation of expression

In the limited number of studies reporting rMVA delivery of *M. tuberculosis* antigen(s), only two promoters have been used; these are the natural 7.5kDa early/late promoter (*P*7.5) and *P*synE/L (summarised in Table 1.5). Immunogenicity and protective efficacy have been demonstrated, but no reference is made to whether promoter activity has been optimal. In contrast, other vaccine fields have undertaken thorough assessment of promoter usage. A model tumour-associated antigen delivered via native vaccinia was evaluated with 11 different promoters to inform rMVA design; a positive correlation between *in vitro* expression levels and protective efficacy was apparent, but strong late expression with PsynE/L was shown to have a negative impact by inducing unfavourable antigen processing characteristics (Bronte *et al.*, 1997). In a study relating to parainfluenza virus, PsynE/L was found to have another detrimental effect, this time on the ability to generate high titre stocks. As an alternative to both PsynE/L and the weaker early/late P7.5, a novel promoter of intermediate strength (mH5) was generated and proven to provide optimal expression of the transgene (Wyatt *et al.*, 1996).

Similar studies are required for novel rMVA vectoring TB antigens. The impact on *in vitro* expression may be expected to be similar, but it is unknown how this will affect the T cell responses thought to protect against TB disease. Investigation is warranted and is the subject of this study.

Table 1.5 Evaluation of rMVA for delivery *M. tuberculosis* antigens. All of the studies listed were performed in mice. Dose refers to the amount of MVA delivered (PFU).

Antigen	Route	Dose & Regimen	Immunogenicity findings	Ref
Psyn (E/L)				
ESAT-6 Ag85A Ag85B HSP65 Mtb39A	s.c	10^7 MM	Antigens were expressed as a fusion. Detectable immunogenicity against each, and protection against Mtb challenge.	(Perera *et al.*, 2009)
P7.5				
MPT64	i.v	10^7 MM, DM	MM gave measurable response, DM response higher.	(Feng *et al.*, 2001)
ESAT-6 MPT63 (fusion)	i.d	10^6 M, MM DM, DDDM	No response from MM. DM gave a response and DDDM was much improved (2 – 10 fold).	(McShane *et al.*, 2001)
Ag85A	i.v	10^6 As above	As above.	(McShane *et al.*, 2002)
Rv0111 (generated by U.Oxf)	i.d	10^6 DM, DDDM	Only DDDM shown to be effective.	Rawkins *et al.*, (pers. comm)

Abbreviations: i.v, intravenous; s.c, sub-cutaneous; i.d, intradermal; D, DNA; M, MVA; U.Oxf, University of Oxford.

Optimised antigen processing

To date, published reports of rMVA delivering TB antigens have only described human tissue plasminogen activator (TPA) signal sequence as a leader and in one instance BVP22 (Yao *et al.*, 2009). The inclusion of TPA has been rationalized by studies demonstrating that it improves expression levels, immunogenicity and protective efficacy (Li *et al.*, 1999; Malin *et al.*, 2000). The exact mechanism by which TPA enhances the efficacy of TB vaccines is

unknown, but it is speculated to involve enhanced secretion and uptake by antigen-presenting cells as a result of increased expression levels (Li *et al.*, 1999). This subsequently leads to MHC Class II presentation and activation of CD4+ T cells, which is an essential component of TB protective immunity.

Protection against TB is now understood to require both CD4+ and CD8+ T-cell activation and for vaccines required to induce cellular immunity generally, phenotypically more diverse responses are evidently preferable; such as those achieved through heterologous prime boosting. Leader sequences promote stimulation of different immune responses and although they are not always discrete and consistent between antigens, leaders do appear to be a tool through which to diversify the response evoked to an antigen.

Not only should the effect of different leader sequences on the immunogenicity of TB antigens be investigated, but also the potential for different leader sequences to be combined in multiple-recombinant constructs, in order to traffic antigen to more than one route simultaneously. This could be evaluated in MVA, but the results would be of relevance to any recombinant vector, e.g. Adenovirus or plasmid DNA, both of which have been used in MVA heterologous prime-boost regimens. Investigation of this approach, using rMVA, is the subject of this study.

1.5 Hypotheses

MVA is an important vector in the field of TB vaccine development, but there have been few studies related to the optimisation of MVA-based vaccines for TB. To investigate the potential for MVA-based TB vaccines to be enhanced, independently of the vectored antigen or antigens, the following hypotheses were investigated:

- Vaccinia promoter selection can influence the immunogenicity and protective efficacy of TB vaccine antigens vectored by MVA.

- The type of immune response evoked by an MVA-vectored antigen can be altered via the addition of leader sequences. Further, by targeting the antigen to multiple pathways, the immune response can be made more diverse.

To test the above hypotheses, specific objectives for the study were to:

1. Design a panel of novel recombinant MVA viruses with which to test each hypothesis.
2. Create the required recombinant cassettes, ready for insertion into the MVA genome.
3. Generate purified stocks of each of the recombinant MVA viruses.
4. Evaluate the impact of vaccinia promoter selection on the immunogenicity and protective efficacy of MVA based vaccines.
5. Investigate the potential for diversifying the immune responses to an antigen through the use of multiple leader sequences.

Chapter 2 Materials and Methods

2.1 Construction of MVA Transfer plasmids

Transfer plasmids were created and purified using standard molecular cloning techniques (Green, 2012).

2.1.1 *E. coli* transformation

Chemically competent *E. coli* TOP10 (ThermoFisher Scientific) were transformed with plasmid DNA according to the manufacturer's instructions. Briefly, 1 µl of purified plasmid DNA or 5 µl of ligation reaction was added to a vial of competent cells. The *E. coli* were transformed via heat shock by incubating them at 42°C for 30 seconds and then transferring them to ice. Pre-warmed SOC medium (250 µl) was added and then the cells were incubated at 37°C, for 1 hour, with shaking at 225 rpm. Following transformation, *E. coli* were spread on agar and incubated at 37°C overnight.

2.1.2 *E. coli* culture

Transformed *E. coli* cells were cultured on Luria-Bertani (LB) agar or in LB broth (BioMérieux) and were selected for according to the antibiotic resistance conferred by plasmid uptake; the medium was supplemented with 100 µg/ml carbenicillin (a stable ampicillin analogue), 25 µg/ml chloramphenicol or 50 µg/ml Zeocin™.

E. coli clones were amplified in liquid culture by transferring a single colony from agar to 5 ml LB broth. Cultures were incubated at 37°C with shaking at 300 rpm, for at least 7 hours and preferably overnight. Liquid cultures were inspected for advanced turbidity before separating *E. coli* from the medium. The bacteria were pelleted by centrifugation at 6000 x g for 10 minutes and the culture medium discarded.

2.1.3 Plasmid DNA purification

Plasmid DNA was extracted from transformed *E. coli* using QIAprep® plasmid Miniprep kits (Qiagen) as per the manufacturer's instructions. Plasmid DNA was eluted in nuclease-free

water and the concentration determined by spectrophotometry (Nanodrop 2000; ThermoFisher Scientific).

2.1.4 Gateway® cloning.

Gateway® cloning was used throughout the study and was carried out according to the Gateway® Technology manual (ThermoFisher Scientific). A summary of the process is presented in Figure 2.1.

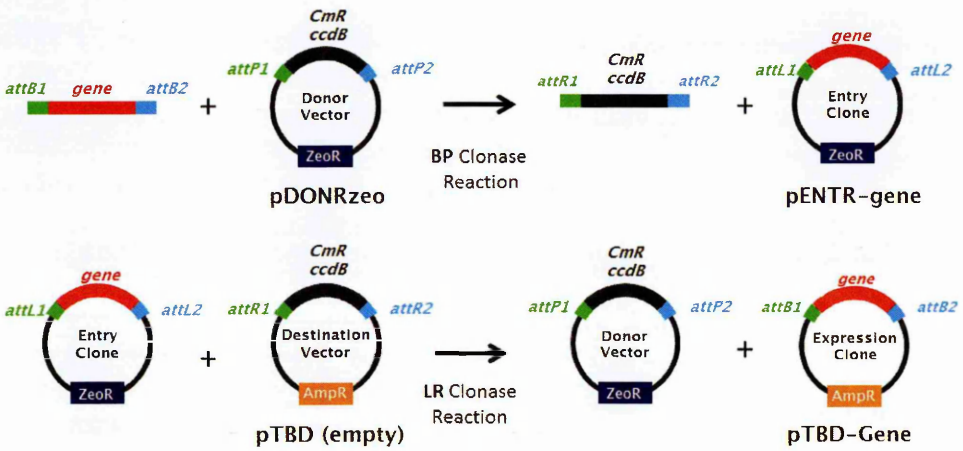


Figure 2.1 Gateway® Cloning. The gene of interest was introduced to the Gateway® system via the addition of *attB* sites to either end of the gene sequence. The gene was transferred to an entry clone (pENTR) by mixing it with pDONRzeo in a BP reaction so that *attB* and *attP* sites would recombine. Each of the MVA transfer plasmids (pTBD) used in the study contained a Gateway® Destination cassette so that it would serve as a destination vector in an LR reaction; the gene of interest was transferred from the Entry clone to the Destination vector via recombination of *attL* and *attR* sites. Throughout, a combination of the *ccdB* lethal gene and antibiotic resistance markers enabled selection of the desired clones. The antibiotic resistance genes used by the system conferred resistance to chloramphenicol (CmR), zeocin (ZeoR) and ampicillin/carbenicillin (AmpR). (Figure adapted from www.thermofisher.com).

The addition of *attB* sites to the gene of interest was achieved via PCR, i.e. inclusion of the *attB* sequence in gene-specific primers (examples are shown in Table 2.1).

Table 2.1 Primers for *attB*-PCR. Each PCR reaction included a forward (F) and reverse (R) primer. The *attB* portion of each primer is shown in capital letters. The gene-specific sequence is shown in lower case.

Primer Name	Primer Sequence (5' to 3')
attB PCR of Rv0111T	
MTB031 (F)	GGGGACAAGTTTGTACAAAAAAGCAGGCTaacggccaacgtacgggctggtc
MTB007 (R)	GGGGACCACTTTGTACAAGAAAGCTGGGTcactggcgacccgcaccgaatc
attB PCR of OVA	
MTB115 (F)	GGGGACAAGTTTGTACAAAAAAGCAGGCTggctccatcg
MTB116 (R)	GGGGACCACTTTGTACAAGAAAGCTGGGTaggggaaaca

As summarised in Figure 2.1, purified PCR products were transferred to the Entry clone and then to the pTBD vector by BP and LR reaction, respectively. Briefly, the PCR product/plasmids were mixed with BP or LR clonase in TE buffer (pH 8.0) and incubated at 25°C for 1 hour. The reaction was stopped by incubation with proteinase K solution at 37°C for 10 minutes. After each BP or LR reaction, *E. coli* TOP10 bacteria were transformed with 1 µl of reaction mix and the desired transformants selected for on LB agar containing antibiotic. The plasmids used during the course of the study are listed in Table 2.2.

Table 2.2 Plasmids propagated and manipulated during the course of the study. Plasmid pLAS-2 was a kind gift from L. S. Wyatt, National Institute of Health (NIH). Plasmids in the pTBD series (TB group Destination plasmids) were originally derived from pLW-44, pLW-24 and pLAS-1 (also kindly donated by L. S. Wyatt, NIH).

Plasmid	Antibiotic resistance marker(s)	Source
pLAS-2	Ampicillin	NIH
pDONRzeo	Zeocin™	ThermoFisher Scientific
Gateway® Destination vectors (prior to insertion of the gene of interest)		
pTBD1	Ampicillin and Chloramphenicol	Laboratory stock
pTBD2	"	"
pTBD3 - 4	"	"
pTBD5 - 14	"	This study

2.1.5 Site directed mutagenesis

Site directed mutagenesis was carried out on plasmids pTBD3 and pTBD4 using QuikChange Lightening Site-Directed Mutagenesis kits (Agilent Technologies) as per the manufacturer's instructions. Primers (Table 2.3) were designed using the manufacturer's online primer design tool. They were required to be 25-45 base pairs in length with base pair changes at or near the centre of the sequence. Mutated DNA was transformed into *E. coli* XL10 Gold ultracompetent cells after digestion of the parent DNA template by Dpn I enzyme.

Table 2.3 Primers designed for site-directed mutagenesis. Base pair changes were made to the indicated plasmids in order to introduce novel unique restriction enzyme sites. Mutations are shown in bold capital letters.

Primer Name	Primer Sequence (5' to 3')
Introduction of AgeI to pTBD3	
QM01 (F)	gcgagaaataatcataaataag**A**ccggtgccaccatgga
QM02 (R)	tccatggtggcaccgg**T**cttatttatgattatttctcgc
Introduction of AgeI to pTBD4	
QM03 (F)	gaagtagaatcataaagaacagt**A**ccggtgccacca
QM04 (R)	tggtggcaccgg**T**actgttctttatgattctacttc
Introduction of EcoRV to pTBD3 and pTBD4	
QM05 (F)	ccgattcagaagaggagccagatct**GAT**atcaaacaagtttgtacaaaaaagc
QM06 (R)	gctttttgtacaaacttgtttgat**ATC**agatctggctcctcttctgaatcgg

2.1.6 Restriction enzyme digest

Restriction enzymes (New England Biosciences) were used according to the manufacturer's instructions. Briefly, 1 unit of enzyme (1 µl) was added to 1 µg DNA (plasmid or PCR product). Each reaction included the appropriate NEB buffer, with or without bovine serum albumin (BSA) as prescribed and was made up to the required volume with nuclease-free water. The reaction was incubated at 37°C for at least 1 hour. For digestion with two enzymes, a double-digest was performed provided enzyme conditions were compatible.

Where they were not, a sequential digest was performed. To prevent re-ligation of digested plasmid, 5' phosphate groups were removed using Antarctic phosphatase (New England Biosciences) as per the manufacturer's instructions.

2.1.7 Separation of DNA fragments by agarose gel electrophoresis

Horizontal gels were prepared by dissolving agarose (1%) in Tris-acetate ethylenediaminetetraacetic acid (TAE) buffer. Sybr® Safe DNA gel stain (ThermoFisher Scientific) was added so that DNA fragments were visible under ultra-violet light for image capture (UVIdoc) or blue-light transillumination for band excision (ThermoFisher Scientific). Samples of DNA were loaded into each gel using gel loading dye and were run in parallel with 1 kb DNA ladder (ThermoFisher Scientific). Electrophoresis was performed at 100 V for up to 1 hour. Fragments requiring excision were extracted using QIAquick® Gel extraction kits (Qiagen) as per the manufacturer's instructions.

2.1.8 Polymerase chain reaction (PCR)

High-fidelity PCR was performed using KAPA HiFi Hotstart readymix (Kapa Biosystems) as per the manufacturer's instructions. Briefly, forward and reverse primers were added at a final concentration of 0.3 μM. Template DNA was present at less than 100 ng (genomic DNA), or between 0.1 and 10 ng (less complex DNA, e.g. fragments). The reaction volume was made up using nuclease-free water. Initial denaturation was performed at 95°C for 2-5 minutes. Cycling conditions were 35 cycles of 98°C for 20 seconds, 65°C for 15 seconds and 72°C for 15 seconds per kilobase (kb). A final extension step of 72°C for 1 minute per kb was performed before cooling the reaction to 4°C. PCR products were cleaned up for use in ligation reactions by gel extraction (Section 2.1.7) or by performing QIAquick PCR purification (Qiagen) as per the manufacturer's instructions.

High-fidelity PCR was applied to generate fragments for transfer plasmid construction. The primers used to create DNA fragments homologous to Deletion site II are listed in Table 2.4. Where possible, primers were 20 – 30 nucleotides long, had a guanidine/cytosine (GC)

content of 40 – 60% and a melting temperature (T_m) between 60 and 70°C. Four nucleotides were added 5' to restriction enzyme recognition sites to promote efficient cutting. Secondary structure was examined using an online tool (http://biotools.nubic.northwestern.edu/OligoCalc.html).

Table 2.4 Primers for creating Del II fragments by high-fidelity PCR. Forward (F) and reverse (R) primers were designed to add unique restriction enzyme recognition sites (underlined). Where the PCR product might have otherwise contained a unique site of value to the cloning process, a two-step PCR reaction (Section 2.1.9) was applied to introduce a mutation (bold, lower case) that would remove it. The template DNA was plasmid pLAS-2 (Table 2.2).

Primer Name	Primer Sequence (5' to 3')
Del II Direct repeat: adding EcoIRCI (F) and XhoI (R)	
MTB95 (F)	TAATGAGCTCGCTTTCTCTCTAGCAAAGATG
MTB96 (R)	TAATCTCGAGGAATCATCCAGTCCACTGAATAG
Del II Left Flank: 2 step PCR removing AgeI, adding SbfI (F) and HindIII (R)	
MTB97 (F)	TAATCCTGCAGGGGATGCGATCATGACGTCC
MTB105 (R)	CGTACAGGACGTAACTATAAACCG**c**TT
MTB106 (F)	CTTGAACAAATATAAA**g**CGG
MTB98 (R)	TAATAAGCTTGGTTTGATCGTTGTCATTTCTCC
Del II Right Flank: Two-step PCR removing HindIII, adding NarI (F) and AscI (R)	
MTB109 (F)	GAAGTCTAAGCAGCTGAAA**t**GCTTTC
MTB110 (R)	GCA TCT TTG CTA GAG AGA AAG C**a**T TTC
MTB107 (F)	TAATGGCGCCCTCCTGAAAAACTGG
MTB108 (R)	TAATGGCGCGCCGAATCATCCAGTCCACTG

2.1.9 Overlap extension (OE) PCR

OE-PCR was applied to generate DNA fragments incorporating multiple PCR products, and/or oligonucleotides. The fragments were combined in a PCR reaction with Kapa HiFi polymerase and subjected to cycling conditions as described in Section 2.1.8. After 15

cycles, the reaction was stopped and primers homologous to the ends of the combined product were added. A further round of 20 cycles was then carried out.

2.1.10 DNA annealing

Custom synthesised oligonucleotides (Table 2.5) were mixed at equimolar concentration in Ligase buffer (New England Biosciences) and incubated at 85°C for 10 minutes. Stepwise cooling was performed in 5°C increments down to 25°C. Each temperature was held for 5 minutes.

Table 2.5 Oligonucleotides for plasmid modification. Custom synthesised DNA fragments (Integrated DNA Technologies) were prepared ready for annealing and ligation; they were designed to have ends compatible with restriction enzyme-digested vector and were 5' phosphorylated.

Oligo Name	Oligo Sequence (5' to 3')
pTBD5/6 to pTBD9/10: Kozak sequence, no leader	
MTB111 (F)	CCGGTGCCACCATGGAT
MTB111r (R)	ATCCATGGTGGCA
pTBD7/8 to pTBD11/12/13/14: N terminal V5 tag	
MTB112 (F)	CCGGTGCCACCATGGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCT ACGGATCTAGAGGG CCCGCGGTTCGAAGAT
MTB112r (R)	ATCTTCGAACCGCGGGCCCTCTAGATCCGTAGAATCGAGACCGAGGAGAGG GT TAGGGATAGGCT TACCCATGGTGGCA
pTBD7/8 to pTBD11/12: C terminal VSVg	
MTB113 (F)	GGCCCGAGCAGCATCGCCAGCTTCTTCTTCATCATCGGCCTGATCATCGGCC TGTTCCTGGTGCTGCGCGTGGGCATCCACCTGTGCATCAAGCTGAAGCACAC CAAGAAGCGCCAGATCTACACCGACATCGAGATGAACCGCCTGGGCAAGTA ACTCGACCTGCA
MTB113r (R)	GGTCGAGTTACTTGCCCAGGCGGTTCATCTCGATGTCGGTGTAGATCTGGC GCTTCTTGGTGTGCTTCAGCTTGATGCACAGGTGGATGCCCACGCGCAGCAC CAGGAACAGGCCGATGA
pTBD7/8 to PTBD13/14: C terminal stop	
MTB114 (F)	GGCCCGTAACTCGACCTGCA
MTB114r (R)	GGTCGAGTTACG

2.1.11 DNA ligation

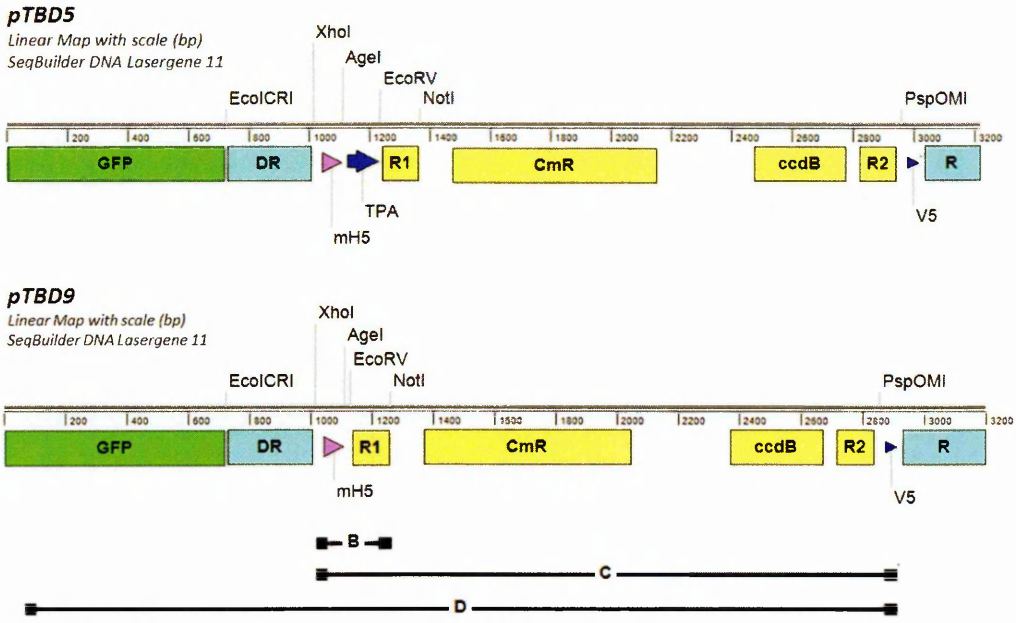
Fragments of DNA were ligated together using the Quick-Ligation™ kit (New England Biosciences) as per the manufacturer's instructions. Briefly, the reaction was set up to include 50 ng of vector with a three-fold molar excess of insert. Quick-Ligation reaction buffer and Quick T4 DNA ligase were added at the required concentrations and the reaction incubated at room temperature for 5 minutes. The reaction mix was chilled on ice prior to transformation of *E. coli* TOP10 (Section 2.1.1). The inserts used in each ligation were derived from high-fidelity PCR (Sections 2.1.8 and 2.1.9) or were otherwise purchased as oligonucleotides and annealed (Section 2.1.10).

2.1.12 DNA sequencing

Sanger sequencing of DNA samples was performed using an external sequencing service (Beckman Coulter Genomics, Takeley, UK). Plasmid DNA or purified PCR products were sent at, or above, the minimum concentration and volume required. Where universal primers were not available, sequences for custom synthesised primers were provided. Primers were designed to bind to the template sequence at approximately 700 base pair intervals, were typically 20-30 nucleotides long and had a GC content between 40 and 60%. Sequences with secondary structure (examined as described in Section 2.1.8) were avoided.

2.1.13 Conversion of pTBD5 to pTBD9

A combination of oligonucleotide custom synthesis, PCR and OE-PCR was applied to the preparation of inserts for the conversion of pTBD5 to pTBD9. A summary of the inserts prepared and their means of preparation is described in Figure 2.2.



Fragments and their means of preparation:

A *17 bp* *(Not shown in the above diagram)*
Annealed oligonucleotides; MTB111/MTB111r (see Table 2.5)
AgeI/EcoRV

B *252 bp* *Custom synthesised by IDT.*
XhoI/NotI

C *1899 bp* *1. TPA to V5 was amplified from pTBD5 by PCR.*
2. TPA was removed from the PCR product by restriction enzyme digest with EcoRV.
3. OE-PCR with fragment B was performed.
XhoI/PspOMI

D *2849 bp* *1. GFP to TPA was amplified from pTBD5 by PCR.*
2. TPA was removed from the PCR product by restriction enzyme digest with AgeI.
3. OE-PCR with fragment B was performed followed by OE-PCR with fragment C.
Eco53KI (EcoICRI isoschizomer)/PspOMI

Figure 2.2 Fragments for the conversion of pTBD5 to pTBD9. Plasmid maps for sections of pTBD5 and pTBD9 are shown. Plasmid conversion using fragment A was unsuccessful. Larger fragments (B-D) were prepared and are illustrated, below which their means of construction is described. Restriction enzymes used for vector/insert digestion are shown underlined.

2.1.14 Construction of the Ovalbumin (OVA) gene

The OVA gene (*Gallus gallus* ovalbumin; NCBI Accession number NM_205152.1), minus the native start, was custom synthesised as three overlapping gBlock fragments (Integrated DNA Technologies) as shown in Figure 2.3. The fragments were joined by OE-PCR and incorporated *attB* sites for recombination into pDONRzeo (see 2.1.4 Gateway® cloning). Amplification was achieved using primers binding to the end of the product (the sequences for primers MTB115 and MTB116 are shown in Table 2.2). As with the other antigens used in this study, the OVA gene was screened for the vaccinia transcription termination sequence TTTTTNT *in silico*, prior to cloning (none were found to be present).

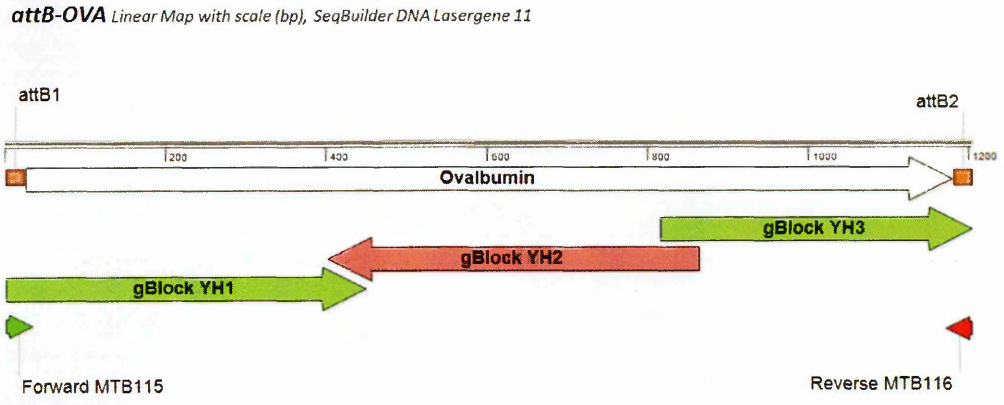


Figure 2.3 Construction of the OVA gene by OE-PCR. In the first step of the reaction, three overlapping gBlock fragments were combined. In the second step of the reaction, the product was amplified by the addition of primers MTB115 and MTB116 (Table 2.2). *AttB* sites were added to the sequence so that it could be used in Gateway® cloning.

2.2 Generation of Recombinant MVA (rMVA)

Recombinant MVA were generated using establish protocols for transfer plasmid mediated construction (Earl *et al.*, 2001a; Earl and Moss, 2001; Earl *et al.*, 2001b; Kremer *et al.*, 2012). Aseptic technique was applied throughout.

2.2.1 Cell culture

Cell line BHK-21 (ATCC CCL-10) was maintained in Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 5 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (all reagents – Sigma Aldrich). Cells were maintained in static tissue culture flasks, incubated at 37°C, 5% CO_2 and 95% humidity. The cell culture medium was changed twice a week and cells were passaged every 7 – 10 days. No more than a total of 10 passages were performed as BHK-21 cells are susceptible to phenotypic drift.

Primary chick embryo fibroblast (CEF) cells were obtained from The Pirbright Institute, UK. Cells were seeded into tissue culture flasks at approximately 2 x 10^7 cells per 175 cm^2 in supplemented MEM, as described above. Flasks were incubated at 37°C, 5% CO_2 and 95% humidity for 24 hours before reducing the incubating temperature to 30°C. Cells were passaged at least once prior to use and from there on were incubated at 37°C.

Throughout, BHK-21 or CEF cells were cultured in multi-well tissue culture plates or in tissue culture flasks with a surface area of 25 cm^2 (T25), 75 cm^2 (T75) or 175 cm^2 (T175). Cells were infected with MVA diluted in supplemented MEM containing 0 or 2% FBS – referred to as '0% MEM' or '2% MEM', respectively. Incubations were performed at 37°C, 5% CO_2 and 95% humidity. Infected cell monolayers were examined by inverted light and/or fluorescence microscopy to monitor the development of cytopathic effects (CPE) and where rMVA was present, the expression of the selection marker - GFP.

2.2.2 Preparation of wild-type MVA stock

A working stock of MVA (ATCC-VR-1508) was prepared by two sequential rounds of amplification on CEF cells. The virus was diluted in 2% MEM and applied to a T75 flask at a multiplicity of infection (MOI) of approximately 0.1. The cells were incubated for 3 days and then harvested by scraping and centrifugation at 1200 x g for 10 minutes. The cell pellet was resuspended in 1-2 ml 2% MEM supernatant and stored at -80°C. Cells were

lysed by three repeat rounds of freeze-thawing and were vortexed and sonicated on each round to promote dissociation of the viral particles. The working stock was created after a second round of amplification performed on eight T175 flasks. The harvested material was diluted down to a working concentration, aliquoted and stored at -80°C for future use. Viral titre was determined as described below.

2.2.3 Determination of MVA titre by immunostaining

CEF cells were seeded into 6 well plates so that they were nearing confluency at the time of use. Viral samples were vortexed and sonicated immediately prior to performing 10-fold serial dilutions in 0% MEM. CEF were infected with a range of dilutions, in duplicate wells, 0.5 ml per well and then incubated for 90 minutes with rocking at 15 minute intervals. After 90 minutes, 0% MEM was removed and replaced with 2% MEM containing 1% low gelling temperature agarose (2-hydroxyethylagarose Type VII; Sigma Aldrich). The pre-molten overlay was allowed to solidify before incubation of the plate(s) for 2-3 days.

The solid overlay was removed from each well immediately prior to immunostaining. Care was taken not to damage the cell monolayer. Anti-vaccinia polyclonal antibody raised in rabbit (AbD Serotec) was added to each well and then detected using VECTASTAIN® ABC-AP Rabbit IgG kit (Vector Laboratories). The kit was used as per the manufacturer's instructions. Briefly, biotinylated anti-rabbit secondary antibody was applied followed by combined avidin/biotinylated-alkaline phosphatase (AP) reagents. Viral plaques were made visible by the addition of Vector Red AP substrate. Plaques were counted under an inverted light microscope (x4) and the number arising from duplicate wells multiplied by the dilution factor to determine the viral titre (PFU/ml) of the original sample.

2.2.4 Generation of rMVA by infection/transfection (I/T) reaction

BHK-21 were grown to 85-90% confluence in a T25 culture flask and then infected with MVA in 0% MEM at an MOI of 0.05. After 90 minutes, the MVA was removed and 5.6 μg of transfer plasmid DNA transfected into the cells using Lipofectamine®, as per the

manufacturer's instructions (ThermoFisher Scientific). After 5 hours, the transfecting medium was removed and replaced with 2% MEM. Two - three days later, BHK-21 were viewed using an inverted light microscope to monitor CPE and GFP expression before harvesting the cell layer into 1 ml of supernatant. The harvested sample was subjected to three rounds of freeze-thawing, with vortexing and sonication before being progressed to plaque purification.

2.2.5 Isolation of rMVA by plaque purification

CEF were seeded into multi-well tissue culture plates and were infected as described for immunostaining (Section 2.2.3). Briefly, material from the I/T step was serially diluted and plated at dilutions of 1:100, 1:1000 and 1:10000. After 90 minutes incubation, the infecting material was removed and replaced with solid overlay. After 2-3 days incubation, viral plaques expressing GFP were visualised using inverted fluorescence microscopy and were picked from the cell layer using a Gilson pipette. Each viral pick was transferred to 0.5 ml 0% MEM for freeze-thawing with vortexing and sonication.

Subsequent rounds of plating were performed as above, but by plating dilutions of 1:10, 1:100 and 1:1000. At each stage, plaques were picked from the highest possible dilution so as to carry over the least amount of parent MVA.

Plaque samples were numbered according to the round of plaque purification and their number, which took into account their lineage e.g. P1 (1.3.2). Where helpful, this was presented schematically, as per the mock example shown in Figure 2.4.

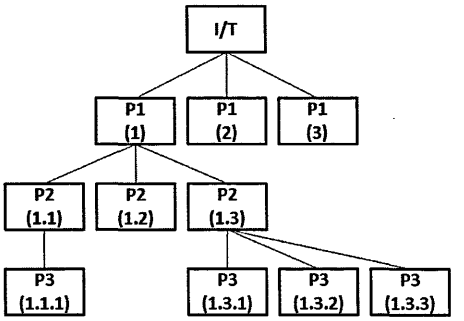


Figure 2.4 A family-tree for rMVA isolation. A mock example is depicted. Following the infection and transfection step (I/T), 3 plaques are isolated - P1 (1), P1 (2) and P1 (3). Plaque sample P1 (1) is propagated and a further 3 plaques are picked. Two of these samples are propagated to yield a further 4 plaques. Samples are labelled according to the round of plaque purification and their number, which takes into account the lineage of the sample.

2.2.6 Generation of markerless rMVA

Following successful isolation of rMVA, viruses generated to express GFP transiently were further passaged to promote removal of the selection marker by an internal recombination event (described in Section 1.3.1). The viral population comprising GFP positive and GFP negative rMVA was propagated on CEF until CPE, but no GFP was visible.

In some instances, GFP expression was observed to persist. This was overcome by infection of CEF in a multi-well plate followed by incubation under a solid 2% MEM overlay (as described in Section 2.2.3). After 2-3 days, areas of the monolayer exhibiting CPE, but no GFP expression, were picked into 0.5 ml 2% MEM for further propagation, thereby expediting GFP removal.

2.2.7 Purification of rMVA by sucrose cushion density centrifugation

Samples of purified rMVA were amplified on CEF cells by serial propagation in culture vessels of increasing size. Large scale amplification was performed using 10 or more T175 flasks. Amplified virus was separated from the cell lysate by density centrifugation. Briefly, the cell pellet was harvested in 10 ml of 10 mM Tris-HCl. The cells were lysed by freeze-thaw cycles, homogenised using a Dounce homogeniser (50 strokes) and sonicated. The

homogenate was layered onto a 36% sucrose solution prepared in 10 mM Tris-HCl and centrifugation performed at 33000 x g, 4°C for 90 minutes. The viral pellet was resusepnded in PBS for aliquoting and titration. All viral material was stored at -80°C.

2.3 *In vitro* characterization of rMVA

2.3.1 Extraction of viral genomic DNA

Viral samples were amplified on CEF in multi-well plates for 2-3 days. Viral genomic DNA (gDNA) was extracted using a Wizard SV Genomic DNA Purification Kit (Promega) as per the manufacturer's instructions. Briefly, the cell culture medium was removed from each infected well and the contents transferred to a mini-column in lysis buffer. Each purification column was subjected to repeat washes before eluting gDNA in nuclease-free water.

2.3.2 Analytical PCR

PCR was performed using KAPA 2G Fast Hotstart readymix (Kapa Biosystems) as per the manufacturer's instructions. Briefly, forward and reverse primers were added at a final concentration of 0.5 µM. Template DNA (viral gDNA) was present at less than 100 ng. Initial denaturation was performed at 95°C for 1 minute. Cycling conditions were 35 cycles of 95°C for 15 seconds, 60°C for 15 seconds and 72°C for 60 seconds. A final extension step of 72°C for 5 minutes was performed before cooling the reaction to 4°C. In each reaction, transfer plasmid DNA was used as a positive control and nuclease-free water in place of DNA as the negative control. The primer pairs used to examine insertion sites and/or the presence of a recombinant cassette are listed in Table 2.6.

Table 2.6 Primers for rMVA analytical PCR. Each primer pair bound to the flanking region of a particular insertion site (Del II or Del III) and/or the recombinant cassette. Primers were obtained from existing stocks or from those created above, i.e. during transfer plasmid construction (Table 2.1 and Table 2.4).

Primer Name	Primer Sequence (5' to 3')
Del III Left flank(F) to Del III Right flank (R)	
MTB93 (F)	CGGCACCTCTCTTAAGAAGT
MTB21 (R)	GTGTAGCGTATACTAATGATATTAG
N-terminal TPA (F) to C-terminal V5 (R)	
MTB01 (F)	GCCACCATGGATGCAATGAAGAGA
MTB34 (R)	TTACGTAGAATCGAGACCGAGGAG
GFP (F) to Del III Right Flank (R)	
MTB92 (F)	CGTAAACGGCCACAAGTTCAGCG
MTB21 (R)	GTGTAGCGTATACTAATGATATTAG
Del II Right flank (F) to Del II Left flank (R)	
MTB109 (F)	GAAGTCTAAGCAGCTGAAATGCTTTC
MTB105 (R)	CGTACAGGACGTAACTATAAACCGCTT
OVA gene start (F) to OVA gene end (R)	
MTB115 (F)	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGCTCCATCG
MTB116 (R)	GGGGACCACTTTGTACAAGAAAGCTGGGTAGGGGAAACA
GFP (F) to Del II Left Flank (R)	
MTB92 (F)	CGTAAACGGCCACAAGTTCAGCG
MTB105 (R)	CGTACAGGACGTAACTATAAACCGCTT

2.3.3 Detection of recombinant antigen expression by Western Blotting

Viral samples were amplified on CEF in multi-well plates for 2-3 days. After removal of the cell culture medium, CEF and viral proteins were harvested in lithium dodecyl sulphate (LDS) buffer containing 10% denaturing reagent (ThermoFisher Scientific). Each sample was further denatured by heating to 90°C for 10 minutes.

Proteins were separated by polyacrylamide gel electrophoresis using NuPage 4-12% Bis-Tris gradient gels (ThermoFisher Scientific). SeeBlue Plus2 pre-stained protein ladder and Magic

Mark XP Western protein standard (both ThermoFisher Scientific) were included in each run and electrophoresis performed at 200 V for up to 1 hour.

Following electrophoresis, protein was transferred to Hybond ECL nitrocellulose membrane (GE Healthcare Life Sciences) by Western Blotting; an XCell II™ Blot module and NuPage Transfer buffer (ThermoFisher Scientific) were used as per the manufacturer's instructions. Blotting was performed at 40 V for 1 hour and successful protein migration was monitored according to movement of the SeeBlue Plus2 ladder from the gel to the membrane.

Immunodetection was performed after blocking with 3% milk powder (Sigma Aldrich) diluted in PBS/Tween 20 (0.05%). The membrane was incubated with anti-V5 monoclonal antibody (AbD Sertotec) and chemiluminescent detection of anti-V5 reactive bands achieved via incubation with anti-mouse HRP conjugate. The application of ECL Plus chemiluminescent reagent and exposure to Hyperfilm ECL (GE Healthcare Life Sciences) was performed as per the manufacturer's instructions.

2.3.4 Semi-quantitative measurement of expression by dot blotting

Viral samples were amplified on CEF and the cells lysed as described above. Material was applied directly to nitrocellulose in 5 µl spots prior to blocking and immunodetection with anti-V5 (as described for Western blotting). Hyperfilm was scanned using a BioRad GS-800 densitometer (BioRad) and optical density measured by ImageQuant TL image analysis software (GE Healthcare) or Quantity One 1D analysis software (BioRad).

2.4 *In vivo* characterization of rMVA

Mouse studies were conducted according to UK Home Office legislation for animal experimentation and were approved by the local ethical committee.

Mouse immunisation, *M. tuberculosis* aerosol challenge and post-terminal sampling were performed by the Biological Investigations Group and the Small Animal Vaccine Evaluation team.

2.4.1 Mouse strain and immunisation

Groups of female C57Bl/6 or CB6F1 mice (8 -10 weeks of age) were immunised with MVA diluted in PBS. A dose of 10^6 or 10^7 PFU was given in 100 µl, intramuscularly (50 µl per hind leg), twice at two week intervals.

BCG Danish (Staten Serum Institut, Denmark) was diluted in Sautons medium as per the vaccine supplier's instructions. Mice were administered a dose of 1 x 10^5 CFU in 100 µl, subcutaneously in the nape of the neck.

Unvaccinated mice were matched for age and sex and received no immunisation.

Post-terminal sampling was performed to obtain whole blood and spleens.

2.4.2 Measurement of cytokine release by ELISpot assay

Splenocytes were harvested and assessed for antigen specific recall responses two weeks after the final immunisation. Cells were isolated via mechanical dissociation of the spleen followed by centrifugation at 500 x g for 7 minutes at 18°C. Red blood cells were lysed with ammonium-chloride-potassium (ACK) buffer (ThermoFisher Scientific). Processing and cell culture was performed aseptically in RPMI medium supplemented with 10% FBS, 5 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.5 µM β-mercaptoethanol and 12.5 mM HEPES buffer (all reagents Sigma Aldrich, except β-mercaptoethanol - ThermoFisher Scientific).

Murine ELISpot assays for IFNγ, IL-4 and IL-17 (MAbtech) were performed as per the manufacturer's instructions. PVDF plates (Merck Millipore) were coated with IFNγ capture antibody. Splenocytes were added at 5 x 10^4 or 2 x 10^5 cells per well. Cells were cultured with restimulating peptides (described below), each present at a final concentration of 2 µg/ml. A mixture of 0.1 µg/ml Phorbol 12-myristate 13-acetate (Sigma Aldrich) and 1 µg/ml Ionomycin (Merck Millipore) was added to positive-control wells. Background IFNγ release was measured in wells containing splenocytes and cell culture medium only. Plates were

incubated at 37°C, 5% CO_2 and 95% humidity for 18 hours priors to removal of the cells for spot detection.

Bound IFNγ was detected via the addition of biotinylated anti-IFNγ followed by streptavidin-AP conjugate. Spots were developed following incubation with AP substrate - BCIP/NBT (MAbtech). Spots were counted using an AID ELISpot reader and results expressed as spot forming units (SFU) per million cells (SFU/10^6) after subtraction of background (media only) responses. Responses greater than background plus two standard deviations were deemed to be positive. Statistical comparison between two groups was performed using unpaired T-tests (Microsoft Excel 2010). Comparison between multiple groups was achieved using one-way ANOVA (GraphPad Prism 6).

Cryopreservation of splenocytes was performed by freezing cells in FBS containing 10% DMSO. Cells were frozen at -80°C in a storage container designed to control the cooling rate (-1°C per minute). Cells were then transferred to the vapour phase of liquid nitrogen storage tanks.

2.4.3 Restimulating peptides and protein

Lyophilised peptides (Mimotopes) representing *M. tuberculosis* strain H37Rv genes Rv0111 and Ag85A (Rv3804c) and were prepared as 15 amino acid oligomers (15 mers) offset by 5/overlapping by 10. Vaccinia peptides (Mimotopes) were based on immunogenic determinants (8-10 mers) for genes B8R, A19L, A47L, A42R and K3L, as described by Tscharke *et al.* (Tscharke *et al.*, 2005). Ovalbumin peptides (Mimotopes) were based on MHC Class I and MHC Class II epitopes; $OVA_{257-264}$ 'SIINFEKL' and $OVA_{323-339}$ 'ISQAVHAAHAEINEAGR', respectively.

All peptides were resuspended in 10% DMSO to a concentration of 8 mg/ml and diluted to a final concentration of 2 μg/ml for cell stimulation. Peptide pools were created by the addition of equal volumes of each peptide.

Whole OVA protein was obtained in the form of purified albumin from chicken egg white (Sigma Aldrich) and was used at the concentration specified. Tuberculin purified protein derivative (PPD) was obtained from the Staten Serum Institute and used at a final concentration of 10 μg/ml.

2.4.4 Measurement of OVA-specific IgG isoforms by ELISA

An in-house enzyme-linked immunosorbent assay (ELISA) was developed for the detection of murine anti-OVA IgG. Reagents were sourced from Sigma Aldrich unless otherwise stated. Serum was separated from whole blood via centrifugation in serum separator tubes (Becton Dickinson) as per the manufacturer's instructions.

Immuno MaxiSorb microtitre plates (ThermoFisher Scientific) were coated with IgG or ovalbumin diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6. Plates were incubated overnight (16+ hours) at 4°C and then blocked with 1% BSA in PBS for at least 2 hours at room temperature. Anti-ovalbumin IgG1 or mouse serum, diluted in PBS, was added at 50 μl per well and incubated for 2 hours at room temperature.

Indirect detection of bound IgG was achieved using biotinylated isotype-specific anti-mouse IgG, followed by ultrasensitive streptavidin-HRP conjugate, both added for 1 hour at room temperature. Measurement of bound IgG was achieved via addition of the chromogenic HRP substrate tetramethylbenzidine (TMB). TMB was incubated for 2 minutes before 1M H_2SO_4 was added to stop the reaction. The plates were read at 450 nm and 690 nm and results for the latter subtracted from the first. Non-specific binding (NSB) was subtracted from all of the results, i.e. the OD measured in the absence of primary antibody (coating IgG or mouse serum) and was determined for a minimum of three wells.

2.4.5 Evaluation of vaccine efficacy after *M. tuberculosis* aerosol challenge

Groups of C57Bl/6 mice (Section 2.4.1) were infected with an aerosolised dose of *M. tuberculosis* strain Erdman. The challenge apparatus and methodology were as previously

described (Clark *et al.*, 2011). Briefly, a suspension of Mtb Erdman was aerosolised in a contained Henderson unit to infect the mice via nose-only exposure. The concentration of the bacteria in the Collison nebuliser was sufficient to provide an inhaled retained dose of approximately 100 CFU. After infection, animals were housed under Advisory Committee on Dangerous Pathogens (ACDP) Hazard Group 3 containment measures and monitored daily for outward signs of ill-health. At four weeks post-infection, post-terminal sampling of the lung and spleen was performed. Whole lung and spleen were homogenised in sterile water and plated on 7H11 OADC selective solid medium (bioMérieux) for enumeration of bacterial load. Groups were compared by unpaired, non-parametric Mann-Whitney test (GraphPad Prism 6).

Chapter 3 Design and construction of novel transfer plasmids

3.1 Introduction

In order to test the study hypotheses (see Section 1.5), several novel rMVA viruses needed to be created. As discussed in Chapter 1.3, there are two principal approaches for generating recombinant MVA: transfer plasmid methodology and BAC technology. Transfer plasmid methodology was selected for the current study on the basis that an existing panel of transfer plasmids was available for use and/or modification. As described in Section 1.3.2, BAC technology confers the principal advantage of negating the need for plaque purification, but is technically demanding to establish (Cottingham, 2012).

The transfer plasmids available at the outset of the study were Gateway® cloning vectors. They are listed in Section 2.1.4, Table 2.2. It was proposed that alteration of this plasmid set via standard cloning techniques would be the most expedient route to obtaining the novel recombinant cassettes required. Before the transfer plasmids could be selected and/or modified, the precise nature of the rMVA viruses needed for the study had to be decided upon. (Generation of the rMVA viruses is described in Chapter 4).

3.1.1 Transfer plasmids for the Vaccinia promoter study

To investigate the impact of vaccinia promoter selection on the immunogenicity and protective efficacy of rMVA vectoring *M. tuberculosis* antigens, it was essential to have viruses identical in every regard except for the vaccinia promoter driving antigen expression. The rationale for the vaccinia promoters and *M. tuberculosis* antigens selected for this aspect of the study is described in more detail in Chapter 5. Briefly, two vaccinia promoters, P7.5 and mH5, and two *M. tuberculosis* antigens (Rv0111 and Ag85A) were chosen. The complete panel of rMVA viruses is listed in Table 3.1. One of the viruses (MVA-mH5-Ag85A) and all of the transfer plasmids were available and so no further cloning work was needed.

Table 3.1 rMVA viruses for the vaccinia promoter study. Existing transfer plasmids were available for the purpose of generating the expression products shown.

Recombinant MVA virus	Expression product	Common Features
MVA-mH5-Ag85A MVA-P7.5-Ag85A	TPA-Ag85A-V5	Insertion site: Del III Selection marker: stable GFP
MVA-mH5-Rv0111 MVA-P7.5-Rv0111	TPA-Rv0111-V5	

Other features conferred by the existing transfer plasmids were in-frame expression of a TPA leader (see Section 1.2.6) at the start (N-terminus) of the polypeptide sequence and in–frame expression of V5 at the end (C-terminus) of the protein. Both fusions are discussed in more detail in Chapter 5. The V5 protein tag, also known as Pk, is a B cell epitope sequence. It is 14 amino acids long and is derived from the P and V proteins of the paramyxovirus of simian virus 5 (Southern *et al.*, 1991). Expression of V5 as an antigen-fusion enables immunodetection of the expression product using anti-V5 IgG, thus, circumventing the need for antigen-specific antibodies.

3.1.2 Transfer plasmids for the Leader Sequences study

To investigate the impact of leader sequences on transgene immunogenicity, including the impact of targeting a recombinant antigen to multiple pathways, two leader sequences were selected for use in two, separate insertion sites: TPA - Del III and VSVg - Del II. The antigen selected for comparison of antigen-fusion effects was the model antigen ovalbumin (OVA). The rationale for the antigen selected, and further explanation for the specific design of the constructs is discussed in Chapter 6. Briefly, the TPA and VSVg fusions were expressed at the N and C terminus of OVA, respectively. At the other terminus, a V5 tag was inserted. For each of the leader sequences under investigation, a corresponding 'no-leader' plasmid was created to enable provision of a no-leader control virus. The viruses required and their expression products, relative to the insertion site, are listed in Table 3.2.

Table 3.2 rMVA viruses required for the leader sequences study. The panel of rMVA comprised single and double-recombinant viruses. For each virus expressing OVA fused to a leader sequence, there was a corresponding 'no-leader' control.

Recombinant MVA virus	Expression product Del II	Expression product Del III
MVA-OVA_{TPA}	-	TPA-OVA-V5
MVA-OVA	-	OVA-V5
MVA-OVA_{VSVg}	V5-OVA-VSVg	-
MVA-OVA	V5-OVA	-
MVA-OVA_{TPA}/OVA_{VSVg}	V5-OVA-VSVg	TPA-OVA-V5
MVA-OVA/OVA	V5-OVA	OVA-V5

To create the transfer plasmids required, Destination vectors pTBD3 and pTBD4 (Section 2.1.4, Table 2.2) were selected for modification. Plasmids pTBD3 and pTBD4 incorporate mH5 and P7.5 for antigen expression, respectively. A summary of their main features is shown in Table 3.3. Also listed are the transfer plasmids that would need to be generated from them in order to create the rMVA listed in Table 3.2. An Entry clone was also needed for insertion of OVA via Gateway® cloning.

Plasmids pTBD3 and pTBD4 carry the GFP selection marker flanked by a direct repeat sequence. Thus, upon transfer to the MVA genome, the marker is excised via a recombination event. All of the transfer plasmids listed in Table 3.3 were required to contain transiently expressed GFP so that single recombinant viruses could be used to generate multiple recombinant viruses, i.e. become markerless for monitoring of insertion of a second recombinant cassette.

Table 3.3 Novel plasmids for the leader sequences study. Each of the transfer plasmids was a Gateway® Destination vector and could receive antigen via recombination with an Entry clone (pENTR). In all of the vectors GFP was flanked by a direct repeat sequence, for transient expression upon transfer to the MVA genome, as described in Section 1.3.

Plasmid	Insertion site	Promoter	N-terminal	C-terminal	Source
Destination vectors (contain a Gateway® cloning site for antigen insertion)					
pTBD3/4	Del III	mH5/P7.5	TPA	V5	Laboratory stock
pTBD5/6	Del III	mH5/P7.5	*TPA*	V5	This study
pTBD7/8	Del II	mH5/P7.5	*TPA*	V5	This study
pTBD9/10	Del III	mH5/P7.5	none	V5	This study
pTBD11/12	Del II	mH5/P7.5	V5	VSVg	This study
pTBD13/14	Del II	mH5/P7.5	V5	None	This study
Entry vector					
pENTR-OVA	n/a	n/a	n/a	n/a	This Study

*Unique restriction enzyme sites

Another objective set for the development of the novel transfer plasmids was that they remain suitable for the study of alternative antigens. For each of the recombinant cassettes required, a pair of plasmids was generated, one incorporating mH5 for transgene expression, the other P7.5, so as to retain each as an option for use in the current and future studies. The B cell epitope tag, V5, was included throughout for immunodetection of the expression product; anti-OVA IgG is available (various commercial sources), but future studies may involve antigens for which antigen-specific IgG is less readily obtainable. It was also considered important that the transfer plasmids be as amenable to further alteration as possible, for example by retaining unique restriction enzyme sites at useful positions. Accordingly, aims for transfer plasmid construction were as described below.

3.2 Chapter aims

- Plan and implement a series of sequential alterations through which to obtain the required plasmids, each derived from pTBD3 or pTBD4.
- Introduce ovalbumin (OVA) to the Gateway® system by creating pENTR-OVA.
- Finalise transfer plasmid construction by transfer of OVA via Gateway® cloning.

3.3 Results

3.3.1 An efficient cloning strategy for construction of the required transfer plasmids

To generate a panel of novel transfer plasmids derived from parent vectors pTBD3 and pTBD4, a series of manipulations (summarised in Figure 3.2) were identified through which to obtain the required constructs as efficiently as possible. In the first instance, pTBD3 and pTBD4 were examined for unique restriction sites allowing for alteration of each flank (L and R), the direct repeat sequence (DR), and N and C terminal fusions (Figure 3.2 A). Interrogation of unique restriction sites revealed all of the above to be excisable, with the exception of the N-terminal fusion (TPA), therefore, the first modification sought to address this limitation by introducing unique restriction enzyme sites.

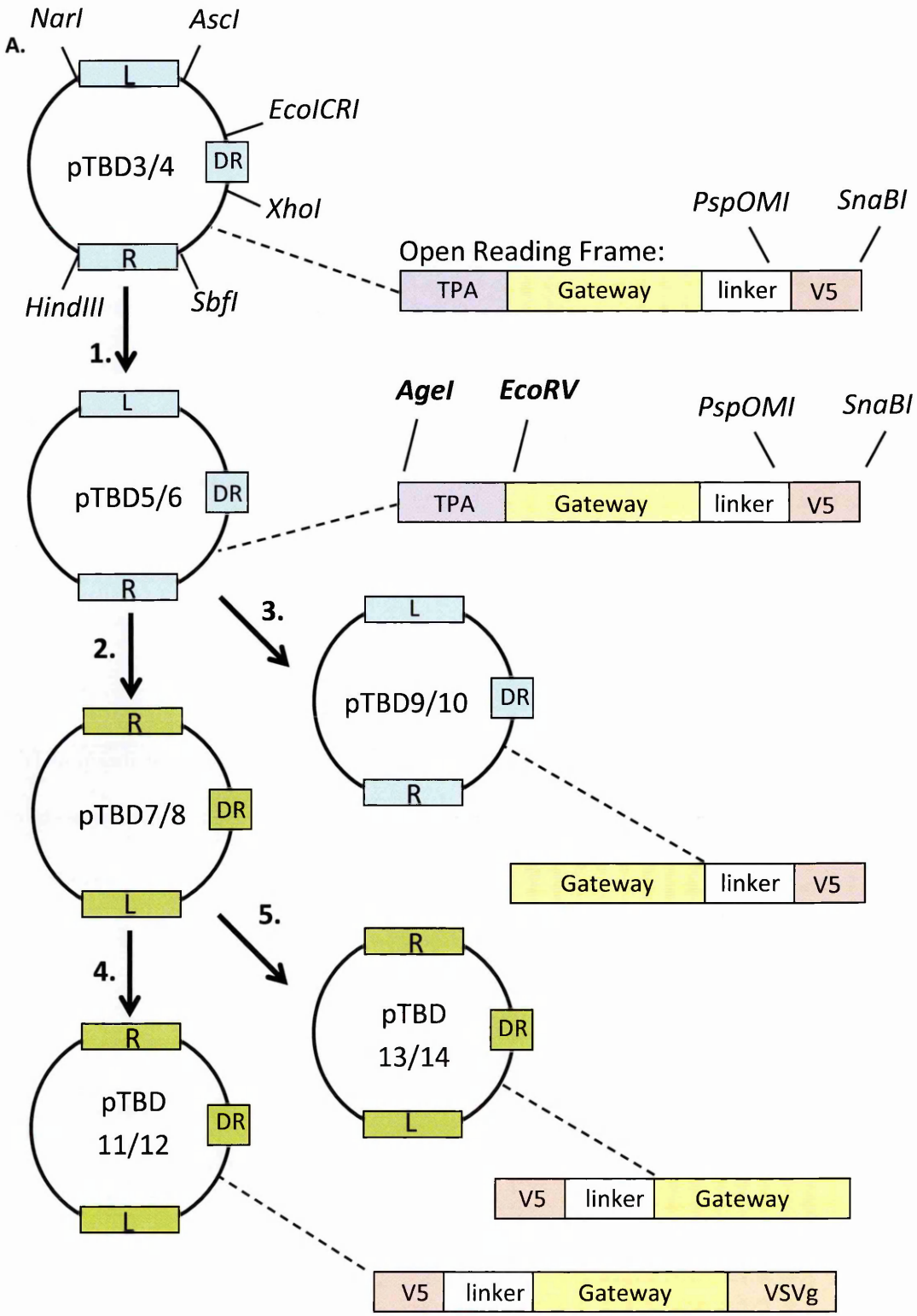


Figure 3.2 Cloning steps for construction of required transfer plasmids. **1.** Unique restriction sites for removal of TPA sequence were introduced by site mutagenesis. **2.** Flanking sequences (L and R) and direct repeat (DR) for Del III (blue) were removed and replaced for sequences targeting Del II (green). **3.** TPA was removed. **4.** TPA and V5 were removed and replaced with V5+linker and VSVg, respectively. **5.** TPA and V5 were removed, and replaced with V5+linker and stop, respectively.

Conversion of pTBD3/4 to pTBD5/6

Unique restriction enzyme sites were introduced via site mutagenesis to enable excision and replacement of the existing N-terminal fusion (TPA). The mutations required were selected by examining the existing sequence against sequences for absent sites. In pTBD3 and pTBD4, the sequence prior to TPA was CCCGGT (A). The sequence after TPA was CCCATCAA (B). Of 39 possible unique recognition sites, 20 were immediately discounted on the grounds of being degenerate, i.e. more than one nucleotide possible at one or more positions (Table 3.4). For the remaining 19 sites, the number of base pair changes needed to convert the existing sequence to the restriction enzyme site was noted.

Introduction of only 1 base pair change permitted the sequence preceding the TPA leader to become the recognition site for restriction enzyme AgeI; site mutagenesis primers were designed accordingly (Section 2.1.5, Table 2.3).

A minimum of 3 base pairs were required to introduce a unique site downstream of TPA. Of 7 possible options, EcoRV was selected on the grounds that the base pairs required to be changed were adjacent. It was also noted that concomitant with the change, one and not two coding changes would be introduced; proline (CCC) to aspartic acid (GAT). Whilst the intervening sequence located between TPA and the gene of interest is not considered biologically significant, it is within the open reading frame. Here, the change of one amino acid is presumed not to have an impact on presentation of the encoded antigen. Site mutagenesis primers were designed accordingly and the required mutations introduced (Section 2.1.5, Table 2.3).

The mutations required to provide AgeI and EcoRV restriction sites were introduced sequentially. Clones were investigated for introduction of the required mutation via analytical restriction digest (Section 2.1.6) with the corresponding enzyme. Plasmid DNA isolated from putative clones was subjected to sequencing across the manipulated area to confirm that pTBD5 and pTBD6 had been successfully generated.

Table 3.4 Restriction enzyme sites absent from pTBD3 and pTBD4. Sequences for absent sites were compared to the DNA sequence before (A) and after (B) the TPA leader. N denotes any base. The number of base pair changes required to introduce the site was recorded. The individual base pairs required to be mutated are shown in bold in square brackets. Some of the changes introduced one* or two**coding changes.

Absent sites	Sequence	Conversion of (A) CCCGGT	Conversion of (B) CCCATCAA
AarI	CACCTGCNNNN/NNNN	-	-
AfeI	AGC/GCT	3	>3
AgeI	A/CCGGT	1 [**C**CCGGT]	>3
AjuI	NNNNNNNNNNNNGAANNNNNNNTTGG	-	-
AjuI'	GAANNNNNNNTTGGNNNNNNNNNNN	-	-
AloI	NNNNNNNNNNNNGAACNNNNNNTCC	-	-
AsiSI	GCGAT/CGC	>3	> 3
AvrII	C/CTAGG	3	> 3
BlpI	GC/TNAGC	-	-
BmtI	GCTAG/C	>3	3 [**C**CC**AT**CAA]**
BpiI	NNNNNNNNNNNNNGAGNNNNNCTC	-	-
BpiI'	GAGNNNNNCTCNNNNNNNNNNNNN	-	-
BsiWI	C/GTACG	>3	> 3
BstEII	GGTNACC	-	-
Bsu36I	CCTNAGG	-	-
CspCI	NNNNNNNNNNNNNCAANNNNNGTGG	-	-
CspCI'	CAANNNNNGTGGNNNNNNNNNNNN	-	-
DraIII	CACNNNGTG	-	-
EcoRV	GAT/ATC	>3	3 [**CCC**ATC]*
FseI	GGCCGG/CC	>3	>3
HpaI	GTT/AAC	>3	>3
NaeI	GCC/GGC	2	3 [**C**CC**AT**CAA]**
NgoMIV	G/CCGGC	2	3 [**C**CC**AT**CAA]**
NheI	G/CTAGC	>3	3 [**C**CC**AT**CAA]**
NruI	TCG/CGA	>3	>3
PacI	TTAAT/TAA	>3	>3
PmeI	GTTT/AAAC	>3	>3
PmlI	CACGTG	3	3 [**C**CC**AT**CAA}**
PpuMI	RGGWCCY	-	-
PshA1	GACNNNNGTC	-	-
PsiI	TTA/TAA	>3	3 [**C**CC**AT**CAA]*
RsrII	CG/GWCCG	-	-
SanDI	GG/GWCCC	-	-
SexAI	A/CCWGGT	-	-
SfiI	GGCCNNNN/NGGCC	-	-
SgrAI	CR/CCGGYG	-	-
StuI	AGG/CCT	>3	>3
SwaI	ATTT/AAAT	>3	>3
Tth111I	GACN/NNGTC	-	-

Conversion of pTBD5/6 to pTBD7/8

A panel of transfer plasmids targeting an alternative site was constructed with a view to introducing inserts at two sites in the MVA genome. The MVA homologous sequences for Del III, L, DR and then R, were replaced sequentially via restriction digest and ligation. Fragments homologous to Del II were obtained by PCR using primers designed to incorporate restriction sites (Section 2.1.8, Table 2.4). During *in silico* preparation of plasmid maps, sequences for Del II L and Del II R were observed to contain AgeI and HindIII, respectively. In order to preserve the uniqueness of these sites within the system, for use in this or future studies, they were removed by overlap extension (OE) PCR (Section 2.1.9) prior to ligation.

For transfer plasmids targeting insertion site Del II, left and right homologous flanks were introduced in the reverse orientation to those targeting Del III (Figure 3.2, Step 2). This was to ensure that the recombinant cassette would be inserted into the MVA genome in the opposite orientation and thereby the same orientation as adjacent open reading frames; a factor potentially linked to the efficiency of transcription (Panicali and Paoletti, 1982). The design, i.e. length of the Del II homologous DNA fragments, matched that of an existing plasmid (pLAS-2), with proven history for insertion of recombinant cassettes at this site in the MVA genome (Earl *et al.*, 2009).

As transfer plasmids pTBD5 and 6 were modified to become pTBD7 and 8, clones were assessed via analytical restriction digest at each stage. Following restriction digest, DNA fragments were separated by agarose gel electrophoresis (Section 2.1.7) to determine the number of fragments and their sizes. An example representing the final manipulation for construction of pTBD7 is shown in Figure 3.3. Sequence data complete for the modified regions confirmed that correct clones were progressed.

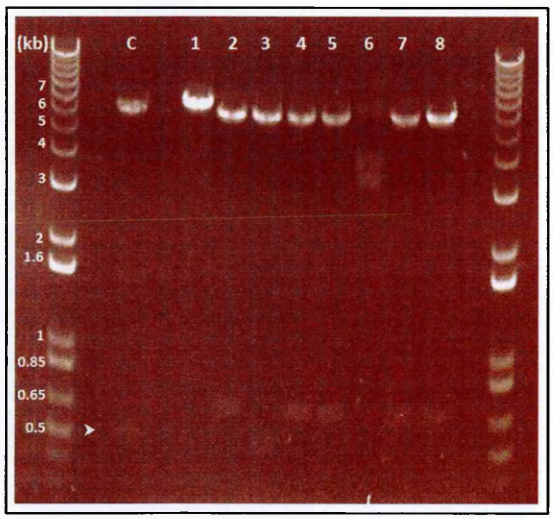


Figure 3.3 Analytical digest of pTBD7 with SbfI and HindIII. Purified plasmid DNA was subject to restriction enzyme digest following insertion of the final Del II homologous sequence: Del II L in place of Del III R. The plasmid, prior to modification, was digested as a control (C) and yielded the expected fragments: 6.3 kb and 522 bp (arrow head). Of 8 clones assessed, 5 appeared to be correct yielding fragments of 6.3 kb and 695 bp: # 2, 4, 5, 7 and 8. Plasmid #2 was selected for sequencing and was progressed to the next stage.

Conversion of pTBD5/6 to pTBD9/10

Transfer plasmids containing antigen with no N-terminal fusion were required for the purpose of creating rMVA 'no-leader' control strains. Plasmids pTBD9 and 10 were created from parent plasmids pTBD5 and 6, respectively, by removal of the existing N-terminal Kozak-TPA sequence, via AgeI/EcoRV digest, followed by ligation of annealed oligonucleotide containing the Kozak sequence only (Figure 3.2 Step 3). Forward and reverse oligonucleotides were designed for ligation directly after annealing, i.e. with ends complementary to the digested plasmid (Section 2.1.10, Table 2.5). Putative clones for pTBD10 were identified by analytical restriction digest; one was selected for sequencing across the modified region and confirmed to be correct. Conversely, putative clones of pTBD9 failed to be identified; the plasmids were variable in size and all were smaller than expected (Figure 3.4).

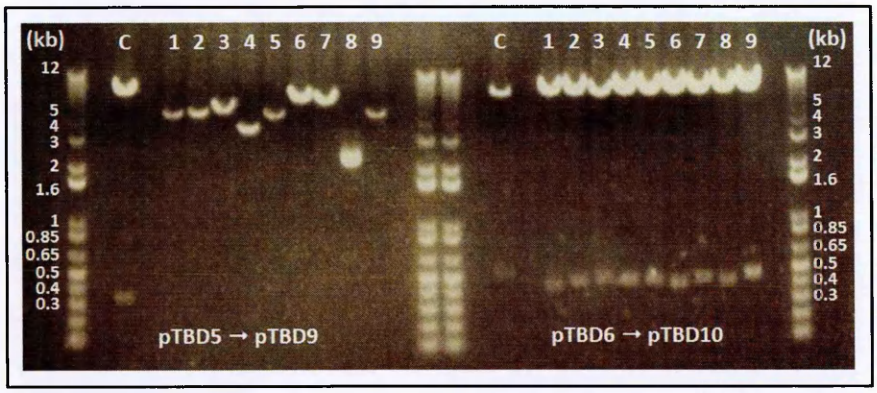


Figure 3.4 Analytical digest of putative pTBD9 and 10 clones with XhoI and NotI. Purified plasmid DNA was subject to restriction enzyme digest. Parent plasmid was digested as a control (C) and in each case yielded the expected fragments: 6.9 kb and 350 bp (pTBD5) or 6.9 kb and 528 bp (pTBD6). Following removal of the TPA sequence, a reduction of 100 bp in the smaller fragment was expected for each plasmid. Putative pTBD10 clone #1 was sent for sequencing and confirmed to be correct.

Repeat attempts to generate pTBD9 produced similar results to those shown in Figure 3.4; attempts to resolve the issue by re-cutting pTBD5, and/or by varying the vector:insert ratio in the ligation reaction (Section 2.1.11), were unsuccessful. Throughout, cut pTBD5 was examined by gel electrophoresis to ensure that the digest was complete and yielding two fragments of the expected size. Annealed oligonucleotides were not re-prepared on account of successful ligation into cut pTBD6 to generate pTBD10.

The characteristics of plasmids pTBD5 and pTBD6 were re-assessed *in silico* and found to be identical in every regard except for the vaccinia promoter included for transgene expression. Thus, successful conversion of pTBD6 to pTBD10 provided evidence for no intrinsic flaw in the design of the cloning step, i.e. AgeI/EcoRV vector digest followed by ligation of MTB111/111r annealed oligonucleotides was proven to work efficiently. To further investigate the reduction in plasmid size observed for pTBD5, one clone (Figure 3.4, #6) was selected for analysis via sequencing (Section 2.1.12). Results revealed that the Kozak-only oligonucleotide had been inserted, but was inserted twice, in two orientations (back-to-back) and that 1.7 kb of the Gateway® cassette was missing: the N-terminal

attachment site (*att*R1), the chloramphenicol resistance gene (CmR) and a portion of the second Gateway® selection marker (ccdB). A schematic of the Gateway® cassette and of the deleted region is shown in Figure 3.5.

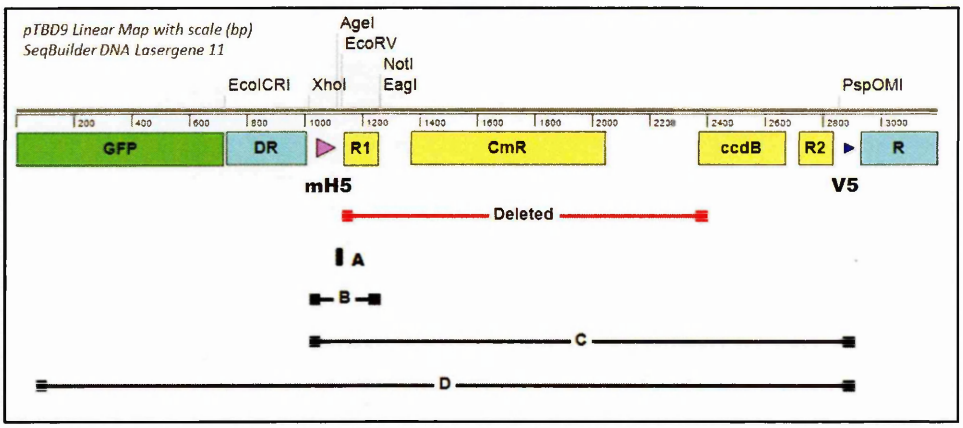


Figure 3.5. Schematic of a portion of plasmid pTBD9. The TPA sequence in pTBD5 was excised by AgeI/EcoRv digest, which would be reinstated upon ligation of a 17 bp oligonucleotide (A). Ligation of fragment A led to a section of the Gateway® cassette (yellow features) being deleted (red bar). Increasingly larger inserts were generated: B (252 bp), C (1900 bp) and D (2850 bp), for insertion into the vector employing restriction enzyme sites: XhoI/Not, XhoI/PspOMI and Eco53KI*/PspOMI, respectively. The features of pTBD9 shown are: selection marker (GFP); direct repeat sequence (DR); mH5 promoter (pink triangle), Gateway cassette recombination sites (R1, R2); chloramphenicol resistance gene (CmR); lethal gene (ccdB); B cell epitope tag V5 (blue triangle); right MVA homologous flank (R). *EcoICRI isoschizomer

It was speculated that other clones, for reasons unknown, may have undergone similar or more extensive deletions. To circumvent the problem, repeat digest/ligations were performed using increasingly longer inserts. In the first attempt, the original oligonucleotide (Figure 3.5, A) was replaced with a longer, custom-synthesised fragment incorporating mH5 and *att*R1 (Figure 3.5, B) with restriction sites XhoI and NotI replacing AgeI and EcoRV, respectively. Following transformation of *E. coli* with ligated DNA, putative clones were screened via analytical digest. The results appeared to confirm successful removal of TPA and ligation of insert B. However, the plasmids had again undergone a

deletion, this time incurring a greater reduction in size: approximately 3 kb (Figure 3.6). An attempt to interrogate the nature of the deletion by sequencing (clone #2) was unsuccessful, presumably on account of primer binding sites no longer being present in the template.

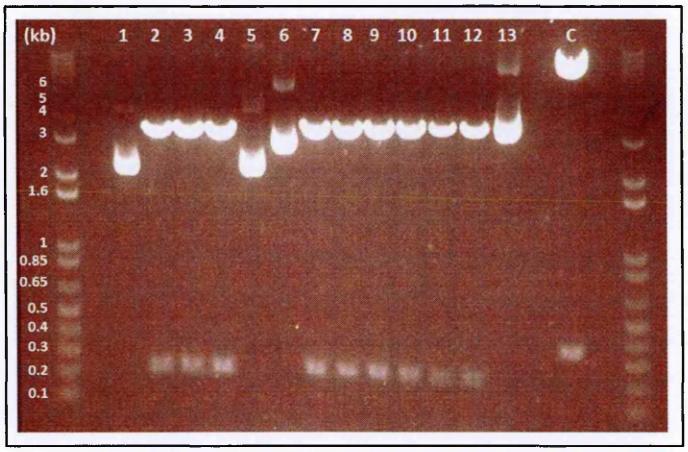


Figure 3.6 Analytical digest of putative pTBD9 clones with XhoI and NotI. Purified plasmid DNA was subject to restriction enzyme digest. Parent plasmid (pTBD5) was digested as a control (C) and yielded the expected fragments: 6.9 kb and 350 bp. Removal of TPA appeared successful for clones 2 - 4 and 7 – 12; a reduction of 100 bp in the smaller fragment was observed. However, the plasmid backbone, which was expected to be the size same as C (6.9 kb), was much reduced.

Two larger inserts were subsequently created. As described in Section 2.1.13 (Figure 2.2), inserts C and D were generated by overlap extension PCR and then insert and parent vector (pTBD5) subjected to the same double or sequential restriction digest. The unique restriction sites employed for inserts C and D were XhoI/PspOMI and Eco53kI/PspOMI, respectively. Both inserts were made with existing primers and so were longer than required; both incurred a reduction in size upon restriction enzyme digestion. Ligation of cut vector with fragments C and D again resulted in a deletion. The apparent reduction in plasmid size was approximately 1 kb, which was less than had been observed with insert B.

Upon sequencing, data were obtained and alignment revealed that a portion of the Gateway® cassette had again been lost.

Until this point, propagation of the Gateway® destination vectors had been in medium supplemented with carbenicillin, as the addition of chloramphenicol had been observed to impede growth. In order to preserve selection pressure on the Gateway® cassette, *E. coli* (TOP10) were transformed with ligation reaction containing inserts A, B C or D and plated on LB supplemented with both antibiotics. After 18 hours incubation, no colonies were observed for A, B and C and only three colonies for D. Each was picked and propagated in LB medium containing carbenicillin and chloramphenicol. Plasmid DNA was subsequently extracted and subjected to two analytical restriction digest reactions: BamHI for excision of CmR to ensure it was present and XhoI/EagI for interrogation of the N-terminal alteration (Figure 3.7). Two putative clones were identified and, on sequencing across the modified region, one was correct and suitable for further use.

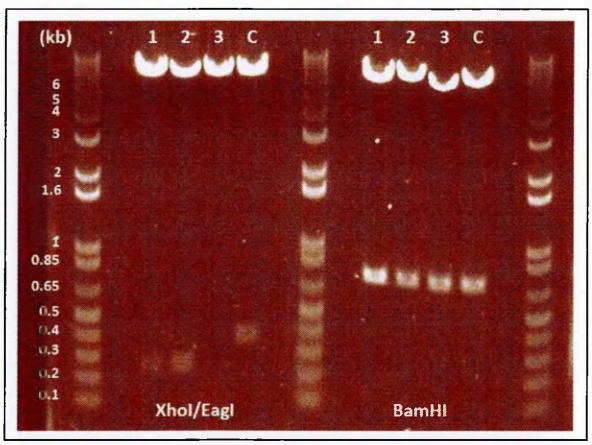


Figure 3.7 Analytical digest of putative pTBD9 clones. Parent plasmid (pTBD5) was included as a control (C). Following restriction enzyme digest with BamHI, fragments of the expected size (6.5 kb (C)/6.4 kb and 702 bp) were observed for all digests with the possible exception of #3, the backbone of which was potentially reduced. Interrogation of the N-terminal modification, for successful removal of the TPA leader showed the expected result for the parent plasmid (6.9 kb and 350 bp) and for clones 1 and 2 (6.9 kb and 245 bp), but not #3.

Conversion of pTBD7/8 to pTB11/12 and pTBD13/14

As summarised in Figure 3.2 (steps 4 and 5), plasmids pTBD7 and 8 were digested with PspOMI and SbfI to remove the existing V5 C-terminal tag. Oligonucleotides providing the required C-terminal modification (MTB113/133r and MTB114/144r, Section 2.1.10, Table 2.5) were annealed and ligated to create pTBD11/12 and pTBD13/14 intermediates, respectively. Conversion to pTBD11/12 and pTBD13/14 was completed by excision of the existing TPA leader via digestion with AgeI and EcoRV, and ligation of annealed oligonucleotides MTB112/112r for introduction of an N-terminal V5 B-cell epitope tag. This identical N-terminal modification, which might have been performed on pTBD7/8 in the first instance, was required to be performed after C-terminal changes and not before on account of deliberate re-introduction of PspOMI – a restriction site exploited for the C-terminal modification. Regardless of the vaccinia promoter present, none of the issues observed with pTBD5 were seen to manifest themselves when modifying the Kozak region: all four plasmids were successfully altered on the first attempt. As above, analytical digest was used to identify putative clones at each step, with sequencing across the modified regions used to confirm that the required changes had been made.

3.3.2 Completion of transfer plasmid construction with insertion of the gene of interest

The ovalbumin gene (OVA) was created by splice overlap extension PCR (Section 2.1.14) and amplified with attB primers. A clean band of the expected size was observed upon separation by gel electrophoresis. Purified PCR product was transferred to pDONRzeo by BP reaction to create pENTR-OVA (Section 2.1.4). All four clones investigated following transformation, carried pENTR-OVA. Sequencing data revealed three out of four clones to contain point mutations within the open reading frame and the fourth to be suitable for progression to LR reaction. Following LR recombination of OVA into each pTBD vector, putative clones were identified by analytical digest and verified by sequencing. In each case, sequence alignment for OVA and the entire open reading frame, inclusive of N and C

terminal fusions was obtained. Plasmid maps, annotated to show the sequenced region, are presented in Appendix I.

3.4 Discussion

In this Chapter, novel transfer plasmids had to be constructed. Specifically, a panel of transfer plasmids with which to generate rMVA expressing OVA for an investigation into the impact of leader sequences on immunological potency. The approach selected was one of modifying existing transfer plasmids that were Gateway® Destination vectors. An additional objective, to be achieved in parallel, was to create a panel of transfer vectors that would be amenable to further alteration, including insertion of alternative antigens.

3.4.1 Alteration of existing vectors via conventional cloning techniques

Conventional cloning techniques such as PCR, restriction enzyme digest and enzyme-mediated ligation were applied to modify the existing transfer plasmids. In the first instance, a plan for making the desired alterations was devised *in silico.* The primary objective for the cloning strategy was to construct the required plasmids as efficiently as possible. Secondly, they were to remain suitable for further alteration, in case additional modification should be required within the scope of this study or in future work.

As described in Section 3.3.1, it was possible to devise and implement such a plan by making use of existing unique restriction enzyme sites, introducing new unique sites where necessary, and preserving them where they might otherwise be removed. This endeavour was assisted by the pre-existence of unique sites around the majority of the regions of interest, e.g. the homologous flanks and direct repeat sequences. This was presumably as a consequence of the provenance of the plasmids; pTBD3 and pTBD4 were derived from pLAS-1, which was originally constructed using restriction digest and ligation techniques (Wyatt *et al.*, 2008b).

Generally, the application of conventional cloning techniques proved an efficient means of completing the desired alterations; the majority of restriction digest and ligation steps were completed successfully, within one attempt and the screening of 8 - 10 putative clones. However, this was not the case in one instance - the conversion of pTBD5 to pTBD9. For this alteration, many repeat attempts and ultimately a change in cloning strategy were necessary to generate the desired vector.

The reason for the difficulty experienced with pTBD9 was not immediately obvious. The planned modification involved removal of the N-terminal TPA leader via AgeI/EcoRV restriction digest followed by ligation of annealed oligonucleotides MTB111/MTB111R. Evidence for the reliability of this step was provided by pTBD10, which was generated from pTBD6 by exactly the same means and in parallel with pTBD5/9 which failed. Furthermore, four other plasmids (pTBD11-14) were successfully modified in the same region by the same restriction digest, albeit followed by ligation with different oligonucleotides (Figure 3.2, steps 4 and 5).

At each repeat attempt of the ligation, cut pTBD5 was examined by agarose gel electrophoresis to ensure that the digest was complete and yielding fragments of the expected size. No abnormalities were found and so no explanation for the truncated products observed after ligation was provided. Scrutiny was applied to the pTBD5 vector. All of the available sequence data relating to the plasmid (laboratory files and sequencing performed in this project) were re-aligned against the *in silico* map to ensure that it was as expected. Indeed, there was alignment with no mismatched base pairs, thus, confirming that the restriction enzyme sites employed were rightly expected to be unique.

The characteristics of plasmid pTBD5 were compared with those of the plasmids which had been successfully modified. Plasmid pTBD5 contains the vaccinia promoter mH5 for expression of the transgene upstream of the region incurring a deletion, whereas plasmid pTBD6 contains the P7.5 promoter. Like pTBD5, plasmids pTBD11 and 13 contain the mH5

promoter for transgene expression; however, they both differ by having an adjacent direct repeat sequence homologous to MVA insertion site Del II. Thought was given to whether the combination of the Del III direct repeat combined with the mH5 sequence might be making the pTBD5 plasmid unique in an unfavourable way; there is evidence for some DNA sequences being prone to adverse rearrangements, through homology-dependent and independent mechanisms (Bzymek and Lovett, 2001; Conley *et al.*, 1986). Further work, beyond the scope of this study, would have been required to determine if something of this nature was the root cause. To circumvent the difficulties encountered, inserts of increasing size were generated which served to move an increasing number of features from the vector to the insert (Figure 3.5, fragment D). This approach was successful, although the number of putative clones remained low.

3.4.2 Alteration of Gateway® Destination Vectors and insertion of OVA

A contributing factor to the difficulty observed in converting pTBD5 to pTBD9 may have been failure to maintain selection pressure for both of the antibiotic resistance markers present; ampicillin resistance on the plasmid backbone and chloramphenicol resistance in the Gateway® cassette. Ultimately, a correct clone was obtained using both antibiotics, i.e. carbenicillin (ampicillin analogue) and chloramphenicol. Of note, the use of carbenicillin alone did not impact upon the success of any of the other cloning steps. Further, use of carbenicillin alone was deemed preferable; inclusion of both antibiotics was observed to impede cell growth (reduced turbidity after overnight culture) and, therefore, the ability to progress the cloning steps expediently.

After insertion of the ovalbumin gene into a Gateway® Entry vector, the antigen was transferred to each of the Destination vectors. The plasmids were then sequenced to confirm integrity of the entire open reading frame (ORF). This was considered an essential prerequisite to the use of the plasmids in the generation of rMVA. Transfer of the recombinant cassette would be monitored by GFP expression, and also via PCR of the MVA

genome across the insertion site, but expression of OVA was dependent upon insertion of the correct sequence in the correct reading frame. In addition, to the ORF sequence data, many other regions of each plasmid were sequenced in the course of confirming that modification has been made successfully. Long term stocks of transformed *E. coli* were deposited for each plasmid, both before and after OVA insertion. The extent of the sequenced regions for the OVA panel of plasmids is shown in Appendix I.

3.4.3 Suitability of the transfer plasmid panel for future work

The transfer plasmids generated are Gateway® Destination vectors and so can be used for the insertion of alternative antigens. The panel offers the ability to append a TPA or VSVg leader sequence, or no leader at all. For each type of plasmid, there is the opportunity to alter the level of antigen expression by employing either P7.5 or mH5 for transgene transcription. All of the plasmids contain a V5 tag in the expression cassette for immunodetection of antigens for which antibody is currently unavailable. If no leader sequence and no V5 tag were required, inclusion of an in-frame stop at the end of the antigen sequence would terminate translation so that no tag was expressed. In addition, unique restriction enzyme sites were retained around regions of interest so that each plasmid would lend itself favourably to further alteration. The position of these unique sites is illustrated in Appendix I.

3.4.4 Alternative approaches to the selected cloning strategy

Gateway® cloning is expedient by virtue of its selection mechanism (Section 2.1.4, Figure 2.1). Conventional cloning, meanwhile, can be time consuming as was the case for the conversion of pTBD5 to pTBD9. An alternative approach might have been to employ custom synthesis of DNA fragments. The current study utilised custom oligonucleotides from 20 – 40 bp (primers) up to 500 bp (for the construction of OVA). During the course of the study, the maximum length of custom-made DNA fragments increased from 500 bp to 2

kb (Integrated DNA Technologies). In future work, greater advantage could be made of the longer oligonucleotides now available.

Custom plasmid synthesis was also a possible alternative, but as with DNA fragments of increasing size, was proportionally more expensive. Ultimately, the majority of the cloning steps progressed efficiently, without delay and at relatively low cost. Thus, the value of being able to alter plasmid features via conventional methods was demonstrated. Another attribute of custom synthesis would have been the ability to generate sequences exactly as desired with no extraneous material or coding changes (applicable where alterations fall within the open reading frame). For the work undertaken in the current study this was not anticipated to have been a compromising factor. However, future work involving multiple changes to a plasmid should use custom synthesis as far as is economically possible.

3.5 Conclusions

Conventional cloning was successfully applied to generate the transfer plasmids required. Subsequently, they were used to generate novel rMVA viruses.

Chapter 4 Generation of novel recombinant MVA viruses

4.1 Introduction

Two panels of rMVA viruses were needed to test the study hypotheses (described in Section 1.5); one for an investigation into the impact of vaccinia promoter selection, the other for an investigation into the influence of leader sequences on transgene immunogenicity. The rMVA virus-panels designed to test each hypothesis and the transfer plasmids required to make them are summarised in Table 4.1. As shown, one virus was already available.

Table 4.1 Summary of the rMVA viruses employed to test each hypothesis. Some of the transfer plasmids (*) were already available. The remainder were constructed within the current study (described in Chapter 3). For the leader sequences study, the set of transfer plasmids incorporating mH5 was selected for creating rMVA. This and the precise design of the inserts used to test each hypothesis are discussed further in Chapters 5 and 6, as indicated.

Recombinant MVA virus	Transfer Plasmid	rMVA source
Vaccinia promoter study (Chapter 5)		
MVA-mH5-Ag85A	*pTBD1-Ag85A	Laboratory stock
MVA-P7.5-Ag85A	*pTBD2-Ag85A	This study
MVA-mH5-Rv0111	*pTBD1-Rv0111	This study
MVA-P7.5-Rv0111	*pTBD2-Rv0111	This study
Leader sequences study (Chapter 6)		
MVA-OVA$_{TPA}$	pTBD5-OVA	This study
MVA-OVA	pTBD9-OVA	This study
MVA-OVA$_{VSVg}$	pTBD11-OVA	This study
MVA-OVA	pTBD13-OVA	This study
MVA-OVA$_{TPA}$/OVA$_{VSVg}$	pTBD5-OVA & pTBD11-OVA	This study
MVA-OVA/OVA	pTBD9-OVA & pTBD13-OVA	This study

4.1.1 Transfer plasmid methodology and plaque purification

Transfer plasmid methodology was selected as the approach for inserting recombinant cassettes into the MVA genome (see Section 3.1). All of the recombinant cassettes contained the GFP gene for use as a selection marker to identify and isolate rMVA plaques. Plaque purification is an established methodology for isolating recombinant viral progeny away from the parent virus (Earl *et al.*, 2001b; Kremer *et al.*, 2012). Following infection of BHK-21 cells with the parent MVA strain and transfection with the appropriate transfer plasmid, cells are incubated for 2-3 days and then the cells and viruses harvested. Virus particles are released from the infected cells by freeze-thaw cycles, and dissociated from the cellular material by vortexing and sonication. Sequential rounds of plaque purification are then performed on CEF (or BHK-21) monolayers. At each round, the cells are cultured under a semi-solid overlay to promote the development of discrete plaques. Recombinant viral progeny are made distinguishable from the parent virus via the use of a selection marker, e.g. GFP, so that they can be physically picked away from the cell monolayer. Where possible, this is done at a distance from areas observed to exhibit cytopathic effects (CPE), but no selection marker expression, so as to reduce the likelihood of co-isolating the parent strain, although, in the earlier rounds of plaque purification this is difficult to achieve.

4.1.2 Generation of markerless rMVA

Recombinant MVA can be generated for stable or transient expression of the selection marker (see Section 1.3.1). Transient expression is achieved by inclusion of an identical DNA sequence either side of the selection marker in the recombinant cassette, the presence of which leads to an internal recombination event through which the marker is 'self-excised' (Wyatt *et al.*, 2008b). To generate a stock of markerless rMVA, plaque purification is carried out in the same way as it is for stable marker expression, i.e. until no parent virus remains, at which point the viral population comprises both marker-positive

and marker-negative lineages. Further passages are then performed until only the markerless population remains.

In the current study, stable GFP expression was selected for the viruses needed to investigate vaccinia promoter selection. For the leader sequences study, transient GFP expression was selected to facilitate the creation of double-recombinant viruses.

4.1.3 Characterisation of novel rMVA and preparation of viral vaccine stocks

Comprehensive methods for preparing and characterising rMVA are available (Earl *et al.*, 2001a; Earl and Moss, 2001; Earl *et al.*, 2001b; Kremer *et al.*, 2012) and relevant aspects are summarised here.

Stocks of rMVA must be characterised to ensure purity of the viral population and stability of the intended insert. This is routinely achieved using analytical PCR; MVA genomic DNA (gDNA) is extracted and amplified across the insertion site, using primers binding to the flanking regions. A single amplicon of the expected size for the recombinant cassette serves to confirm a homogenous population of rMVA. Smaller products, dependent upon their exact size, are indicative of truncated inserts and/or the presence of native MVA.

Faithful expression of the transgene can be confirmed by immunostaining of the cell monolayer with transgene-specific antibodies. An alternative means of confirming transgene expression is to subject infected cell lysate to Western blotting (separation of protein by SDS PAGE followed by transfer to protein-blotting membrane for immunodetection).

Prior to immunisation studies, amplified stocks of rMVA must be separated from the host cell lysate because an unpurified preparation will contain recombinant antigen generated in the course of viral replication. Combination-administration of virally vectored antigen with recombinant antigen enhances antigen-specific immune responses (Hutchings *et al.*, 2007). This is a valid strategy for enhancing the immunological potency of rMVA-based vaccines,

but to make an interpretable assessment of rMVA immunogenicity alone, extraneous recombinant antigen must be removed by density centrifugation. There are established protocols for purifying vaccinia stocks by pelleting through a layer of sucrose solution.

Before a purified stock of rMVA can be evaluated at a specified concentration, the stock must be titrated to enumerate the number of plaque forming units per millilitre (PFU/ml): serial dilutions of the stock are plated on permissive cells, under a solid overlay in multi-well plates. After 2-3 days incubation, plaques are counted and PFU/ml calculated according to the dilution factor. An alternative method for quantifying MVA viral particles is to titrate for determination of tissue culture infectious dose 50 ($TCID_{50}$). This is the concentration of virus required to kill 50% of cells. This method is more labour intensive as a greater number of counts must be performed to accurately determine the proportion of cells infected over several dilutions. With either method, plaques must first be made visible for counting as MVA does not form lytic plaques. This may be achieved by virtue of the selection marker, or can otherwise be accomplished by immunostaining of the cell monolayer using anti-vaccinia, or anti-transgene antibodies.

It is only following satisfactory preparation and characterisation of rMVA viruses that their relative attributes can be fairly evaluated and compared. Accordingly, aims for this Chapter were as follows.

4.2 Chapter Aims

To assess the impact of vaccinia promoter strength on the immunogenicity and protective efficacy of rMVA vectoring M. tuberculosis antigens (described in Chapter 5):

- Create and amplify stocks of the rMVA viruses listed in Table 4.1, each derived from wild-type MVA and stably expressing GFP.
- Characterise stability and purity via PCR.
- Confirm antigen expression via Western blot.
- Generate purified stocks of known titre.

To assess the influence of leader sequences on transgene immunogenicity, including their combined effect in a multiple recombinant (described in Chapter6):

- Create and amplify markerless stocks of the rMVA viruses listed in Table 4.1, each derived from wild-type MVA or from a single rMVA virus expressing OVA.
- Characterise stability and purity via PCR for one (single rMVA) or both (double rMVA) inserts.
- Confirm antigen expression via Western blot.
- Generate purified stocks of known titre.

4.3 Results

Not all of the rMVA required for the study were amenable to plaque purification. The outcome for each virus is summarised in Table 4.2.

Table 4.2 Generation of rMVA viruses for the current study. For those that were insufficiently stable, a purified viral stock could not be prepared.

Recombinant MVA virus	Transfer Plasmid	Outcome
Vaccinia promoter study		
MVA-mH5-Ag85A	pTBD1-Ag85A	Laboratory stock
MVA-P7.5-Ag85A	pTBD2-Ag85A	Purified
MVA-mH5-Rv0111	pTBD1-Rv0111	Unstable
MVA-P7.5-Rv0111	pTBD2-Rv0111	Purified
Leader sequences study		
MVA-OVA_{TPA}	pTBD5-OVA	Purified
MVA-OVA	pTBD9-OVA	Purified
MVA-OVA_{VSVg}	pTBD11-OVA	Purified
MVA-OVA	pTBD13-OVA	Purified
MVA-OVA_{TPA}/OVA_{VSVg}	pTBD5-OVA & pTBD11-OVA	Unstable
MVA-OVA/OVA	pTBD9-OVA & pTBD13-OVA	Unstable

For those rMVA that did appear to be stable, scrutiny was applied to the integrity of the recombinant insert and the ability of the rMVA to express the transgene. Where viruses could not be purified this was further examined, as far as practicable, to understand the root cause and to determine next steps.

4.3.1 Generation of novel rMVA expressing either Ag85A or Rv0111

The novel rMVA viruses required for the vaccinia promoter study (Table 4.1) were created using transfer plasmids as described in Section 2.2. To characterise the viruses generated, virus samples were amplified and examined via PCR and Western blot, as described in Section 2.3.

Recombinant MVA expressing antigen under the control of P7.5

Recombinant MVA viruses expressing Ag85A or Rv0111 under the control of the weaker, P7.5 promoter were both readily purified: both MVA-P7.5-Ag85A and MVA-P7.5-Rv0111 were isolated away from wild-type MVA after three rounds of plaque purification. Representative results for analytical PCR and Western blot are shown in Figure 4.1.

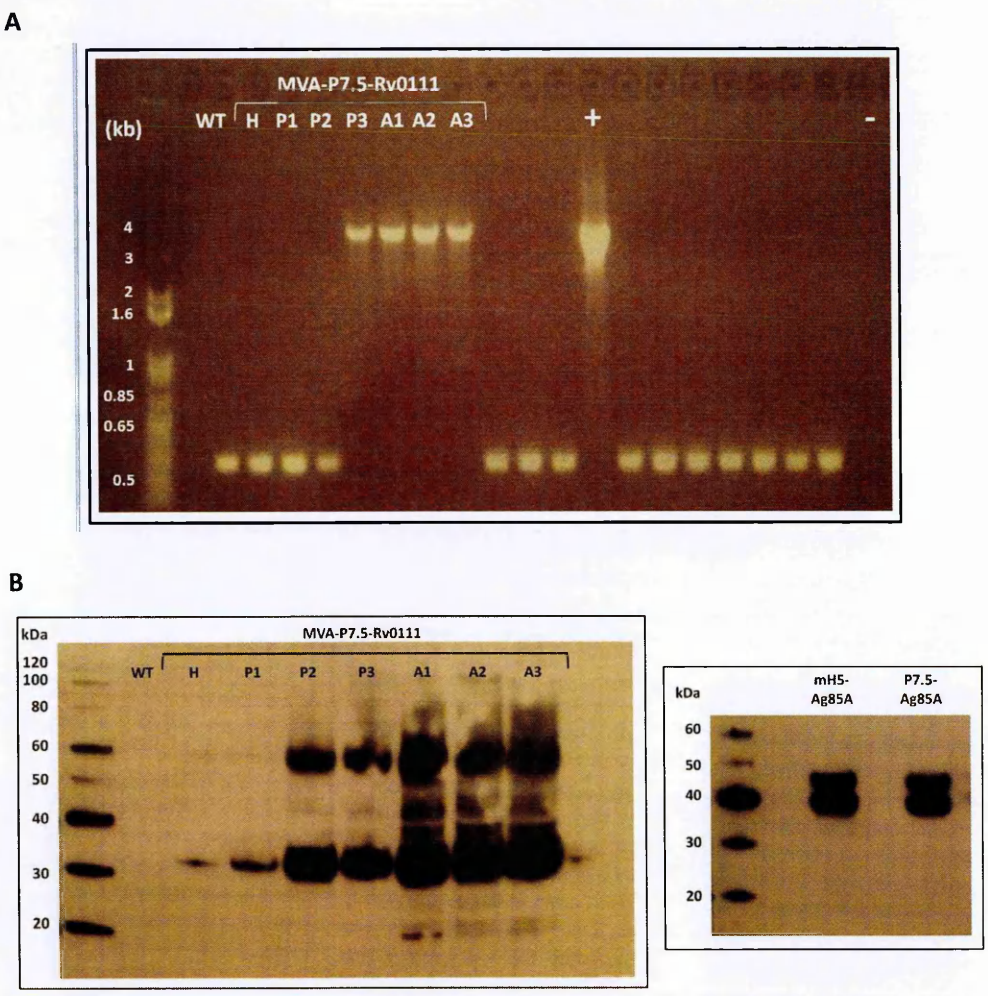


Figure 4.1 *In vitro* characterisation of novel recombinant MVA. A) Wild-type MVA (WT) and MVA-P7.5-Rv0111 virus samples were amplified on CEF cells and gDNA extracted for analytical PCR of the insertion site. PCR products were subsequently separated by gel electrophoresis. Transfer plasmid DNA was used as template in the positive control (+) reaction and yielded a product of the expected size. Water was used in place of DNA in the negative control (-) reaction and did not produce an amplicon. B) Infected CEF cell lysate was separated by SDS PAGE and then transferred via Western Blot to nitrocellulose for immunodetection with anti-V5.

The amplicons generated after PCR of the insertion site provided evidence for the presence of either wild-type MVA (549 bp) or rMVA (MVA-P7.5-Rv0111; 3901 bp). Results for MVA-P7.5-Ag85A are not shown. The failure of the PCR to yield two products, indicative of a mixed population, was speculated not to occur, or to occur rarely, because of preferential, more efficient amplification of the smaller target. Each rMVA virus was considered to be pure when wild-type MVA was no longer detectable.

For both MVA-P7.5-Ag85A and MVA-P7.5-Rv0111, evidence for antigen expression was provided by Western blot; the presence of anti-V5 immunoreactive bands demonstrated that the C-terminal V5 tag had been expressed (Figure 4.1B). In the case of MVA-P7.5-Rv0111, the expected size of the product was 86.3 kDa. Within the cell lysate, a number of breakdown products were detected. The expected size of the V5-tagged expression product for MVA-P7.5-Ag85A was 44.5 kDa. A band of this size and a smaller product were seen. In both cases, the presence of anti-V5 immunoreactive bands confirmed that the recombinant antigen was being expressed as translation had occurred through to the C-terminus. No V5-immmunoreactive product was seen in CEF infected with wild-type MVA (Figure 4.1A). However, a non-specific band of approximately 70 kDa was observed if the ECL hyperfilm was exposed to the blot for longer time periods.

Having been successfully purified, MVA-P7.5-Ag85A and MVA-P7.5-Rv0111 were available for further amplification and titration (discussed below).

Recombinant MVA expressing antigen under the control of mH5

When rMVA viruses expressing Ag85A or Rv0111 under the control of the stronger mH5 promoter were generated, only the Ag85A construct was amenable to plaque purification: MVA-mH5-Ag85A was isolated from wild-type MVA after four passages (this information was obtained from laboratory records). Meanwhile, the MVA-mH5-Rv0111 virus remained unpurified after three attempts to perform the infection/transfection and subsequent plaque purification.

The apparent instability of MVA-mH5-Rv0111 was investigated further by amplifying all of the available infection/transfection harvests and plaque picks on CEF monolayers. After two days incubation, cell layers were examined for CPE and GFP expression and then harvested for characterisation by PCR and Western blot. A sample of wild-type MVA (WT-MVA) and samples relating to MVA-P7.5-Rv0111 (which had been successfully purified), were included in the experiment for comparison.

For each sample, the presence of CPE and GFP was arbitrarily scored to reflect the relative levels visible within the cell layer. After processing of cell lysate for determination of antigen expression, Western blots were scored similarly with results ranging from no V5-immunoreactive bands visible (-) to a strong V5-positive product (+++). In some cases a faint band may have been visible, but was difficult to discern above background (-/+). After extraction of MVA gDNA, two PCR reactions were performed and their products visualised after separation by gel electrophoresis. Primers for the 'L/R' PCR reaction bound to the left and right flanks of the Del III insertion site. The reaction was observed to yield either a small product, interpreted to confirm the presence of wild-type MVA (WT), or a large product observed to correspond to the size of the intended recombinant insert (Rec). As described above, the L/R PCR reaction did not yield two products, as might be expected for a mixed population of wild-type and recombinant virus. This was speculated to be because of preferential, more efficient amplification of the smaller wild-type target whenever it was present. Therefore, in order to investigate the presence of rMVA by PCR, a reaction employing primers annealing within the recombinant cassette was implemented; the forward primer annealed within the TPA leader sequence (N-terminal antigen fusion) and the reverse primer annealed within the V5 tag (C-terminal antigen fusion) (for a full description of the oligonucleotides, see Section 2.3.2, Table 2.6). All of the results obtained for WT-MVA, MVA-P7.5-Rv0111 and MVA-mH5-Rv0111 are summarised in Table 4.3.

Table 4.3 Characterisation of viral samples arising in failed attempts to generate MVA-mH5-Rv0111. After two days amplification on CEF cells, the relative levels of CPE and GFP expression were recorded. Cell lysates were harvested and processed for interrogation by PCR and Western blot. Samples relating to wild-type (WT) MVA (the parent strain) and MVA-P7.5-Rv0111 (P7.5) were analysed in parallel.

Sample	Promoter	Description	CPE	GFP	V5 WB	PCR L/R	PCR TPA/V5
1	N/A	WT-MVA	+++	-	-	WT	-
2	P7.5	I/T	+++	-	+	WT	+
3		P1	+++	+	+	WT	+
4		P2	+	++	++	WT	+
5		P3	+	+++	++	Rec	-
6		A1	++	++++	+++	Rec	++
7		A2	+++	++++	+++	Rec	++
8		A3	+++	++++	+++	Rec	+
9	mH5	I/T	+++	-	+	WT	+
10	(attempt 1)	P1	+++	+	-	WT	-
11	mH5	I/T	+++	-	+	WT	+
12	(attempt 2)	P1	++	+	-	WT	-
13		P2	+++	++	-	WT	-
14		P3	++	+++	-	WT	-
15	mH5	I/T	+++	-	+	WT	+
16	(attempt 3)	P1	+++	+	-/+	WT	-/+
17		P2	++	-	-	WT	-/+
18		P3	++	-	-	WT	-

Abbreviations: CPE, cytopathic effects; GFP, green fluorescent protein, V5 WB, anti-V5 Western blot; PCR, polymerase chain reaction; L/R, forward and reverse primers binding within the Left (L) and Right (R) flank; TPA/V5, forward and reverse primers binding within the TPA leader and V5 tag; I/T, infection/transfection sample, P1, 'plaque pick' sample & round of purification; A1, 'amplification' sample & round of amplification; WT, wild-type; Rec, recombinant.

The results observed for WT-MVA and MVA-P7.5-Rv0111 were as predicted. WT-MVA induced CPE, gave no GFP expression, was detectable as wild-type (WT) when the insertion site was amplified (L/R PCR) and gave a negative result when an attempt to amplify a

section of the recombinant insert was made (TPA/V5 PCR). MVA-P7.5-Rv0111 induced CPE to a level consistent with the amount of MVA expected to be present; a decreasing amount as the recombinant was isolated away from the parent strain and then an increasing amount as the purified recombinant was amplified. The level of GFP expression increased as the recombinant was isolated and amplified, as did V5 expression. Native MVA was detectable until the strain was purified, which took three rounds of plaque purification. There was evidence for the recombinant insert (TPA-V5) being present at all times except for the sample derived from the third plaque pick (P3). This is presumed to be an anomalous result; an alternative explanation might be lack of gDNA template, but sufficient virus, measurable by the number of GFP expressing plaques, had been present.

Given that many of the MVA-mH5-Rv0111 amplified samples expressed GFP, but gave little (-/+) or no (-) evidence for the TPA to V5 section of the recombinant cassette, another PCR reaction was performed using a forward primer binding near the start of the GFP gene and a reverse primer binding in the right flank (GFP/R, Section 2.3.1, Table 2.6). Results revealed a product for all samples except for the negative control, sample No. 1 and sample No. 18 (Figure 4.2).

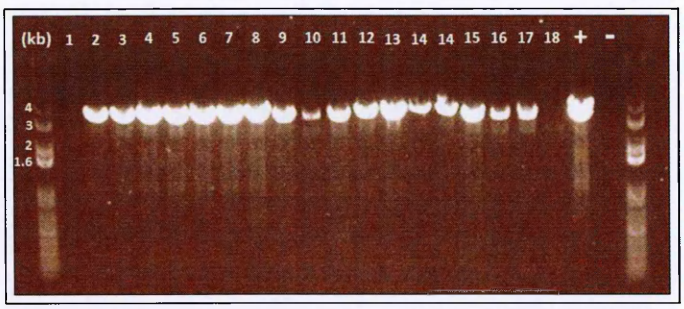


Figure 4.2 Interrogation of the insertion site following failed attempts to generate MVA-mH5-Rv0111. Products of the GFP/R PCR reaction were separated by gel electrophoresis (see table 4.3 for a description of the samples; 1-18). Transfer plasmid DNA was used in the positive control (+) reaction and gave a product of the expected size (3190 bp). Water was used in place of DNA template to provide a negative control (-). Only one product was observed for each reaction. NB. Sample 14 appears twice because lane-to-lane leakage was observed when loading the gel. The positive control band was very bright, but on close inspection was not considered to be two bands merging.

The absence of a product for the WT-MVA control (sample No. 1) was expected and confirmed that without a recombinant cassette, specifically the GFP gene, the forward primer would not be complementary to the viral gDNA template and no amplicon would be generated. A product of 3190 bp was expected for all of the samples related to MVA-P7.5-Rv0111 and this was observed, matching the product in the positive control for which the template had been the MVA-P7.5-Rv0111 transfer plasmid.

For the MVA-mH5-Rv0111 samples where GFP expression had been observed, but there was no evidence for the recombinant cassette upon TPA/V5 PCR, it was speculated that a truncated product might arise. Instead, all of the MVA-mH5-Rv0111 samples gave yield to a PCR product the same size as that in the positive control. This was investigated further by having these PCR products sequenced (Section 2.1.12).

Sequence files returned for the GFP-R PCR products were analysed against sequence maps for the corresponding transfer plasmid (pTBD1-Rv0111 or pTBD2-Rv0111). The results are summarised in Table 4.4 where samples are confirmed as having the expected sequence ('correct') or an unexpected sequence ('wrong'). It was not deemed necessary to send all of the samples relating to MVA-P7.5-Rv0111 for sequencing (-).

MVA-P7.5-Rv0111 was successfully purified with no anomalous PCR results and so sequence data relating to the amplicon derived from this construct were expected to be correct. Indeed, they aligned with no mismatched base pairs.

Sequence data for the MVA-mH5-Rv0111 3rd attempt (Samples 15-17) were also correct and this strain had shown a weak TPA-V5 product suggesting that perhaps the required rMVA virus was present, but at low titre, eventually becoming undetectable by the third round of plaque purification (P3, Sample No. 18).

Table 4.4 Sequence analysis for GFP-R PCR products. Purified PCR products were sequenced and compared to the expected sequence, to determine which were correct.

Sample	Promoter	Description	Sequence Data
1	N/A	WT-MVA	No product
2	P7.5	I/T	Correct
3		P1	Correct
4		P2	-
5		P3	-
6		A1	-
7		A2	-
8		A3	Correct
9	mH5 (attempt 1)	I/T	Correct
10		P1	Correct
11	mH5 (attempt 2)	I/T	Correct
12		P1	Wrong
13		P2	Wrong
14		P3	Wrong
15	mH5 (attempt 3)	I/T	Correct
16		P1	Correct
17		P2	Correct
18		P3	No product

Evidence for the intended recombinant virus having been present, but again at low titre, was also observed for the MVA-mH5-Rv0111 1st attempt (Samples 9-10), which gave the correct sequence despite a TPA-V5 PCR product and V5 expression not being detectable.

Sequence data for the MVA-mH5-Rv0111 2nd attempt showed that an aberrant recombinant had been generated. Transfer plasmid backbone had been incorporated which replaced the majority of the Rv0111 antigen, but which gave, coincidentally, a GFP-R PCR product of the expected size. Evidently, this recombinant was stable in nature, explaining why GFP expression was observed to increase with successive plaque purification rounds, just as it did for MVA-P7.5-Rv0111.

Overall, an assessment of the 3 attempts to generate MVA-mH5-Rv0111 concluded that it may have been made successfully, but was only ever present at very low levels and was unable to propagate such that it could be isolated away from wild-type MVA. Characteristics of the Rv0111 transgene were reviewed to investigate why MVA-mH5-Rv0111 could not be purified.

Truncation of Rv0111 to enable generation of stable MVA-mH5-Rv0111 virus

Rv0111 is predicted to contain 10 transmembrane domains (Figure 4.3) and so this was considered a likely cause for its instability (Krogh *et al.*, 2001). It was speculated that a truncated version of the gene, eliminating the majority of the predicted transmembrane helices, may enable a stable MVA-mH5-Rv0111 virus to be generated. Accordingly, a truncated Rv0111 sequence, $Rv0111_{361\text{-}685}$ (Rv0111T), was amplified from *M. tuberculosis* strain H37Rv gDNA. The rationale for the length of Rv0111T was to eliminate as many predicted transmembrane helices as possible while retaining immunogenic epitopes (murine and human T cell epitopes have been identified, as discussed in Section 1.4.2).

Rv0111T was amplified from the *M. tuberculosis* genome using primers incorporating attB sequences, so that the gene could be rapidly transferred to the required MVA transfer plasmid via Gateway® cloning (Section 2.1.4). Upon completion, the transfer plasmid was sequenced to ensure that there were no mutations in the open reading frame. A transfer plasmid map annotated to show the sequenced regions is presented in Appendix II. Once the transfer plasmid was confirmed to be correct, the infection/transfection reaction in BHK-21 was repeated. In the subsequent attempt to isolate recombinant virus, MVA-mH5-Rv0111T was successfully isolated and native MVA confirmed to be absent (by PCR) after three rounds of plaque purification. Evidence for recombinant antigen expression was also obtained via Western blot (not shown).

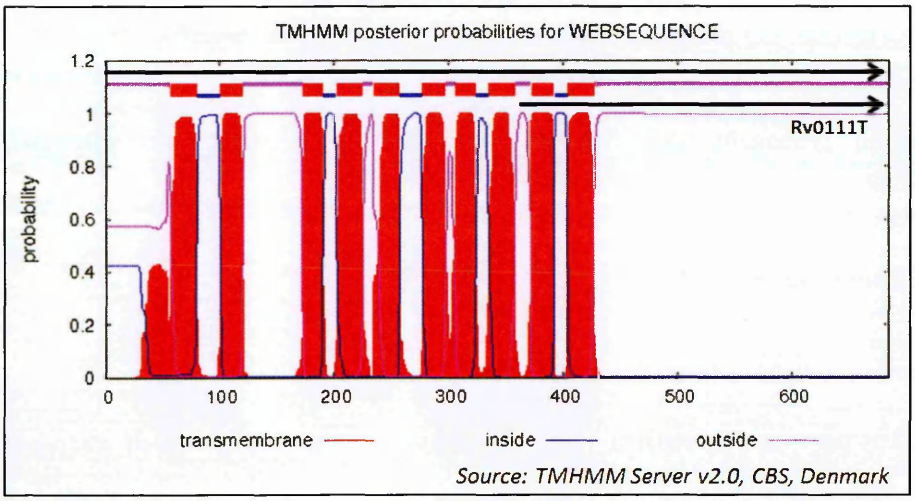


Figure 4.3 Rv0111 transmembrane helices The Rv0111 amino acid sequence (685 residues) was entered into a server for transmembrane helices prediction (TMHMM Server 2.0, Centre for Biological Sequence Analysis, Denmark). Arrows show the length of Rv0111 before (2-685) and after (361 – 685) truncation.

Amplification of MVA-Ag85A and MVA-Rv0111 virus stocks

Each of the recombinant viruses successfully isolated away from wild-type MVA were subsequently amplified by propagation through culture vessels of increasing size, culminating in a large-scale amplification involving multiple tissue culture flasks. Cell lysate was harvested and viral particles separated from host cell and vaccinia-derived protein by sucrose-cushion density centrifugation (Section 2.2.7).

Titration on CEF monolayers was performed in order to determine the titre (PFU/ml) of the purified stocks (Section 2.2.3). In parallel, purified material was amplified and harvested for a further round of PCR and Western blot characterisation, to ensure that it was of suitable quality for subsequent studies (Section 2.3). A summary is provided in Table 4.5.

Table 4.5 Purified stocks for an investigation into vaccinia promoter selection

Summary for sucrose cushion purified material	MVA-P7.5-Ag85A	MVA-mH5-Ag85A	MVA-P7.5-Rv0111	MVA-mH5-Rv0111T
No. of passages taken to isolate recombinant MVA	3	3	3	3
Total no. of passages (plaque purification & amplification)	7	8	7	8
Stability of insert confirmed by PCR	✓	✓	✓	✓
Expression product detected via Western blot	✓	✓	✓	✓

4.3.2 Generation of markerless rMVA expressing ovalbumin (OVA)

The novel rMVA viruses required for the leader sequences study (Chapter 6) were created and characterised as described above, with the addition that after isolation of recombinant virus further passages were performed to remove transiently expressed ('self-excising') GFP (Section 2.2.6). In light of the findings for MVA-mH5-Rv0111, where an erroneous recombinant had been purified, a panel of analytical PCR reactions was devised for robust analysis of the recombinant insert at each round of plaque purification. Expression was investigated as before, using anti-V5 for immunodetection of the recombinant antigen following Western blot of infected CEF cell lysate.

An expanded PCR panel for virus characterisation

Using the transfer plasmids created in Chapter 3, four recombinant MVA viruses expressing OVA were constructed. Between them, recombinant cassettes were introduced at two sites in the MVA genome, Deletion site II (Del II) and Deletion site III (Del III). For all, the selection marker was transiently expressed by virtue of homologous sequences flanking the GFP gene. The PCR reactions that were implemented are summarised in Figure 4.4. Existing primer pairs (previously applied to the characterisation of MVA-Rv0111) were employed for Del III specific reactions - L/R and GFP/R. For the remainder, it was possible

to establish primer pairs using oligonucleotides originally synthesised for construction of the transfer plasmids (sequences are listed in Section 2.3.2, Table 2.6).

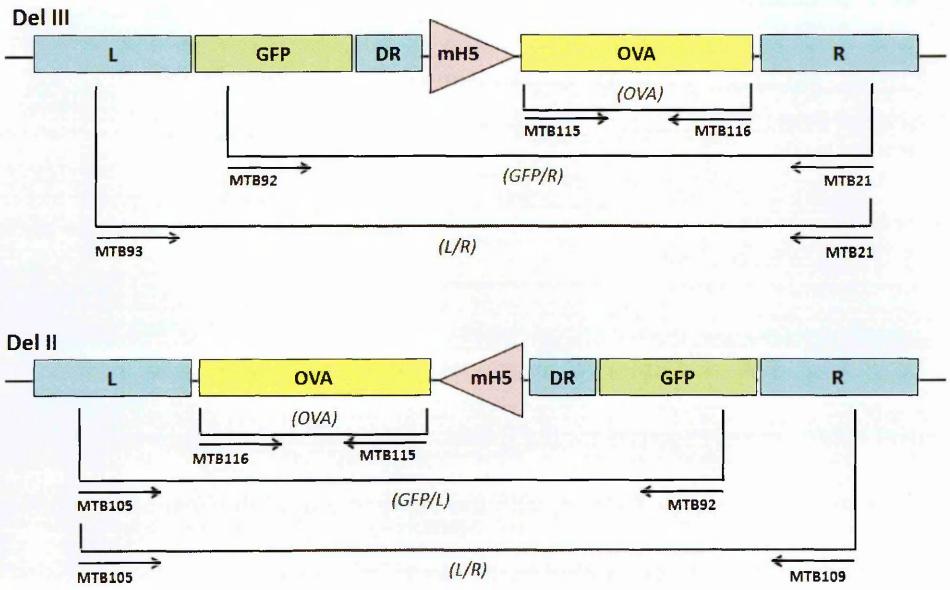


Figure 4.4 Primer pairs for characterisation of recombinant MVA expressing OVA For each insert site, analytical PCR was employed to detect wild-type vs recombinant virus (L/R) and to detect GFP in the context of the insertion site (GFP/R and L/GFP). A PCR test targeting OVA, within the recombinant cassette, was also implemented. As described in Chapter 3, the recombinant cassette for Del II is introduced in the opposite orientation. (Abbreviations: L, left flank; R, right flank; DR, direct repeat; GFP, green fluorescent protein; OVA, ovalbumin; mH5, modified vaccinia promoter H5).

The transient GFP system leads to the generation of antigen-free rMVA

Following the infection/transfection process, samples were plated for isolation of GFP-positive plaques. After the first round of plaque picking, harvested plaques were amplified for extraction of MVA gDNA and characterisation by PCR. Results pertaining to each of the four viruses provided evidence for a mixed population comprising recombinant and wild-type virus. In addition, a third population was identified; it was observed that some of the virus was recombinant, contained GFP, but had an insert smaller than was expected for the intended recombinant cassette. The size of the insert was consistent with a recombinant

virus containing the GFP gene only (MVA-GFP). It was reasoned that this would occur on account of an undesired homologous recombination event involving the direct repeat sequence (Figure 4.5B). Further, it was speculated that a fourth population could arise in which OVA, but not GFP would be transferred to the MVA genome (Figure 4.5C).

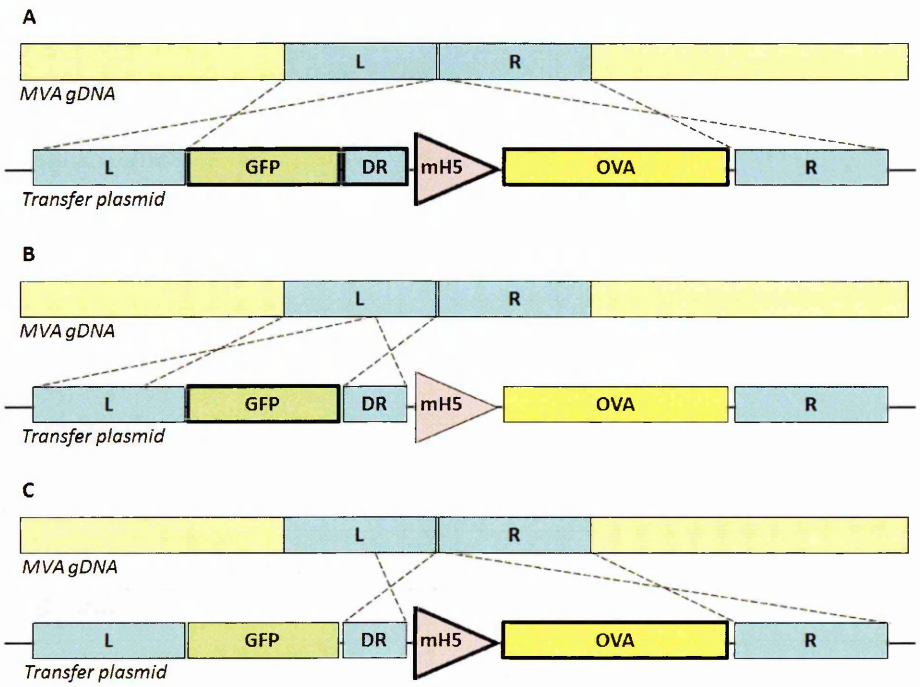


Figure 4.5 Putative homologous recombination events between MVA gDNA and a transient-marker transfer plasmid. A) The desired recombination event involves the regions flanking the insertion site. B) The presence of a direct repeat (DR) sequence, homologous to the end of the preceding flank (L) makes possible an alternative recombination event in which only the GFP gene is transferred. C) It may also be possible for the antigen, but not GFP, to recombine into the MVA genome. For each scenario, features of the transfer plasmid transferring to MVA gDNA are shown with a bold outline.

For each of the viral populations that could emanate from the infection/transfection process (Figure 4.5) the length of the amplicon expected to arise in each PCR reaction was determined (Table 4.6).

Table 4.6 PCR reactions for virus characterisation and expected products (bp). Viral populations and the transfer plasmid(s) from which they could arise are shown. For some virus-PCR combinations, no product was expected (-). For viruses in which GFP was transiently expressed, a reduction in PCR product size upon GFP loss was anticipated (shown in parentheses).

Virus	Target and primer pairs				
		Del III		Del II	
	OVA	**L/R**	**GFP/R**	**R/L**	**GFP/L**
	MTB115 MTB116	MTB93 MTB21	MTB92 MTB21	MTB109 MTB105	MTB92 MTB105
Wild-type MVA	-	549	-	750	-
pTBD5 or pTBD9-OVA (Del III), pTBD11 or pTBD13-OVA (Del II)					
MVA-GFP	-	1599	1071	1739	1622
pTBD5 or pTBD9-OVA (Del III), pTBD11 or pTBD13-OVA (Del II)					
MVA-Ag only	1205	2069	-	2274	-
*pTBD**5**-OVA*					
MVA-OVA$_{TPA}$ (Del III) (without GFP)	1205	3119 (2069)	2591 (-)	750	-
*pTBD**9**-OVA*					
MVA-OVA (Del III) (without GFP)	1205	3014 (1964)	2486 (-)	750 (n/a)	-
*pTBD**11**-OVA*					
MVA-OVA$_{VSVg}$ (Del II) (without GFP)	1205	549	-	3262 (2274)	3159 (-)
*pTBD**13**-OVA*					
MVA-OVA (Del II) (without GFP)	1205	549	-	3115 (2127)	3012 (-)

For all four MVA-OVA viruses, the decrease in amplicon size expected to occur following loss of transiently expressed GFP gene was also calculated. In some instances, no PCR product was expected, because the homologous sequence for one or both primers would be absent from the template, or because primers would bind, but at separate insertion

sites (Del II and Del III are separated by 130 kb of genomic DNA, the PCR reaction is optimised for an amplicon of approximately 3 kb). It was noted that, should antigen-only recombinant MVA arise directly from the infection/transfection process (Figure 4.5C), it would be genetically identical to the intended construct following GFP loss and so indistinguishable upon PCR. In each PCR test, transfer plasmid DNA was included as a positive control and water used in place of DNA to provide a negative control.

Isolation of markerless rMVA expressing OVA

MVA-OVA$_{TPA}$ (Del III) and MVA-OVA (Del III) were both purified after two rounds of plaque purification. A schematic summarising the viral samples harvested for each virus and their status with regards to recombinant cassette insertion and transgene expression, as determined by PCR and Western blotting, is shown in Figure 4.6. Viral sample nomenclature is described in Section 2.2.5.

MVA-OVA$_{VSVg}$ (Del II) and MVA-OVA (Del II) were both purified after 3 and 4 rounds of plaque purification, respectively. A schematic summarising the viral samples harvested for each virus and their status with regards to recombinant cassette insertion and transgene expression is shown in Figure 4.7.

Representative PCR and Western blot data for the four MVA-OVA viruses are shown below. For ease of reference throughout, the viruses are referred to by a single digit, corresponding to the transfer plasmid used to create them.

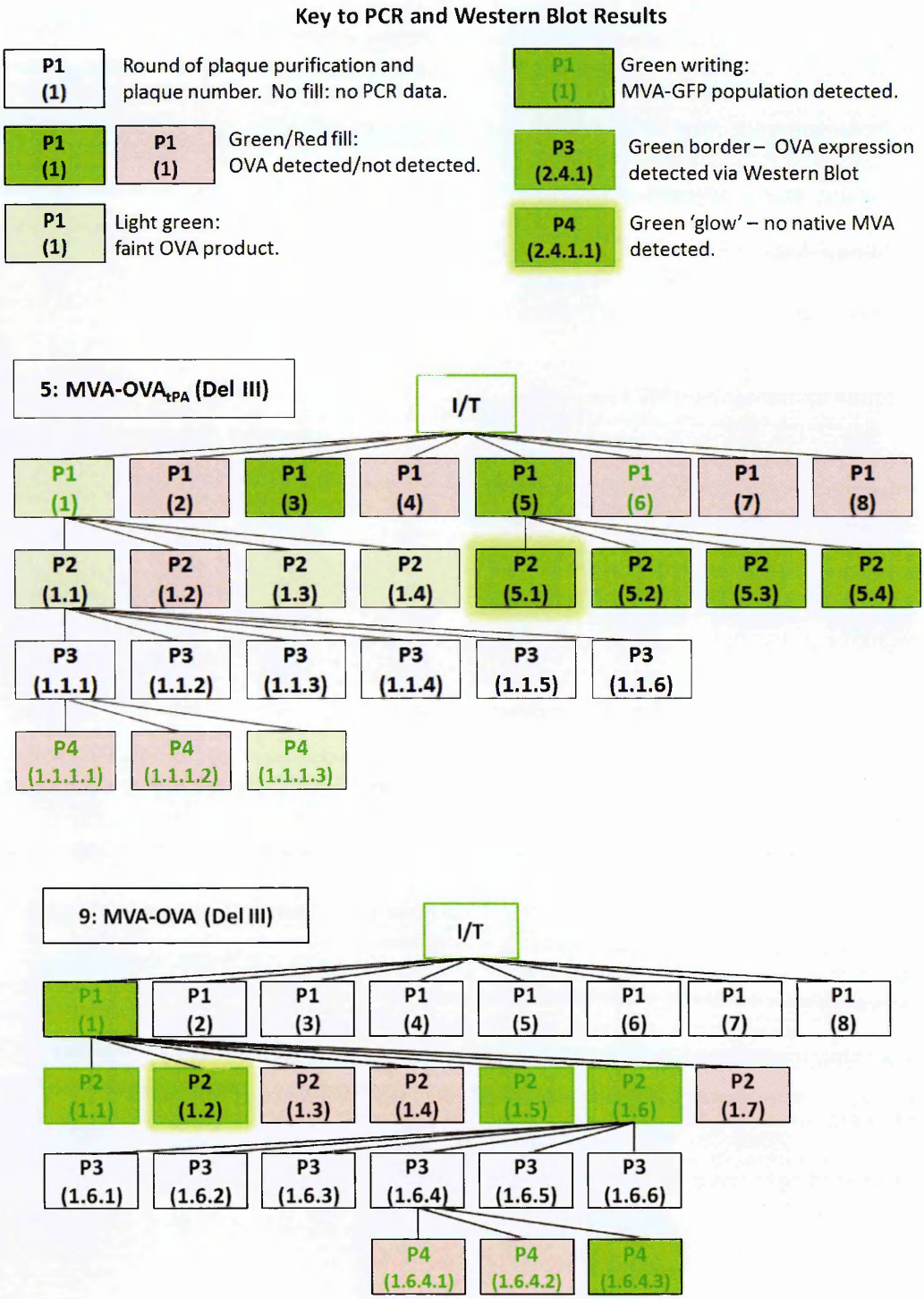


Figure 4.6 Plaque purification of MVA-OVA$_{TPA}$ (Del III) and MVA-OVA (Del III). Plaque-pick samples, their propagation and the results of *in vitro* characterisation (PCR and Western blot) are summarised. Numbers adjacent to the virus name indicate the pTBD plasmid used to generate the virus e.g. pTBD5-OVA for MVA-OVA$_{TPA}$ (Del III). Not all picks were analysed by Western blot and only positive results for expression are shown. Abbreviations: I/T, infection/transfection reaction; P1 (1), Plaque pick, round of plaque purification and plaque sample number(s) (the latter shown in parentheses).

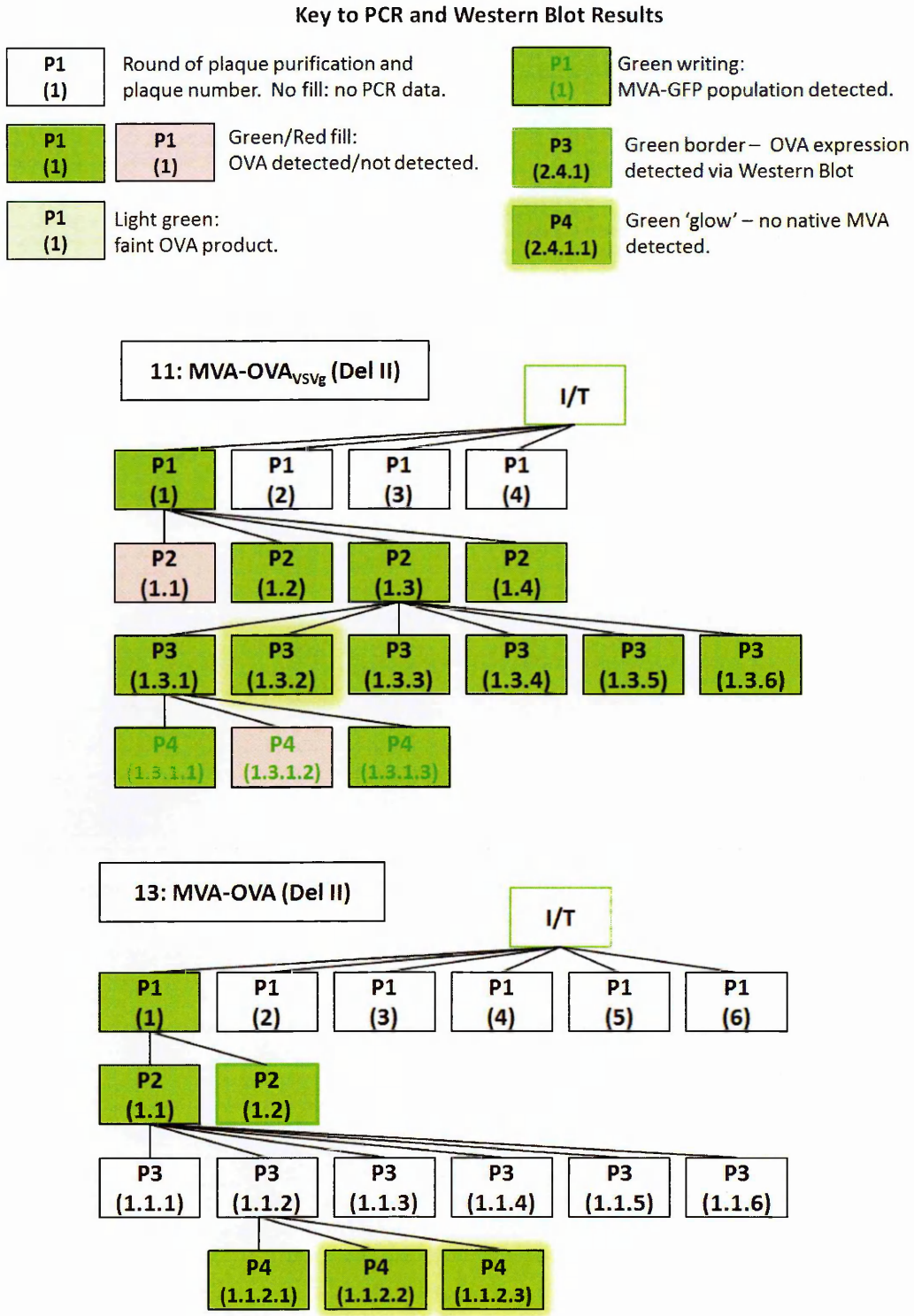


Figure 4.7 Plaque purification of MVA-OVA$_{VSVg}$ (Del II) and MVA-OVA (Del II). Plaque-pick samples, their propagation and the results of *in vitro* characterisation (PCR and Western blot) are summarised. Numbers adjacent to the virus name indicate the pTBD plasmid used to generate the virus e.g. pTBD5-OVA for MVA-OVA$_{TPA}$ (Del III). Not all picks were analysed by Western blot and only positive results for expression are shown. Abbreviations: I/T, infection/transfection reaction; P1 (1), Plaque pick, round of plaque purification and plaque sample number(s) (the latter shown in parentheses).

Rationalised processing of genomic DNA samples for PCR

For all four MVA-OVA viruses, all plaque picks were amplified and viral genomic DNA (gDNA) harvested, but to accommodate the extended panel of PCR reactions, stepwise analysis of the samples was implemented. After the first round of plaque purification (P1), one plaque sample for each virus was initially analysed using the primer pairs described in Figure 4.4 and Table 4.6. The PCR products arising from reactions 'OVA', 'L/R' and 'GFP/R' are shown in Figure 4.8.

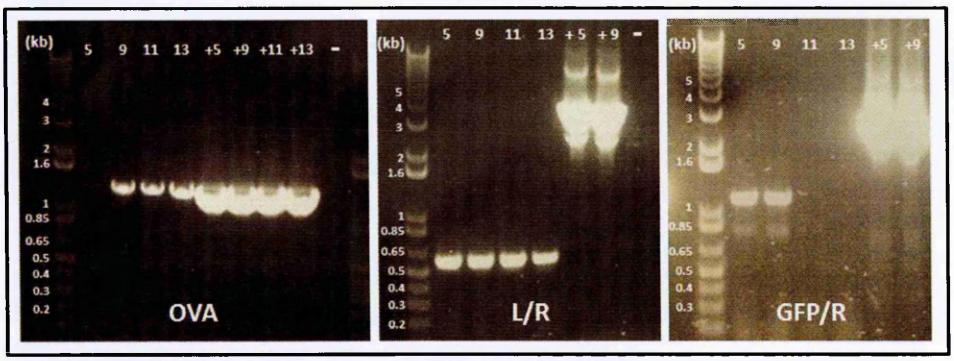


Figure 4.8 Analytical PCR of MVA-OVA samples collected in the first round of plaque purification. Virus from plaque-pick samples was amplified for extraction of gDNA and characterisation of the insert site and recombinant cassette via PCR. For each virus, DNA from the corresponding transfer plasmid was used as template in the positive control reaction (+). Water was included in place of DNA in the negative control (-). For ease of reference, the plaque samples were referred to by a single digit, corresponding to the transfer plasmid used to create them (shown in Table 4.4).

Across the PCR reactions, negative controls produced no amplicon. The products observed for each of the positive controls following the 'OVA' reaction were close to the expected size and appeared to have migrated further on account of having been run at high concentration. For both '5' (MVA-OVA$_{TPA}$ Del III) and '9' (MVA-OVA Del III), the OVA reaction confirmed that a viral population carrying the ovalbumin gene (1205 bp) was present; a faint band was visible for virus derived from pTBD5. Plaque samples for '11' (MVA-OVA$_{VSVg}$ Del II) and '13' (MVA-OVA Del II) also contained ovalbumin-positive gDNA.

Following reaction 'L/R', the positive control (transfer plasmid) DNA yielded bands consistent with each plasmid having undergone a recombination event, excising the selection marker in the same manner anticipated for the recombinant viral gDNA. Thus, they provided an indication of the expected products, both with and without GFP. As expected, amplification across the insertion site revealed the presence of wild-type MVA (549 bp) and this product was amplified in preference to larger amplicons derived from the recombinant gDNA, known to be present in the sample because of the 'OVA' result. The 'L/R' reaction targets insertion site Del III and so plaque samples for '11' and '13' yielded bands consistent with a native genotype and this would have been expected even had they been purified recombinant virus.

Amplification of the GFP gene in the context of insertion site Del III (reaction 'GFP/R') preferentially highlighted a recombinant population containing GFP only (MVA-GFP) for both '5' (MVA-OVA_{TPA} Del III) and '9' (MVA-OVA Del III). There was also evidence for a non-specific band (~850 bp) faintly visible in each of the positive controls.

It was noted that in both the 'L/R' and the 'GFP/R' reactions, the positive control template also revealed a non-specific product larger than the recombinant cassette (> 4kb), likely to be plasmid DNA carried over from the PCR reaction on account of the template being insufficiently dilute. This was remedied in future reactions by further dilution of the plasmid DNA.

Overall, for both of the Del III constructs, investigation by PCR revealed the presence of at least three virus populations - wild-type MVA, MVA-GFP and MVA carrying the ovalbumin gene. The latter was potentially present with and without the GFP gene. As summarised in Figure 4.7, the results for '11' (MVA-OVA_{VSVg} Del II) and '13' (MVA-OVA Del II), some of which can be seen in Figure 4.8, revealed the presence of wild-type MVA and MVA-OVA (with and without GFP). There was no evidence for a MVA-GFP population.

The P1 plaque samples characterised by PCR were also investigated for evidence of antigen expression via Western blot of infected cell lysate. The results did not reveal an anti-V5 reactive band for any of the viruses (not shown). Whilst the ovalbumin gene had been detected upon PCR, this was considered a plausible outcome on account of the potentially very low amount of recombinant virus present. Material from each of the infection/transfection steps did provide evidence for V5 immunoreactivity.

Plaque samples from the first round of plaque isolation were subsequently diluted and plated for further plaque isolation. In subsequent rounds of PCR analysis, more plaques were analysed, but the PCR reactions were performed in stages to down-select those worthy of further investigation, e.g. those containing the ovalbumin gene.

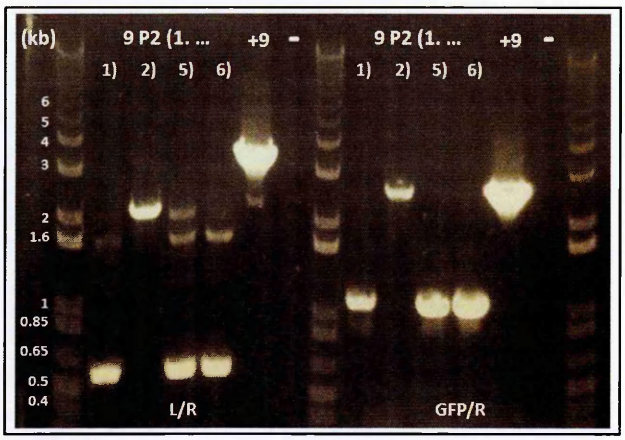
Purification of MVA-OVA (Del III)

At the second round of purification (P2), PCR was performed as above and revealed that MVA-OVA (Del III), derived from pTBD9, had been isolated efficiently. Of seven P2 plaque samples harvested and characterised, four were confirmed by PCR to contain virus carrying the ovalbumin gene (not shown): P2 (1.1), P2 (1.2), P2 (1.5) and P2 (1.6). Reaction 'L/R' and 'GFP/R' were subsequently performed on these four samples. Results are shown in Figure 4.9A.

In three of the pTBD9-derived P2 samples (1, 5 and 6), amplification across the insertion site ('L/R') was able to demonstrate a mixed population - wild-type MVA (549 bp), MVA-GFP (1599 bp) and GFP-free MVA-OVA (1964 bp). The ability of the reaction to detect larger inserts in addition to the native product, which might have otherwise been preferentially amplified, was attributed to an increase in the proportion of recombinant viral populations. The relative intensity of each band was not interpreted beyond that as the amplification efficiency for PCR products of different size is not anticipated to be equal. For P2 (1.2), the 'L/R' reaction provided evidence for GFP-free MVA-OVA and possibly a

very faint band for the recombinant cassette with the GFP gene. Wild-type MVA was not detectable.

A



B

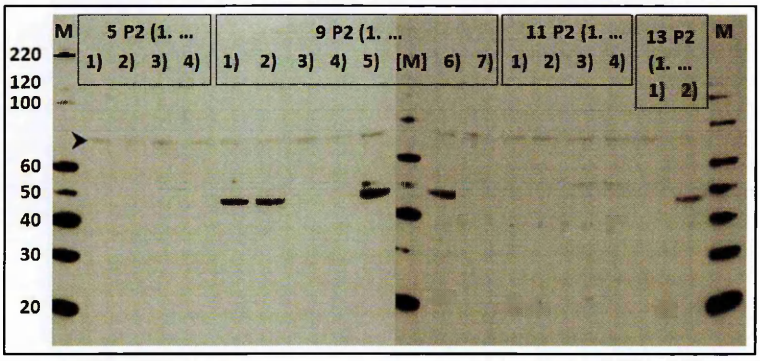


Figure 4.9 Characterisation of MVA-OVA samples collected in the second round of plaque purification (P2). A) Viral samples relating to '9' (MVA-OVA Del III) were amplified for extraction of gDNA and characterisation of the insert site and recombinant cassette via PCR. DNA from the corresponding transfer plasmid was used as template in the positive control reaction (+9). Water was included in place of DNA in the negative control (-). B) All 4 of the samples relating to those investigated by PCR were observed to express recombinant antigen on Western blot (expected product size, 47.8 kDa). All contain a non-specific product of approximately 70 kDa (arrow head) observed to be a feature of MVA-infected CEF cell lysate (negative control not shown).

In support of the 'L/R' PCR result, reaction 'GFP/R' confirmed the presence of MVA-GFP in the P2 (1.1), P2 (1.5) and P2 (1.6) samples. For sample P2 (1.2), it provided evidence for the desired recombinant only, inclusive of the GFP gene. Hence, between the 2 reactions it was

apparent that both a GFP-positive and GFP-negative population of MVA-OVA (Del III) was present. This was expected as visual inspection of the cell layer prior to extraction of gDNA had shown GFP-positive plaques.

At the second round of plaque purification, those samples in which the ovalbumin gene was detectable by PCR also demonstrated expression by Western blot (Figure 4.9B). At this stage, plaque sample P2 (1.2) was selected for further passaging for removal of the GFP gene.

Purification of MVA-OVA$_{TPA}$ (Del III)

As summarised in Figure 4.6, further plaque purification of sample P1 (1) was carried out, but at the fourth round of purification (P4) the MVA-GFP (no OVA) population was shown to persist. Ultimately, further plaque samples from round 1 were investigated to select an alternative for progression, specifically, a plaque sample in which MVA-GFP was not apparent. As described above for MVA-OVA (Del III), the required recombinant was then isolated and, again, by the second round. Sample P2 (5.1) was selected for further passage for removal of the GFP gene.

Purification of MVA-OVA$_{VSVg}$ (Del II) and MVA-OVA (Del II)

As described above, one plaque sample for each virus was analysed at the first round of plaque isolation (Figure 4.8). Both of the recombinant MVA viruses went on to be purified from these samples after three and four rounds of plaque purification, respectively. The PCR reactions applied throughout were 'OVA' (as used for the Del III constructs), and 'R/L' and 'GFP/L' which were Del II specific. An example of the results obtained at the fourth round of plaque purification (P4) for MVA-OVA (Del II), derived from pTBD13, are shown in Figure 4.10. Sample P4 (1.1.2.2) was selected for further passage to remove the GFP gene, while for MVA-OVA$_{VSVg}$ (Del II), derived from pTBD11, sample P3 (1.3.2) was selected (as shown in Figure 4.7).

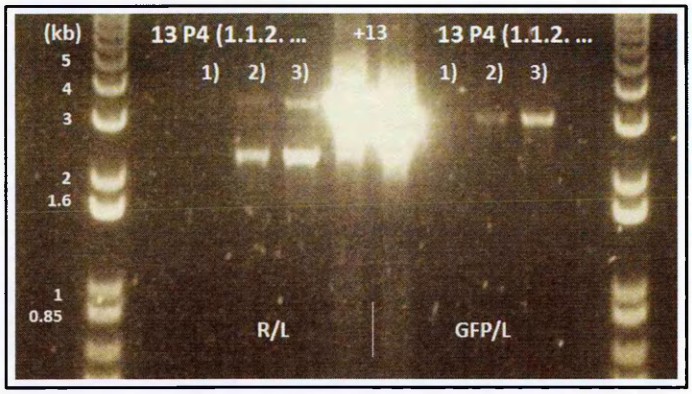


Figure 4.10 Analytical PCR of MVA-OVA samples collected in the fourth round of plaque purification. Viral samples relating to MVA-OVA (Del II), derived from pTBD13, were amplified for extraction of gDNA and characterisation of the insert site by PCR. After amplification across the Del II insertion site (R/L), two of the three pTBD13-derived MVA-OVA plaques were observed to contain a mixture of the required recombinant, with and without the GFP gene. No wild-type MVA was detected. Amplification of the insertion site using a primer binding within the GFP gene (GFP/L) revealed the required rMVA only and no MVA-GFP, i.e. rMVA with no OVA antigen. All of the samples were observed to contain the ovalbumin gene (results not shown).

Removal of transiently expressed GFP and amplification of virus stocks

After isolation of each of the MVA-OVA viruses away from wild-type MVA, the purified samples were subjected to serial passage to remove the GFP gene. As described in Section 2.2.6, the removal of GFP by serial passage was assisted by culturing CEF under a solid overlay so that discrete plaques expressing GFP could be physically removed prior to harvesting the cell layer. Where a GFP-positive population of MVA-OVA was found to persist, the approach of picking discrete foci of GFP-free cells was taken to maximise the likelihood of amplifying GFP-free recombinant virus only. The number of passages required before each virus no longer exhibited GFP expression is summarised in Table 4.7. At each subsequent round of amplification cell monolayers were screened to ensure that no GFP positive cells were visible. Upon PCR analyses of the final, purified rMVA stock, no GFP-positive PCR products were observed.

Table 4.7 Purified stocks for an investigation into leader sequences. Purified, titrated stocks were subjected to PCR of the insertion site to reveal products of the expected size for pure, GFP-free recombinant MVA-OVA. Expression was also confirmed via Western blot.

Sucrose cushion (SC) purified stocks:	MVA-OVA$_{TPA}$ (Del III)	MVA-OVA (Del III)	MVA-OVA$_{VSVg}$ (Del II)	MVA-OVA (Del II)
No. of passages taken to isolate MVA-OVA (+GFP)	2	2	3	4
No. of additional passages taken to isolate MVA-OVA (- GFP)	2	5	4	2
Total no. of passages (purification, GFP removal and amplification)	9	11	11	11
Stability of insert confirmed by PCR	✓	✓	✓	✓
Expression product detected via Western blot	✓	✓	✓	✓

Following isolation of GFP-free MVA-OVA, each of the viruses was amplified through culture vessels of increasing size and then purified by sucrose cushion purification (Section 2.2.7). The concentration of the purified stocks (PFU/ml) was determined by titration on CEF in multi-well plates (Section 2.2.3). All of the titrated stocks were subjected to a final, further round of characterisation by PCR and Western blot to ensure that the material was of suitable quality for subsequent studies (summarised in Table 4.7). The results for the Western blot are shown in Figure 4.11. Each virus produced a V5 reactive band of the expected size. MVA-OVA$_{TPA}$ (Del III) ('5') also produced a larger band, possibly a dimer of the expected product. Overall, the results confirmed that all four MVA-OVA viruses were suitable for further use, to create double recombinants (below) or for comparison of their relative immunogenicity (Chapter 6).

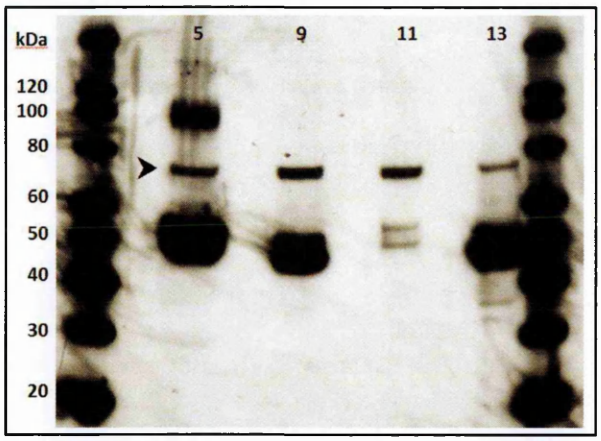


Figure 4.11. Expression of Ovalbumin by four MVA-OVA viruses. Infected CEF cell lysate was separated by SDS PAGE and transferred to nitrocellulose for immunodetection with anti-V5. Viruses are referred to by the number of the pTBD plasmid used to create them (see Table 4.6). From left to right, the expected size for the product was 51.6, 47.8, 53.9 and 48.3 kDa. All contain a non-specific product of approximately 70 kDa observed to be a feature of MVA-infected CEF cell lysate (arrow head). The presence of OVA as a doublet is expected due to the formation of different glycosylated forms.

4.3.3 Instability of double recombinant MVA-OVA

The MVA-OVA viruses above were generated to be markerless so that, as single recombinants, all would have the potential to be transformed into a double recombinant virus via insertion of a second, separate recombinant cassette. The infection/transfection reactions carried out are listed in Table 4.8. MVA-OVA_{TPA} (Del III) and MVA-OVA (Del II) were selected to serve as the parent strains (because they had been available first). The viruses were diluted for infection of BHK-21 at the required MOI (see section 2.2.4). Subsequently, the infected cells were transfected with transfer plasmid to create double recombinant MVA viruses (summarised in rows 1 and 2 of Table 4.8).

Table 4.8 Creation of double recombinant MVA-OVA viruses. The names given to the double recombinant viruses reflect the order in which the antigens with leader sequences have been inserted.

I/T	Infecting MVA parent strain	Transfected transfer plasmid	Resultant double recombinant
1	MVA-OVA_{TPA} (Del III)	pTBD11-OVA_{VSVg} (Del II)	MVA-OVA_{TPA}/OVA_{VSVg}
2	MVA-OVA_{VSVg} (Del II)	pTBD5-OVA_{TPA} (Del III)	MVA-OVA_{VSVg}/OVA_{TPA}
3	MVA-OVA (Del II)	pTBD9-OVA (Del III)	MVA-OVA/OVA

A modified PCR panel for characterisation of double recombinant MVA-OVA

Due to pre-existence of the recombinant cassette in the first insertion site, new primer pairs were incorporated into the analytical PCR protocol to detect OVA in the context of the flanking regions (Table 4.9). As before, amplification across the insertion site was performed to detect the presence of multiple populations: wild-type MVA, MVA-GFP and MVA-OVA (with and without GFP). Throughout, both insertion sites were analysed to ensure stability of the first insert.

Table 4.9 PCR characterisation of double recombinant MVA and expected products (bp) Amplification from the OVA gene to each of the flanks means that one amplicon will include the GFP gene and reduce in size upon its loss (shown in parentheses).

Viral inserts	Target and primer pairs			
	Del III		Del II	
	OVA/R MTB115 MTB21	**L/OVA** MTB93 MTB116	**OVA/L** MTB116 MTB109	**R/OVA** MTB105 MTB115
Existing insert (from pTBD5):				
MVA-OVA$_{TPA}$ (Del III)	1417	1859	-	-
Second insert (from pTBD11)				
MVA-OVA$_{VSVg}$ (Del II) (without GFP)	-	-	2590 (1602)	1852 (no change)
Existing insert (from pTBD13):				
MVA-OVA (Del II)	-	-	1627	1705
Second insert (from pTBD9))				
MVA-OVA (Del III) (without GFP)	1417 (no change)	2802 (1754)	-	-

Isolation of MVA-OVA$_{TPA}$/OVA$_{VSVg}$

Two attempts were made to generate a double recombinant virus expressing OVA fused to two different leader sequences. Following the first infection/transfection reaction (Table 4.8, I/T No.1) propagation of the harvested I/T sample gave rise to only two plaques, on two separate occasions (Figure 4.12A). A promising isolate was identified at the second round

of plaque purification, (P2) 2.2, but was found to be devoid of the required virus in the subsequent round (P3). Throughout, the recombinant cassette in the first insertion site (Del III) was present and of the expected size and so appeared to be stable. The inability to isolate MVA-OVA_{TPA}/OVA_{VSVg} on this occasion was considered likely to stem from the inadequate number of plaques arising from the I/T-step; the number of BHK-21 cells visibly expressing GFP prior to collection of the sample had been less than expected.

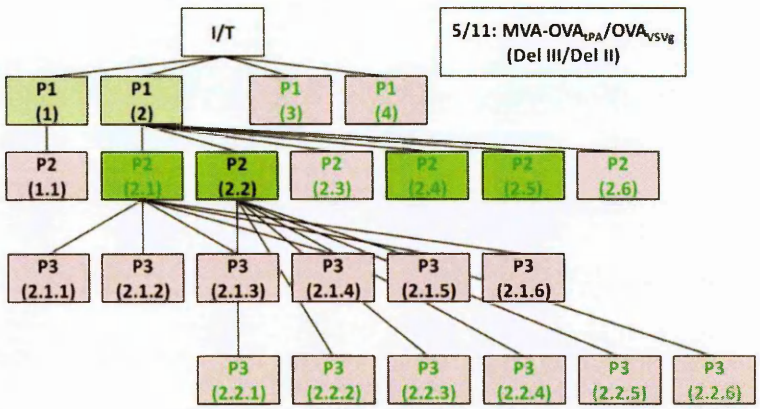
To obtain the required double recombinant MVA, two alternative strategies were attempted in parallel: the same infection/transfection was reattempted using increasing concentrations of the infecting virus. The infection/transfection was also repeated using the alternative single-recombinant MVA as the recipient strain (Table 4.8, I/T No. 2).

Of the two approaches, the latter appeared to yield more putative plaques, but by the second round of plaque purification the ovalbumin gene at the first insertion site (Del II) was no longer detectable via PCR. Indeed, there was no product at all for PCR reactions incorporating primers binding to the Del II region. This result was confirmed at the third round of purification (P3), where six out of six plaque samples were shown to be purely recombinant gDNA with regards to Deletion site III (Figure 4.12B).

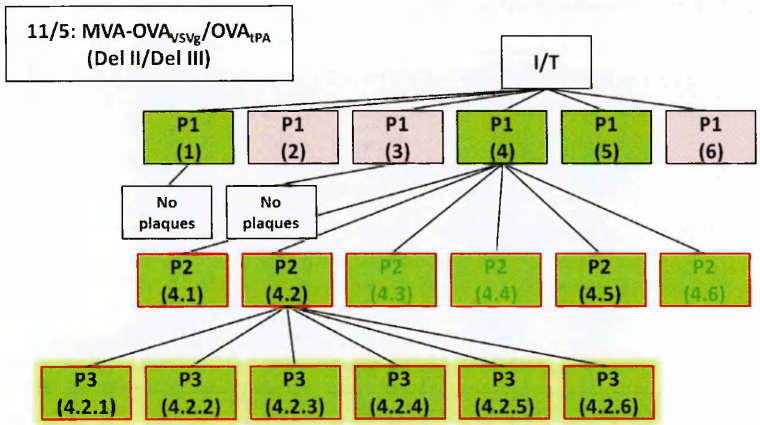
Isolation of MVA-OVA/OVA

As outlined in Table 4.8 (I/T No. 3), MVA-OVA/OVA was created by introducing a second recombinant cassette into the MVA genome at Deletion site III. After four rounds of plaque purification, no more virus of native genotype remained for the second, Del III insertion site (P4 (5.1.1.1), Figure 4,12C), but by this round of plaque purification the insert at Deletion site II had been lost. Again, no PCR products were observed for reactions incorporating primers binding to the Del II region. This was confirmed in further passages which would have otherwise served to amplify the virus.

A) I/T No.1



B) I/T No.2



C) I/T No. 3

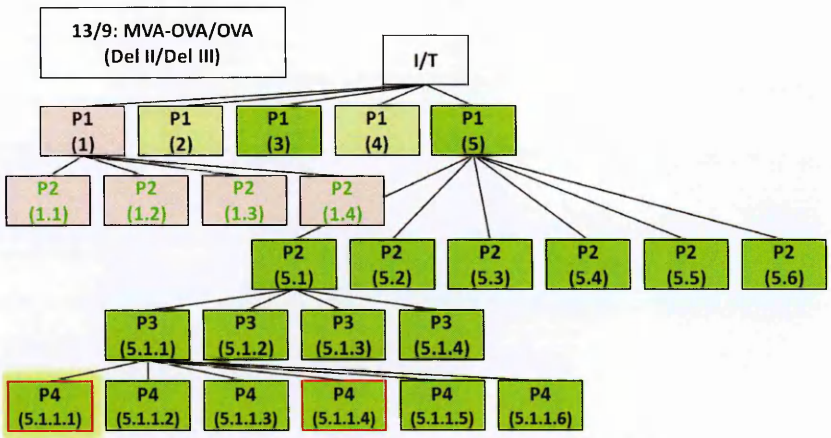


Figure 4.12 Plaque purification and propagation to create MVA-OVA double recombinant viruses. Plaque-pick samples, their propagation and the results of PCR characterisation for the second insertion site are summarised. Numbers adjacent to the virus name indicate the first and second pTBD plasmid used to create the virus e.g. pTBD5-OVA and pTBD11-OVA for MVA-OVA$_{TPA}$/OVA$_{VSVg}$ (Del III/Del II)). Abbreviation and Key: as shown in Figure 4.6 and Figure 4.7. Red outline: no PCR product for first (Del II) insert.

In summary, while the single recombinant MVA viruses expressing OVA were successfully purified, neither of the double-recombinant viruses was successfully isolated within the time available. In both cases, the insert at Deletion site II was lost during the additional passages needed to insert the second recombinant cassette.

4.4 Discussion

As discussed in Section 3.1, transfer plasmid methodology is one of two approaches available to generate rMVA; the other alternative is to apply BAC technology. The transfer plasmid system was selected for the current study because of the advantages conferred by its ease of set-up and implementation. The main disadvantage of the transfer plasmid system is the need to plaque-purify recombinant viral progeny.

4.4.1 Plaque purification of rMVA

After each infection/transfection (I/T) reaction the BHK-21 cell layer was harvested and subjected to sequential rounds of plaque isolation on CEF cells. It was possible to monitor the success of each I/T step by inspecting the BHK-21 cell layer for cells expressing GFP. The detection of adequate GFP expression proved an important prerequisite to undertake successful plaque purification.

Plaque purification was very efficient for MVA-P7.5-Ag85A and MVA-P7.5-Rv0111, taking only three rounds to complete. In contrast, the process was more challenging for MVA-mH5-Rv0111; the insert was unstable at the higher expression level, thus providing a growth advantage to non-expressors. Analytical PCR reactions were fundamental to determining what had occurred in this instance and demonstrated the importance of applying PCR tests at every round of plaque purification to ensure that only appropriate isolates were progressed.

Characterisation of rMVA by PCR

For each novel rMVA, amplification of gDNA at the site of antigen insertion was vital to ensure that the recombinant cassette was present, had not been truncated and to identify the point at which native MVA was no longer contaminating the recombinant viral stock. The use of primers that bound either side of the insertion site might have had the potential to simultaneously detect multiple populations, e.g. wild-type and recombinant viral DNA. This occurred in some instances, but more often the reaction was observed to demonstrate a bias towards smaller products. Rather than presenting a significant limitation, this feature worked in favour of the overall objective which was to ensure that no WT-MVA remained. However, it did mean that other reactions had to be implemented in order to more fully examine each recombinant cassette.

For samples pertaining to MVA-mH5-Rv0111, additional PCR reactions specific for the recombinant antigen were able to demonstrate that it was absent beyond the early purification rounds, despite continued presence of the GFP gene on one occasion. These analyses were essential to being able to conclude that the virus was insufficiently stable to be purified.

Improved stability was observed for the single rMVA viruses expressing OVA; following antigen insertion, viral replication was able to occur and the rMVA viruses were readily isolated. However, each of these viruses was generated to express GFP transiently and the ability for an unwanted rMVA population to arise was revealed to be an inherent complication for this system; the transient-marker recombinant cassette was able to mediate transfer of the GFP gene alone.

Additional PCR tests were able to clarify which viral populations were present within a given sample but, in terms of expedient rMVA preparation, the application of an extended panel of reactions was time consuming. The apparent propagation of plaque samples that were either unsuitable according to PCR data, or not required (as shown in Figures 4.6, 4.7

and 4.12), was a reflection of the need to continue propagation whilst waiting for PCR results to become available. For double recombinant MVA the number of required PCR tests was again increased, but was vital in demonstrating the instability of recombinant viruses at their first insertion site (discussed further below).

In summary, the difficulties presented by plaque purification were arguably off-set by the relative ease with which they could be interrogated by analytical PCR, but more time was required for transient marker expression and/or the creation of double recombinants. Ultimately, some rMVA were readily isolated and PCR was an expedient means of confirming purity and stability of the recombinant insert.

Characterisation of rMVA by Western Blot

In addition to characterisation by PCR, transgene expression was investigated using anti-V5 antibodies. During plaque purification, detection of an expression product lent support to PCR findings in some instances. However, in the earlier rounds of plaque purification transgene expression might not have always been detectable on account of the low level of rMVA present. Thus, the main benefit of characterisation by Western blot was not to support the selection of appropriate plaque isolates, but to verify faithful transfer of the recombinant antigen for effective transcription and translation. This appeared to be particularly pertinent at certain stages of rMVA preparation. Accordingly, material arising from each I/T step was analysed for early assurance of successful antigen insertion and, later, expression from the amplified stocks was checked as a precursor to performing further rMVA studies.

The manner in which the expressed recombinant products manifested was varied. MVA expressing Rv0111 produced V5-positive proteins that were a range of sizes, including a dominant band of approximately 30 kDa. The Rv0111 gene encodes a membrane-bound acyltransferase with 10 transmembrane domains; thus, it is hydrophobic and insoluble. This was considered a likely cause for the range of anti-V5 immunoreactive products

observed. In support of this conclusion it was calculated *in silico* that a C-terminal fragment of Rv0111, devoid of transmembrane domains, would give rise to a V5-positive protein of approximately 30 kDa.

Both Ag85A and OVA are soluble, and MVA expressing each of these proteins both produced two - three discrete V5-positive products upon Western blot. Eukaryotic expression of Ag85A has been reported to culminate in glycosylated and non-glycosylated forms, thus, the protein appears as two products when separated according to size (Huygen *et al.*, 1996). Ovalbumin is also reported to appear as a doublet because of the existence of mono and di-glycosylated products (Suzuki *et al.*, 1997).

Immunodetection with anti-V5 antibodies was applied throughout despite the availability of transgene-specific antibodies in some instances, e.g. anti-OVA. This was in order to demonstrate the flexibility of the system for investigating alternative recombinant antigens, as had to happen with Rv0111 which was truncated to promote stability under higher transcriptional regulation. It was noted that in the presence of degraded antigen, as seen for Rv0111, greater assurance of successful translation was provided by the inclusion of a C-terminal V5 tag, i.e. to confirm translation of the entire open reading frame. For those rMVA expressing an N-terminal V5 tag, e.g. MVA-OVA (Del II), it was reassuring to see V5-reactive products of the expected size.

With regards to the use of rMVA as viral vaccine vector, the appearance of the expression product after separation by SDS PAGE may be less relevant than the ability to detect it at all. This is because novel vaccines for use against TB (and other important diseases) are primarily being developed to induce cell-mediated immunity, i.e. T cell responses to processed antigen peptides and not B cell responses to conformational epitopes.

4.4.2 rMVA viruses for an investigation into Vaccinia promoter selection

Of the four rMVA viruses required for this aspect of the study, only three were successfully purified. The fourth, MVA-mH5-Rv0111, was apparently unstable and could not be isolated. MVA expressing Rv0111 under the control of the weaker, P7.5 promoter was readily purified and so instability of MVA-mH5-Rv0111 was attributed to heightened levels of Rv0111 expression. An rMVA virus expressing a truncated Rv0111 gene was generated (MVA-mH5-Rv0111T) and was readily isolated. As this version of the recombinant antigen excluded the majority of the protein's putative transmembrane domains, evidence for their being the cause of, or a contributing factor to, the instability was provided. A link between transmembrane regions and rMVA instability has been reported previously (Wyatt *et al.*, 2009). The validity of undertaking a comparison of promoter effects for a full length antigen versus a truncated version is discussed in Chapter 5.

4.4.3 rMVA viruses for an investigation into the use of Leader sequences

Six rMVA expressing OVA needed to be created, two of which would contain recombinant cassettes at two insertion sites. To facilitate insertion of a second recombinant cassette, all of the viruses were created to express GFP transiently so that markerless rMVA could be obtained. The single-recombinant viruses were apparently stable and were successfully purified, while the double-recombinant viruses were not. As discussed above, the transient marker system led to less efficient rMVA purification, but this would not appear to have contributed to the instability observed for the double-recombinants. There was some evidence to suggest that this may have been associated with the maintenance of a recombinant cassette in Deletion site II.

Deletion site II confers inferior stability

The single recombinant MVA expressing OVA comprised two strains employing Deletion site III for insertion of the recombinant cassette and two strains utilising Deletion site II. All were readily purified within 2-4 rounds of plaque purification. Prior to the amplification of

the viruses, further passages were performed to remove the transiently expressed, 'self-excising' GFP gene. By the time that viral purification was undertaken, all of the rMVA had undergone between 9 – 11 passages. Purified viral stocks were plated to determine their titre and at the same time, all were satisfactorily examined by PCR to confirm purity and stability of the recombinant inserts. Subsequently, some of the viruses were used to create double recombinant MVA-OVA, none of which were successfully isolated.

MVA-OVA_{TPA} (Del III) was used for insertion of a second recombinant cassette in Deletion site II. By the time of abandoning the attempt, the parent virus had been passaged 14 times in total and the recombinant cassette in Deletion site III was still present.

MVA-OVA_{VSVg} (Del II) and MVA-OVA (Del II) were both used for insertion of a second recombinant cassette in Deletion site III. By the time of their 14^{th} and 16^{th} total passage, respectively, the insert in Del II had been lost. The PCR tests employing primers binding to the Del II region gave no product at all.

The six major genomic deletion sites of MVA have been used routinely as regions for insertion of exogenous DNA. There is some evidence in the literature to suggest that Deletion site II may confer inferior stability for HIV antigens (Wyatt *et al.*, 2009) and in this example the use of intergenic regions was favoured, i.e. insertion between essential regions so that spontaneous deletions would be lethal. Many rMVA studies have employed OVA as a model antigen and this gene was not anticipated to induce spontaneous loss in the Del II region. There was insufficient time within the course of the current study to confirm conclusively if one or both insertion sites would incur stability issues over a protracted number of passages. There was also insufficient time to rectify this issue. The impact on the overall objectives and the value in assessing those rMVA-OVA that were successfully purified is discussed in Chapter 6, as are alternative approaches to successfully generating the double-recombinant MVA.

4.5 Conclusions

Transfer plasmid methodology was an efficient means of generating novel rMVA, including markerless rMVA, provided it was supported by a suitable and sufficient number of PCR tests to characterise viral progeny.

The model antigen ovalbumin was inserted in two sites of the MVA genome and may have had a greater tendency to induce spontaneous deletions when inserted into Deletion site II, as opposed to Deletion site III. Further work would be needed to determine whether this was the case.

Characterisation by PCR identified those rMVA that were sufficiently stable and pure to be used in further studies, while Western blot was applied to confirm each was able to express the recombinant antigen. The viruses available for future work are listed in Table 4.10.

Table 4.10 rMVA available for further study. All of the rMVA listed are single recombinant viruses expressing a product from either Del II or Del III.

Recombinant MVA virus	Expression product Del II	Expression product Del III
Vaccinia Promoter Study		
MVA-P7.5-Ag85A	-	TPA-Ag85A-V5
MVA-mH5-Ag85A	-	TPA-Ag85A-V5
MVA-P7.5-Rv0111	-	TPA-Rv0111-V5
MVA-mH5-Rv0111T	-	TPA-Rv0111T-V5
Leader Sequences Study		
MVA-OVA$_{TPA}$	-	TPA-OVA-V5
MVA-OVA	-	OVA-V5
MVA-OVA$_{VSVg}$	V5-OVA-VSVg	-
MVA-OVA	V5-OVA	-

The rMVA listed in Table 4.10 were subsequently used to test the hypotheses of the study (described in Section 1.5). Thus, the impact of vaccinia promoter selection on the immunogenicity and protective efficacy of TB vaccine antigens vectored by MVA was investigated and is described in Chapter 5, while the potential for leader sequences to

diversify the quality of the immune response evoked to antigens vectored by rMVA was studied and is described in Chapter 6.

Chapter 5 Does vaccinia promoter selection have the potential to alter the efficacy of rMVA TB vaccines?

5.1 Introduction

Vaccine antigen expression in rMVA based vaccines is achieved via insertion of the transgene downstream of a vaccinia promoter. The vaccinia promoter sequence contains binding sites for transcription initiation factors and determines both the strength and timing of transcriptional activity. To achieve high levels of transgene expression, promoters combining early and late (E/L) regulatory elements are routinely used. Optimised, synthetic promoters combining early and late elements are available (PsynE/L), but as discussed in Section 1.2.4, they may compromise virus stability.

One of the first vaccinia promoters to be employed for the expression of foreign genes was the naturally occurring early/late promoter P7.5. This promoter is still widely used, but alternatives offering greater levels of transgene expression are available for selection, provided they do not compromise stability. In an attempt to create a promoter of intermediate strength to PsynE/L and the weaker P7.5, the naturally occurring H5 promoter was modified (mH5) to provide optimised expression and stability (Wyatt *et al.*, 1996). Recombinant Vaccinia virus studies have shown that the timing and magnitude of transgene expression also influences the nature of transgene-specific immune responses; early expression is critical for the induction of cell mediated immunity (discussed in Section 1.2.4). In this regard, the attributes of mH5 are highly desirable as early expression in particular is increased relative to H5.

5.1.1 Promoter effects on antigen expression, immunogenicity and protective efficacy

There have been at least three reports describing head-to-head comparison of vaccinia promoters P7.5 and mH5, using rMVA expressing the same antigen. In a study involving the luciferase gene fused with a T-cell epitope sequence, promoters P7.5 and mH5 gave rise to

similar levels of expression over 24 hours, but the mH5 construct was able to induce a higher frequency of CD8+ antigen-responding T cells (Orubu *et al.*, 2012). In a pair of rMVA viruses expressing the model antigen ovalbumin, mH5 drove higher levels of expression than P7.5, but there was little change in the induction of CD8+ T cell responses to the immunodominant ovalbumin epitope. Meanwhile, responses to subdominant CD8+ epitopes were increased suggesting that mH5 may alter immunodominance hierarchy (Becker *et al.*, 2014). Of more relevance to the TB field, a dual rMVA expressing an HIV antigen (HIVA) and Ag85A was described in which mH5 and PsynE/L were compared to P7.5. There was evidence for increased expression by mH5 as compared to P7.5, established *in vitro* for a single infecting dose only, and increased expression did appear to correlate with increased immunogenicity. However, the results were complicated by simultaneous delivery of two separate antigens and investigation of some of the responses in the context of boosting a previously administered recombinant BCG strain. Further, the impact of increased immunological potency on protective efficacy was not assessed (Hopkins *et al.*, 2011). Overall, these studies did not define the relationship between transgene expression and the immunological potency and efficacy of the rMVA under test.

5.1.2 Optimisation of vaccinia promoter selection for TB antigen delivery

MVA85A is the only vaccinia-based TB vaccine candidate to have entered human trials and until recently, was the most advanced TB vaccine candidate under clinical development (see Section 1.4.3). Unfortunately, results for a Phase IIb efficacy study in infants were unable to demonstrate improved protection relative to the placebo control (Tameris *et al.*, 2013). Research efforts for MVA85A have since focused on alternative delivery routes and immunisation regimens. Aerosol delivery has been investigated in rhesus macaques and was proven to induce equivalent antigen-specific responses to intradermal delivery, with the added benefit that anti-vector immune responses were reduced (White *et al.*, 2013). Similar findings were subsequently reported for humans (Satti *et al.*, 2014) and most

recently, another Phase I clinical study of MVA85A was conducted to assess its ability to boost Adenovirus vectoring TB vaccine antigens (Sheehan *et al.*, 2015).

MVA85A delivers a single *M. tuberculosis* antigen (Ag85A) fused to a TPA leader sequence and utilises vaccinia promoter P7.5 for recombinant antigen expression (McShane *et al.*, 2002). The P7.5 construct showed potent immunogenicity in early clinical trials and demonstrated efficacy in pre-clinical testing. Modification/improvement of the construct may have initially been deemed unnecessary, but given the lack of efficacy observed in humans, rational optimisation might now be warranted. The impact of replacing P7.5 for mH5 should be determined in a manner consistent with assessing whether the efficacy of MVA85A, and other MVA-based TB vaccines, can be improved via optimisation of antigen expression.

5.1.3 Design and generation of rMVA for the current study

Vaccine candidate Rv0111 has induced promising levels of protective efficacy in pre-clinical small animal models (see Section 1.4.2), but has unfavourable characteristics from a formulation perspective; the amino acid sequence of the protein contains 10 putative transmembrane domains (Vipond *et al.*, 2006a; Vipond *et al.*, 2006b). MVA is a potential vector for Rv0111 delivery, but if confronted with stability issues the extent to which increased expression should be pursued is unclear. Therefore, a vaccinia promoter optimisation study for TB antigens is needed to clarify the potential benefits. The vaccine antigens selected for the current study were Ag85A and Rv0111. The vaccinia promoters selected for comparison were the promoter included in the current MVA85A clinical candidate and a promoter optimised for expression and stability - P7.5 and mH5, respectively.

5.1.4 Evaluation of protective efficacy for novel TB vaccines

Pre-clinical evaluation of TB vaccines occurs through a hierarchy of animal models where immunogenicity testing is routinely performed in mice and preliminary efficacy studies in mice and guinea pigs (McShane and Williams, 2014). As discussed in Section 1.4.2, TB vaccines need to induce Th-1 immunity and so a key readout for murine immunogenicity studies is antigen-induced IFNγ release. To evaluate protective efficacy, immunised animals are challenged with an aerosolised dose of *M. tuberculosis* (Mtb) and bacterial load in the lung and spleen determined at a set time point post-infection. Vaccine efficacy is measured according to the reduction in Mtb colony forming units (CFU) retrieved from the organs, relative to untreated and treated controls. The standard positive control in such tests is the current BCG vaccine. Dependent upon the requirements of the experiment, the test vaccine may be evaluated alone or as a booster vaccine to BCG. For ethical (and economical) reasons it is appropriate to restrict the pre-clinical efficacy testing of novel TB vaccines to those demonstrating adequate immunogenicity in a relevant model. The aims for this Chapter, described below, were implemented accordingly.

5.2 Chapter Aims

The objective for this Chapter was to test the hypothesis that vaccinia promoter selection can influence the immunogenicity and protective efficacy of TB vaccine antigens vectored by MVA. Specific objectives for this chapter were to:

For each pair of MVA:

- Investigate relative levels of antigen expression.
- Characterise viral fitness and stability.
- Measure immunological potency.
- Evaluate protective efficacy.

5.3 Results

In order to make a head-to-head comparison of vaccinia promoter effects rMVA identical in every regard except for the vaccinia promoter driving transgene expression were required. This was achieved with respect to the configuration of promoters in relation to the inserted antigen; the early and late transcriptional start sites for P7.5 and mH5 were all within a 100 bp of the Kozak sequence in which the translational start resides (detailed information regarding the sequence of each recombinant cassette is presented in Appendix III.). However, not all of the rMVA were able to be purified with the full length antigen sequence. The four rMVA vaccines constructed for the study were MVA-P7.5-Ag85A, MVA-mH5-Ag85A, MVA-P7.5-Rv0111 and MVA-mH5-Rv0111T, with the latter carrying a truncated Rv0111 gene (described in Chapter 4).

5.3.1 rMVA expressing Rv0111 under the control of P7.5 and mH5

MVA-P7.5-Rv0111 and MVA-mH5-Rv0111T antigen expression levels

Sucrose-cushion purified and titrated stocks of each of the above viruses were used to infect CEF cells at a range of concentrations. After two days, the cells were harvested in denaturing buffer for determination of antigen expression by dot blot (Section 2.3.4). The results demonstrated a dose dependent increase in the amount of V5 present in the infected cell lysate for each virus (Figure 5.1).

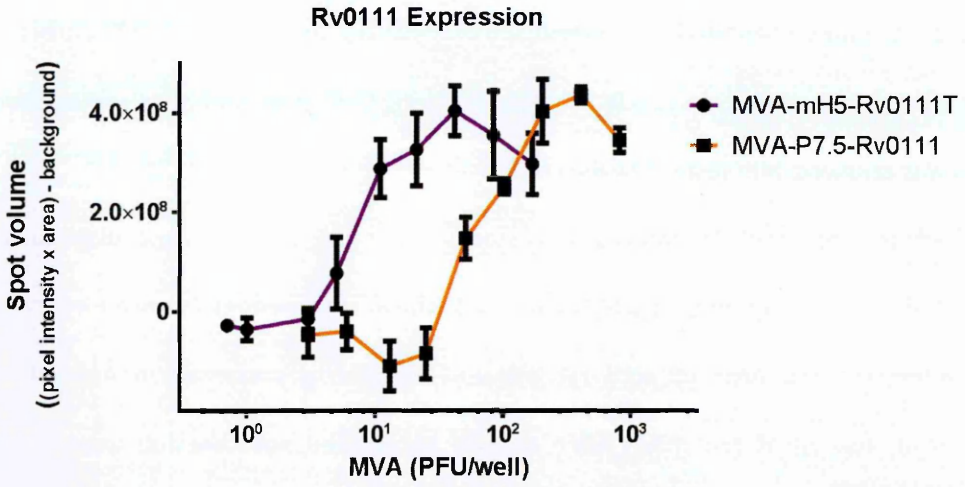


Figure 5.1 Expression of Rv0111 by rMVA. CEF cell monlayers were infected with rMVA in triplicate wells. After two days, cell lysate was transferred to nitrocellulose for detection of Rv0111 using anti-V5 and anti-mouse-HRP. Following incubation with ECL Plus chemiluminescent reagent, nitrocellulose was exposed to Hyperfilm ECL and densitometry performed to measure spot volume using ImageQuant TL image analysis software (GE Healthcare). Results for spot volume minus background (mean +/- SEM) were plotted against the concentration of infecting virus (PFU/well).

The relative expression levels of the rMVA viruses were compared. There was clear separation of the exponential region for each curve. The results provided evidence for MVA-mH5-Rv0111T being able to achieve an equivalent level of expression to MVA-P7.5-Rv0111 after infection of the cells with approximately 10 fold less virus. As stated above (Section 5.2), a comparison of vaccinia promoter effects should ideally characterise rMVA viruses that are identical in every regard except for the promoter under test. Therefore, the rate of viral replication was investigated to determine whether the increased expression observed for MVA-mH5-Rv0111T was due to superior viral fitness, conferred by the expression of a truncated Rv0111 gene.

Relative fitness of MVA-P7.5-Rv0111 and MVA-mH5-Rv0111T

In the above experiment, an additional well of CEF cells was infected with each serial dilution of rMVA. When cells were harvested for analysis by dot blot, the additional well

was harvested for titration and determination of rMVA concentration (PFU/ml). The results enabled dot blot densitometry data to be re-plotted against PFU/well as measured at the end of the incubation period (Figure 5.2).

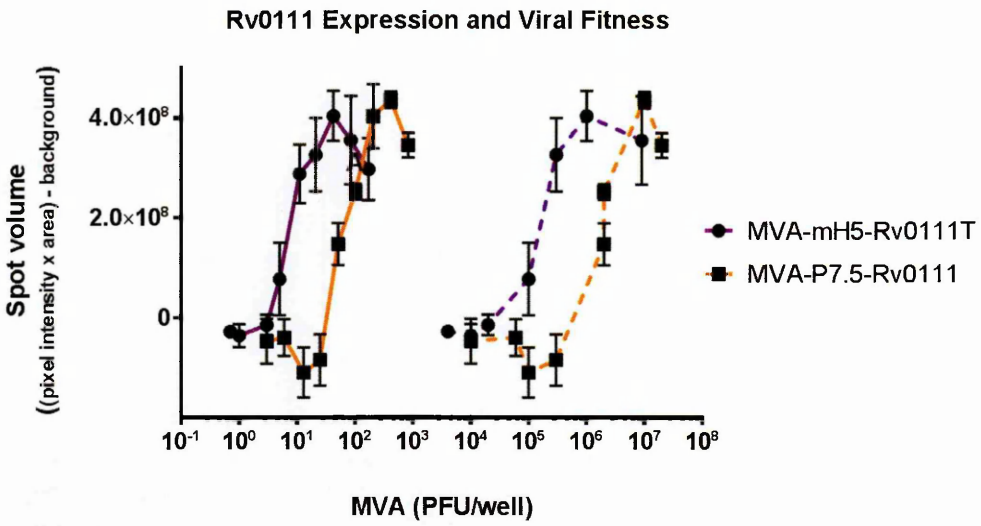


Figure 5.2 Amplification of rMVA expressing Rv0111. Data were as described for Figure 5.1. The densitometry results (mean ± SEM) were plotted against the concentration of infecting virus (solid line) and in addition, the viral titre at the end of the experiment (dashed line).

After two days incubation, titration of the infected CEF cell lysate revealed that the number of PFU per well had increased for both viruses across the range of infecting concentrations. There was little difference in the degree to which each of the viruses had been amplified. The average fold increase for MVA-mH5-Rv0111T and MVA-P7.5-Rv0111 were similar (2.6×10^4 and 1.8×10^4, respectively).

The similar level of amplification observed for MVA-mH5-Rv0111T and MVA-P7.5-Rv0111 was inconsistent with the observation that MVA-P7.5-Rv0111 plaques regularly appeared to express more GFP (visualised during routine inspection of the cell layer by inverted fluorescent microscopy). By way of a further simple estimate of viral fitness, titres for individual plaques were determined as reported for rMVA generated during the development of mH5 (Wyatt *et al.*, 1996). Two representative plaques for each rMVA

expressing Rv0111 were isolated and diluted for determination of PFU per plaque. The results confirmed that the growth rate for the two rMVA was similar and that the rate of replication for MVA-P7.5-Rv0111 might have been closer to the parent virus (Figure 5.3).

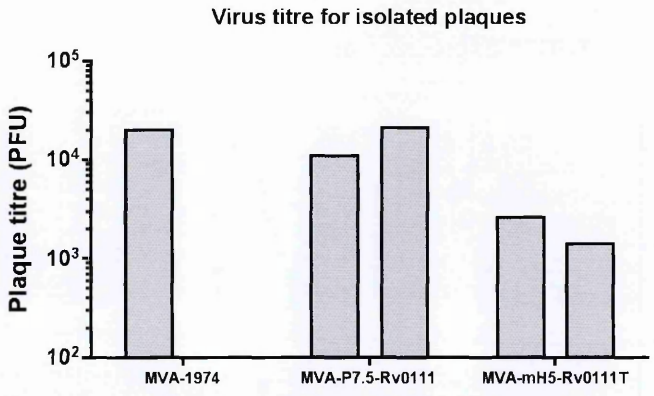


Figure 5.3 Plaque titre estimates for rMVA expressing Rv0111. CEF cells were infected with each of the three viruses. Three days later, representative plaques were isolated and titrated for determination of their concentration. Cell monolayers were immunostained with anti-vaccinia to make viral plaques visible for counting. Data are the mean for duplicate counts.

MVA-P7.5-Rv0111 and MVA-mH5-Rv0111T Th-1 immune responses

To investigate the impact of transgene expression on antigen immunogenicity, mice were immunised with each rMVA to determine antigen-specific Th-1 immune responses. After two doses of MVA, splenocytes were harvested for restimulation *ex vivo* with overlapping antigen-specific peptides representing Rv0111T (as described in Sections 2.4.2 and 2.4.3). MVA-mH5-Rv0111T induced a measureable antigen-specific Th-1 response (Figure 5.4). Responses to MVA-P7.5-Rv0111 were not significantly increased above background levels of IFNγ release.

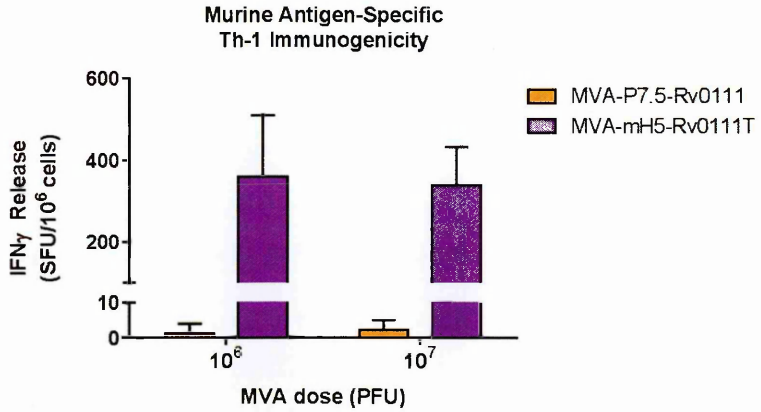


Figure 5.4 Immunogenicity of rMVA virus expressing Rv0111. Groups of C57Bl/6 mice (n=5) were immunised intramuscularly with rMVA at a dose of 1 x 10^6 or 1 x 10^7 PFU, twice, at two weeks apart. Two weeks after the final immunisation, splenocytes were harvested for restimulation with overlapping peptides representing Rv0111T. The number of cells induced to release IFNγ was measured by ELISpot assay (Mabtech). Results were plotted as the number of spot forming units (SFU) per million cells (mean +/- SEM) after subtraction of background, i.e. SFU/10^6 measured in the presence of cell culture medium only.

No dose dependent effects were seen for the two doses of MVA-P7.5-Rv0111 and MVA-mH5-Rv0111T administered (1 x 10^6 and 1 x 10^7 PFU). Variability within the assay was greater than that seen on other occasions and a repeat assay would be required to determine whether this observation was genuine. Poor antigen recall responses for MVA vectoring Rv0111 under the control of promoter P7.5 had been seen previously; an rMVA construct prepared outside of the current study was only measurably immunogenic when administered as part of a heterologous prime-boost regimen (See Section 1.4.3, Table 1.5). The rationale for administering an MVA-only regimen in the present study was to ensure that only MVA-induced and, thereby, promoter-mediated effects were investigated.

Given that MVA-P7.5-Rv0111 was so weakly immunogenic following an MVA-only vaccination regimen, it was deemed inappropriate to repeat the immunogenicity test, or progress this pair of rMVA viruses to a protective efficacy study in which mice would be immunised and then infected with *M. tuberculosis*.

5.3.2 rMVA expressing Ag85A under the control of P7.5 and mH5

Recombinant MVA carrying the same Ag85A expression cassette were successfully isolated and purified. Subsequently, a more in-depth comparison of their features was carried out. In the first instance, the expression and fitness experiment performed for rMVA expressing Rv0111 was modified to provide detailed time-course information. CEF were infected with rMVA expressing Ag85A to enable the relationship between dose, fitness and expression to be defined.

Relative fitness of MVA-mH5-Ag85A and MVA-P7.5-Ag85A

Purified stocks of rMVA were diluted to infect CEF cell monolayers at approximately 10^4 PFU per well, equivalent to an MOI of 0.1 PFU/cell. Sufficient multi-well plates were infected to permit harvesting of cell lysate at set time points from triplicate wells. One sample from each time point, for both viruses, was titrated in parallel to obtain 3 sets of viral growth data (Figure 5.5). The concentration of MVA (PFU/ml) was determined by counting GFP-expressing plaques (made possible because the selection marker had been inserted for stable expression).

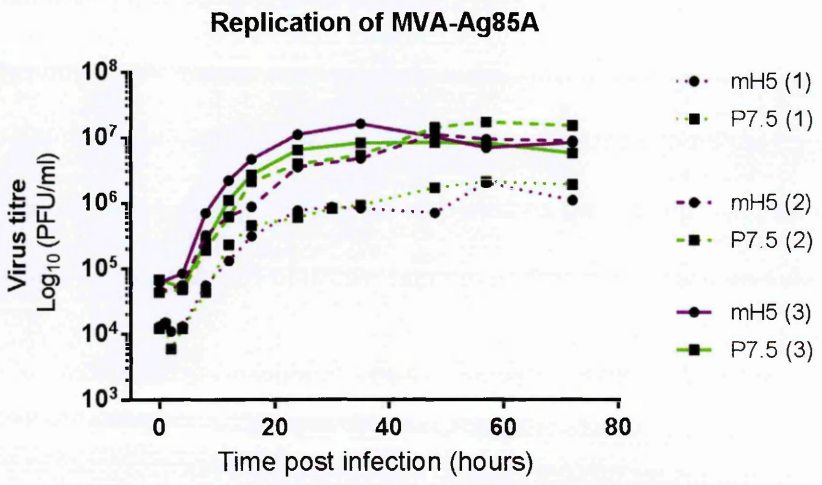


Figure 5.5 Growth curves for rMVA expressing Ag85A. CEF monolayers were infected with MVA at an MOI of 0.1 PFU/cell and then harvested at set time points in triplicate. The titre of each sample was determined by titration on CEF cells and counting of GFP-expressing plaques.

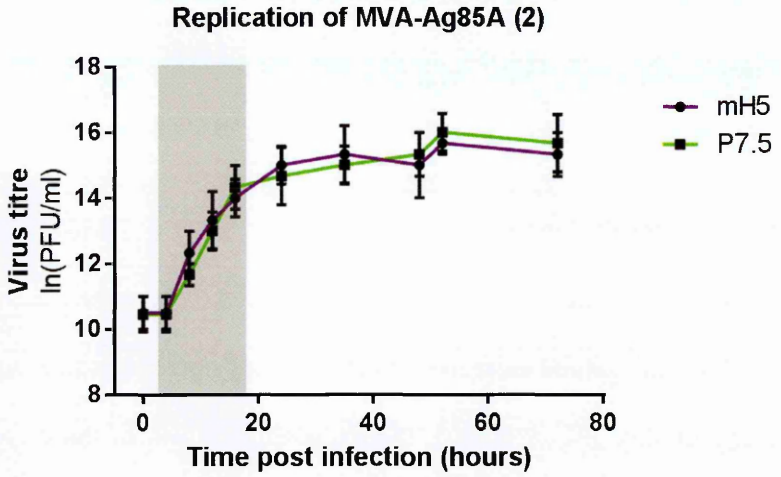
Each pair of viral replication curves was very similar. Overall, the first set of titration data was lower. This was later attributed to a slight difference in the density of the CEF cells used for titration. The wider reaching implications for this observation are discussed in Section 5.4.1.

A statistical method for the comparison of viral growth curves was applied to investigate differences in the rate of viral replication for the two viruses (Wang and Bushman, 2006). In the first instance, the natural logarithm (ln) of mean values for virus titre was calculated and plotted against time (Figure 5.6A). The exponential region of the curves was then selected, by eye, for analysis by linear regression. Data falling within the exponential region were replotted to permit the fitting of curves (Figure 5.6B). The slope of each curve was calculated and statistically compared (GraphPad Prism). The results confirmed that there were no statistically significant differences between the rates of replication for MVA-mH5-Ag85A and MVA-P7.5-Ag85A (summarised in Table 5.1).

Table 5.1 Analysis of viral replication by linear regression. Growth curves for rMVA expressing Ag85A (exponential region only) were analysed to find the line of best fit and to statistically compare the slopes (GraphPad Prism). Slope constants and 95% confidence intervals for the slope are shown. The slope was equivalent to the rate of change in virus titre per hour (ln(PFU/ml)/hr). Statistical comparisons were made between pairs of growth curves for which viral titres has been determined in parallel. None of the differences were statistically significant ($P > 0.05$).

MVA-Ag85A	Slope ln(PFU/ml)/hr	95% Confidence Interval	P value
Plot 1 (dotted line)			
mH5	0.2568	0.1537 – 0.3599	0.236
P7.5	0.3162	0.1645 – 0.4678	
Plot 2 (dashed line)			
mH5	0.2218	-0.0251 – 0.4687	0.185
P7.5	0.3142	0.2854 – 0.3431	
Plot 3 (solid line)			
mH5	0.3285	0.0917 – 0.5653	0.942
P7.5	0.3332	0.2207 – 0.4458	

A



B

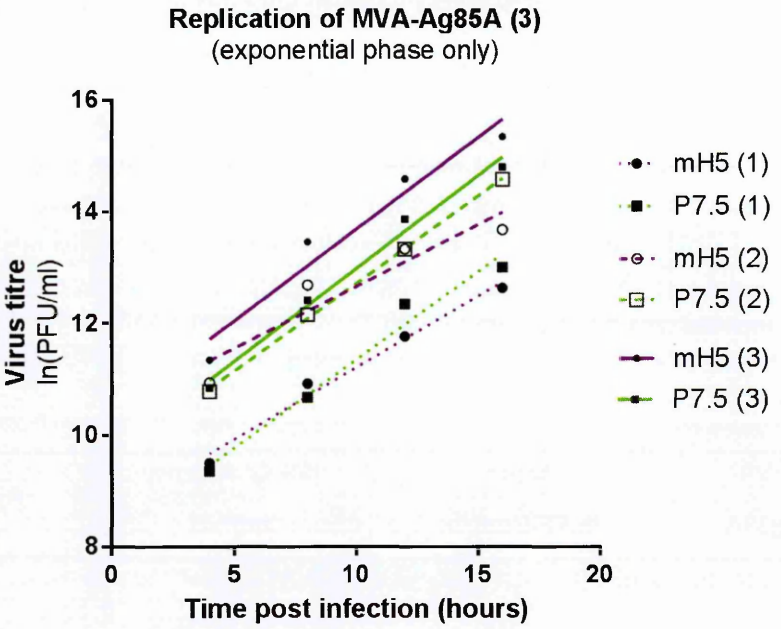


Figure 5.6 Statistical comparison of viral growth curves for rMVA expressing Ag85A
A) The natural logarithm (ln) of virus titre was calculated and plotted against time and then the exponential region of the data was selected by eye (grey box). B) Individual replicates from the exponential region were plotted for analysis by linear regression (GraphPad Prism). The line of best fit was added to each data set and statistical comparison carried out (Table 5.1).

MVA-P7.5-Ag85A and MVA-mH5-Ag85A antigen expression levels

In parallel with the comparison of viral rates of replication, samples were harvested for measurement of Ag85A expression over time. Cell lysate samples were investigated for presence of the V5 tag by dot blot, as described in Section 2.3.4.

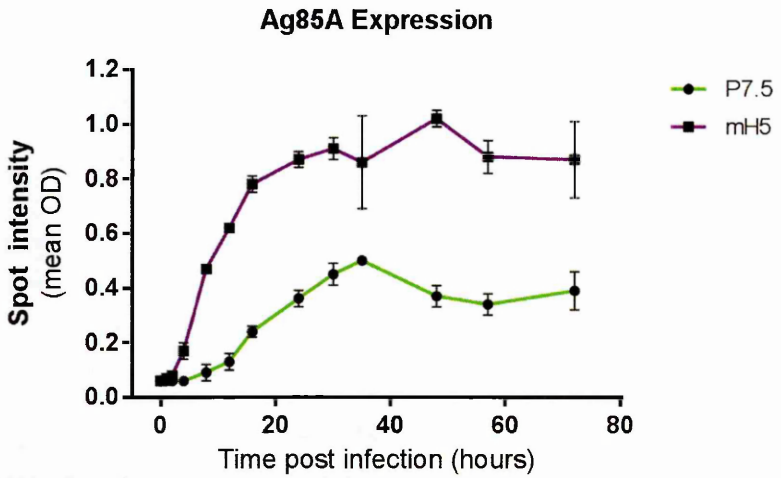


Figure 5.7 Expression of Ag85A by rMVA. CEF monolayers were infected with MVA at an MOI of 0.1 PFU/cell and then harvested at set time points. Cell lysate was transferred to nitrocellulose to create triplicate spots before immunodetection with anti-V5 and anti-mouse-HRP. Following incubation with ECL Plus chemiluminescent reagent, nitrocellulose was exposed to Hyperfilm ECL and densitometry performed to measure the mean optical density (OD) of each spot using Quantity One 4.6.9 ID analysis software (Bio-Rad). Results (mean +/- SEM) were plotted against the time of sample harvest.

From as early as four hours post-infection, MVA-mH5-Ag85A generated measurably more Ag85A than MVA-P7.5-Ag85A. Values for spot intensity (mean +/- SEM) were 0.171 +/- 0.032 and 0.062 +/- 0.002, respectively. The difference between these values was statistically significant (T test, P = 0.03).

The expression of Ag85A from both viruses appeared to plateau at around 30 hours post-infection. Viral concentration (PFU/ml) had been determined for a separate set of samples that were infected and harvested in parallel (Figure 5.5 and Figure 5.6A). Growth curves

had revealed that viral amplification had also reached, or was nearing, a plateau at 30 hours post-infection. To better understand the amount of Ag85A produced in the context of the amount of virus present, the data were replotted against virus titre (Figure 5.8). MVA-P7.5-Ag85A was able to produce equivalent amounts of recombinant antigen to MVA-mH5-Ag85A when present at approximately a 10-fold greater concentration.

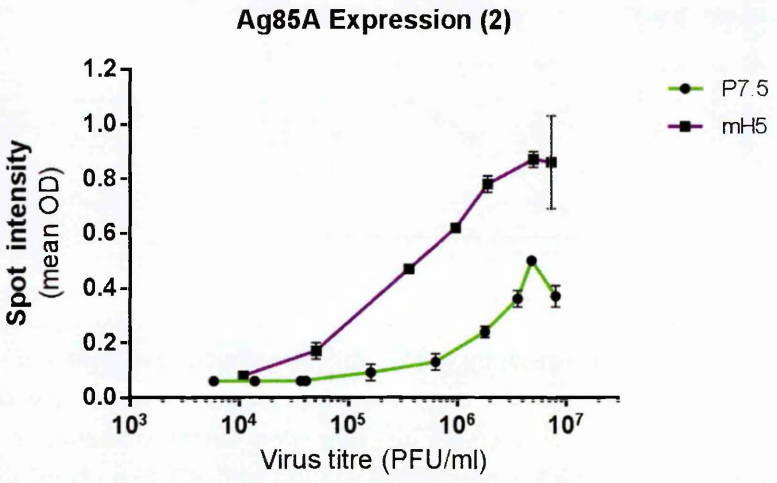


Figure 5.8 Dose dependent expression of Ag85A by rMVA. Densitometry data are as described in Figure 5.7. The results were plotted against viral concentration (PFU/ml), equivalent to total PFU because the titrated samples had a volume of 1 ml.

MVA-P7.5-Ag85A and MVA-mH5-Ag85A Th-1 immune responses

The immunogenicity of MVA-P7.5-Ag85A and MVA-mH5-Ag85A were compared in mice. A vector-only control group was included in the study to monitor for MVA-mediated effects. Following immunisation, splenocytes were harvested for measurement of antigen-specific Th-1 immune responses (Figure 5.9).

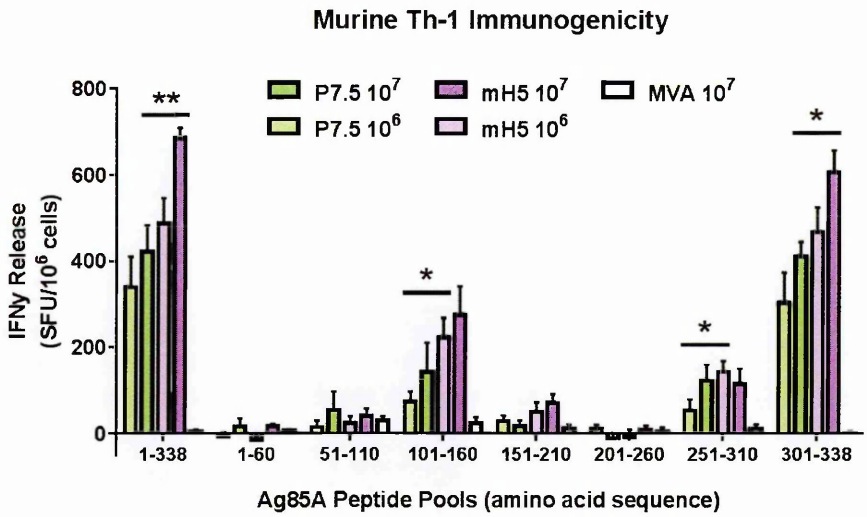


Figure 5.9 Immunological potency of rMVA expressing Ag85A. Groups of C57Bl/6 mice (n=5) were immunised intramuscularly with rMVA (1 x 10^6 or 1 x 10^7 PFU) expressing Ag85A under the control of vaccinia promoter P7.5 or mH5. Another group of mice received wild-type MVA (1 x 10^7 PFU). All immunisations were given twice at an interval of two weeks. A further two weeks after the final immunisation, splenocytes were harvested for restimulation with overlapping Ag85A peptides of 15 amino acids, overlapping by 10. Peptide pools representing the whole antigen (66 peptides; amino acids 1-338) or sections from the N to C terminus were applied (up to 10 peptides). The number of cells induced to release IFNγ was measured by ELISpot assay (Mabtech). Results were plotted as the number of spot forming units (SFU) per million cells (mean +/- SEM) after subtraction of background, i.e. SFU/10^6 measured in the presence of cell culture medium only. Results were compared by t-test (*$P < 0.05$, ** $P < 0.01$). The data are representative of two separate mouse experiments, one of which was subjected to repeat analysis on cryopreserved cells (shown) to confirm reproducibility of the results.

Only groups receiving rMVA expressing Ag85A were able to demonstrate Ag85A-specific immune responses. There was no detectable IFNγ release in samples derived from mice immunised with wild-type MVA (MVA 10^7). The highest responses overall (fractionally) were to the 'Whole Antigen' peptide pool. Results for the other peptide pools provided evidence for at least three immunodominant epitopes residing within amino acids 301-338, 101-160 and 251-310 (listed in decreasing order of magnitude). Responses for each rMVA virus were generally dose dependent and higher where Ag85A was expressed under the

control of the mH5 promoter; the immunogenicity of MVA-P7.5-Ag85A approached that of MVA-mH5-Ag85A when used at a 10-fold higher dose.

In order to investigate anti-vector T-cell responses, splenocytes were restimulated with Vaccinia virus immunodominant CD8+ epitopes: B8R, A19L, A47L, A42R and K3L (Tscharke *et al.*, 2005). The peptides induced IFNγ release and demonstrated dose dependent responses for the rMVA expressing Ag85A, which had been administered at two different doses (Figure 5.10).

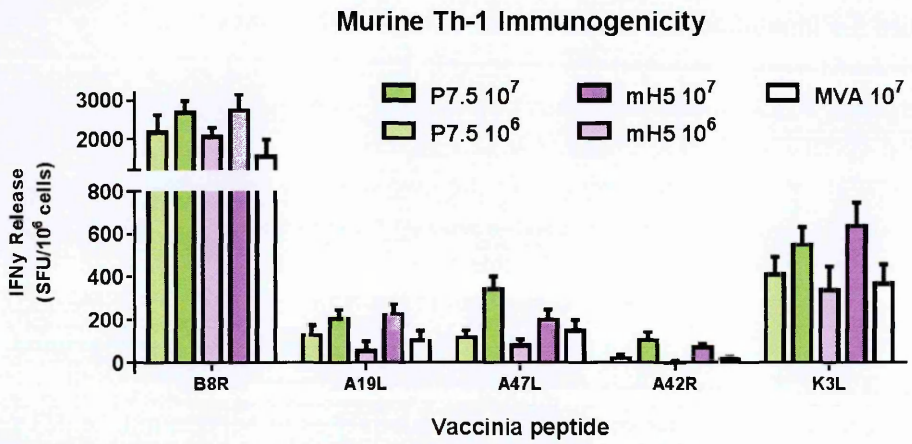


Figure 5.10 Immunes responses to vaccinia derived peptides. Groups of C57Bl/6 mice (n=5) were immunised intramuscularly with rMVA (1 x 10^6 or 1 x 10^7 PFU) expressing Ag85A under the control of vaccinia promoter P7.5 or mH5. Another group of mice received wild-type MVA (1 x 10^7 PFU). All immunisations were given twice at an interval of two weeks. A further two weeks after the final immunisation, splenocytes were harvested for restimulation with vaccinia derived peptides (8-10 residues). The number of cells induced to release IFNγ was measured by ELISpot assay (Mabtech). Results were plotted as the number of spot forming units (SFU) per million cells (mean +/- SEM) after subtraction of background, i.e. SFU/10^6 measured in the presence of cell culture medium only. The data are representative of results observed for fresh and cryopreserved cells (shown).

Responses for mice immunised with wild-type MVA provided evidence for the actual dose given being closer to 10^6 PFU as opposed to the intended 10^7 PFU. Having confirmed that the four rMVA expressing Ag85A were all immunogenic and able to induce Th-1 antigen-specific immune responses, their protective efficacy was evaluated in mice.

MVA-mH5-Ag85A affords equivalent protection to MVA-P7.5-Ag85A at a 10 fold lower dose

Groups of C57Bl/6 mice were immunised with rMVA and wild-type MVA as described above. Whilst a proportion of each group was used for antigen-recall studies (Figures 5.9 and 5.10) the remainder went on to be challenged with an aerosolised dose of *M. tuberculosis* (Section 2.4.5). In addition to the groups immunised with recombinant and wild-type MVA, the study was set up to include unvaccinated and BCG controls, to provide negative and positive control data, respectively. A schematic of the study vaccination and challenge schedule is shown in Figure 5.11.

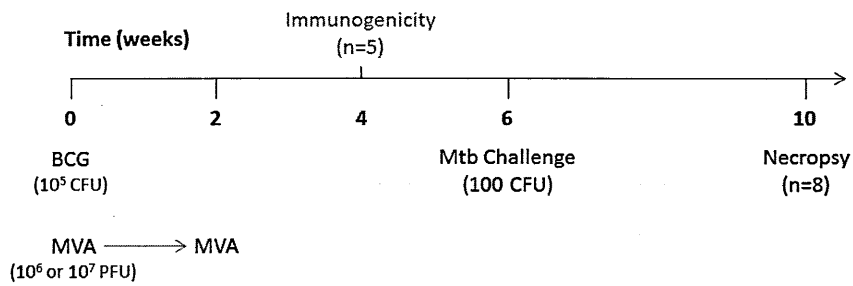


Figure 5.11 Murine efficacy study schedule. Groups of C57Bl/6 mice (n=8) were immunised intramuscularly with rMVA (1×10^6 or 1×10^7 PFU) or native MVA (1×10^7 PFU), twice, at two weeks apart. Mice in the BCG control group were immunised subcutaneously with *M. bovis* BCG Danish (SSI, Denmark) at a dose of 1×10^5 CFU. Six weeks after the first immunisations, vaccinated and unvaccinated mice (n=8) were challenged with aerosolised Mtb to provide an inhaled retained dose of approximately 100 CFU. Four weeks after infection, the mice were necropsied and lung and spleen removed. Organs were processed for determination of Mtb bacterial load.

Lungs and spleens were harvested for determination of bacterial load at four weeks post-challenge. The organs were processed for serial dilution and plating on selective medium as described in Section 2.4.5. Total CFU per organ was calculated for each animal and plotted against a log scale (Figure 5.12).

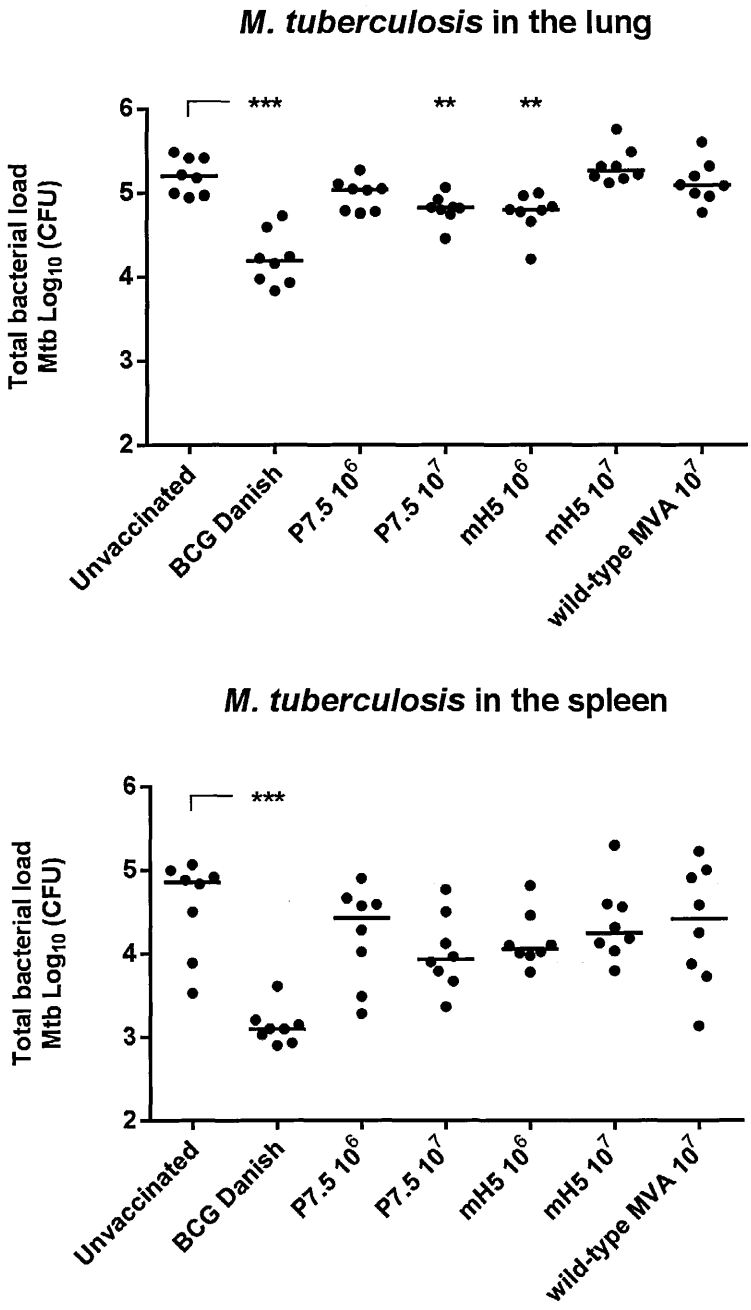


Figure 5.12 Protective efficacy of rMVA expressing Ag85A. Groups of C57Bl/6 mice (n=8) were vaccinated with *M. bovis* BCG Danish (SSI, Denmark), rMVA expressing Ag85A under the control of vaccinia promoter P7.5 or mH5, or wild-type MVA. MVA was given at a dose of 1 x 10^6 PFU or 1 x 10^7 PFU. Immunised mice were challenged with *M. tuberculosis* and four weeks later the bacterial load in the lung and spleen was determined. Results for individual mice and group median were plotted against a logarithmic scale. Statistically significant reductions relative to the unvaccinated control are highlighted (**P < 0.01, ***P< 0.001; Mann-Whitney, GraphPad Prism).

The data for the negative (unvaccinated) and positive (BCG) controls were as expected. There was a 1 – 2 log reduction in the median bacterial load for the BCG group compared to the unvaccinated group and in both organs this difference was statistically significant (Mann Whitney P<0.001). No statistically significant reductions were seen in the lung or spleen for the vector-only control group (wild-type MVA).

MVA-P7.5-Ag85A was able to induce modest reductions in the bacterial load of the lung and spleen and did so dose dependently. At a dose of 1 x 10^7 PFU, a statistically significant reduction relative to unvaccinated controls was observed in the lung (Mann Whitney P = 0.0011). MVA-mH5-Ag85A was able to achieve the same result using a dose that was 10 fold lower (P= 0.0011). Curiously, in mice immunised with a higher dose of MVA-mH5-Ag85A (1 x 10^7 PFU), protective efficacy in the lung was reduced such that bacterial levels were equal to those seen in the unvaccinated control. However, there may have been reduced dissemination of Mtb to the spleen since the median value CFU was 0.6 $\log_{10}$ lower in the MVA-mH5-Ag85A group than the unvaccinated control.

Stability of MVA-P7.5-Ag85A and MVA-mH5-Ag85A over multiple passages

To confirm that any benefits conferred by increased antigen expression were not offset by decreased stability of the recombinant cassette, the rMVA were characterised over an extended number of passages on CEF cells. The expression, fitness and immunogenicity of the MVA-P7.5-Ag85A and MVA-mH5-Ag85A, described above, had been evaluated after 10-11 passages, as this was the total number of passages required to purify and amplify the viruses (see Section 4.3.1, Table 4.5). Further passages were undertaken up to a total of 20. After each, analytical PCR of the insertion site (Figure 5.13) and Western blot for detection of the expression product (not shown) was performed. The results confirmed no change in the insert size or expression product.

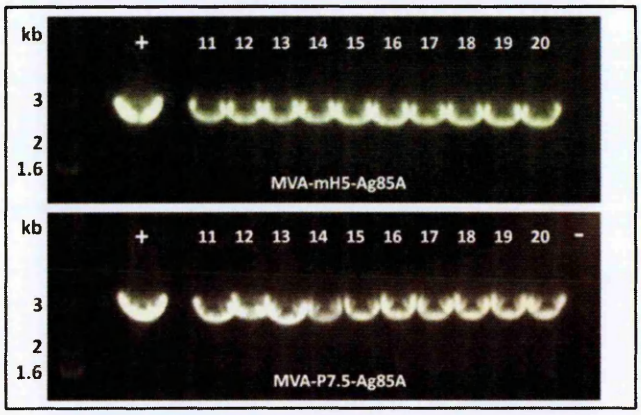


Figure 5.13 Analytical PCR of the Del III insertion site containing Ag85A. MVA-mH5-Ag85A and MVA-P7.5-Ag85A were passaged up to 20 times. After each passage a sample of infected CEF cell lysate was subjected to gDNA extraction and interrogation of the insertion site via PCR. The PCR was performed using primers binding either side of the insertion site. Amplicons were separated by gel electrophoresis. Transfer plasmid DNA was used in the positive control (+) reaction for each construct and gave a product of the expected size (MVA-mH5-Ag85A, 2680 bp; MVA-P7.5-Ag85A, 2863 bp). Water was used in place of DNA template to provide a negative control (-). A single band of the same size in all reactions confirmed that the insert was present and had not been truncated.

5.4 Discussion

A head-to head comparison was undertaken for two pairs of rMVA expressing a TB antigen under the control of vaccinia promoter P7.5 or mH5. MVA-P7.5-Rv0111 and MVA-mH5-Rv0111T were compared, but with the caveat that the expression products were not identical. The rMVA expressing the Ag85A gene were identical in every regard and so a more comprehensive comparison was undertaken.

5.4.1 Promoter effects on fitness and stability

As discussed above in Section 5.1, and in Section 1.2.4, vaccinia promoter selection must balance optimised expression with stability of the recombinant insert. Heightened levels of antigen expression may lead to increased immunological potency, but this is only beneficial provided the vaccine is sufficiently robust for product-scale manufacture. Mtb gene Rv0111 was so unstable under the control of mH5 that the desired recombinant could not be isolated. A truncated version of the gene was stable and immunogenic and so may offer

a viable alternative. Optimised expression for a truncated HIV antigen is being pursued as a clinical candidate (Wyatt *et al.*, 2009; Wyatt *et al.*, 1996). Expression of Ag85A under the control of mH5 did not impact negatively on viral fitness or on stability of the recombinant insert.

An important observation arising from the titration of viral samples was that differences in the estimated titre were most heavily impacted upon by technical variability in the plating of the virus, i.e. by differences in the cell monolayers upon which they were plated (Figure 5.5). For this reason the viral titres of rMVA that were destined to be compared, in this Chapter and in Chapter 6, were always titrated in parallel.

5.4.2 Promoter effects on antigen expression

The first comparison of P7.5 with mH5 was made upon initial modification of the naturally occurring early/late promoter H5 and expression investigated using β-galactosidase production by rVV-LacZ (Wyatt *et al.*, 1996). A five-fold increase in antigen expression was measured at a specified MOI, but not over a range of doses. Similarly, other published comparisons of P7.5 and mH5 have described expression following infection at one dose and over a time-course, e.g. as performed for expression of the luciferase reporter gene (Orubu *et al.*, 2012) and the model antigen ovalbumin (Becker *et al.*, 2014). In each of these examples the difference in expression was less pronounced, and it was not possible to gauge whether equivalent levels of expression might have been achieved by only a slight change in viral titre.

In the current study, antigen expression was investigated via dot blot to provide semi-quantitative data; the amount of antigen expressed was not defined, but characterised for each pair of rMVA in parallel to enable relative levels of antigen expression to be compared. Further, results were obtained across a range of doses. Both MVA-mH5-Rv0111T and MVA-mH5-Ag85A produced an equivalent amount of antigen to their P7.5 counterpart, but at a 10-fold lower concentration. In both cases, the rate of viral amplification was confirmed

not to have influenced the result. The data provided evidence for optimised expression having the potential to provide optimal vaccine antigen delivery at a reduced dose of viral vector to an extent not previously reported. It should be noted that upon inoculation *in vivo*, rMVA expression will be limited to one round of viral protein production as the virus is replication deficient. However, relative differences in the strength of antigen expression are still expected to be manifest.

5.4.3 The correlation between *in vitro* expression and *in vivo* immunogenicity

The induction of Th-1 immunity in C57Bl/6 mice was measured to assess the immunological potency of each rMVA. After two doses of rMVA, *ex vivo* antigen-recall responses were measured by murine IFNγ ELISpot assay. The immunogenicity of each pair of rMVA viruses appeared to correlate with their relative levels of antigen expression. In each experiment, splenocytes were restimulated with overlapping peptides representative of the amino acid sequence of the recombinant antigen. Peptides were 15 residues in length and overlapped by 10 residues and so were designed to evoke CD4+ and CD8+ T cell responses (Rodda, 2002).

Splenocytes from MVA-P7.5-Rv0111-immunised mice did not release IFNγ in the presence of Rv0111T peptides, but equivalent samples from MVA-mH5-Rv0111T–immunised mice demonstrated an antigen-specific recall response. Thus, evidence to support increased immunogenicity of the construct containing mH5 was obtained. The results highlighted the importance of including Vaccinia-derived peptides in future experiments, in order to demonstrate successful and equivalent delivery of the vector. Responses to rMVA expressing Ag85A were investigated more fully.

The number of antigen-responding T cells correlated with the immunising dose after immunisation with MVA-P7.5-Ag85A and MVA-mH5-Ag85A. Consistent with the observation that MVA-P7.5-Ag85A expressed an equivalent amount of recombinant antigen to MVA-mH5-Ag85A when used at a 10 fold higher dose, the P7.5 and mH5 constructs

induced a similar number of IFNγ secreting cells after two inoculations with 10^7 and 10^6 PFU, respectively.

The profile of the response to Ag85A was investigated using a whole-antigen peptide pool and sub-pools representing the length of the Ag85A amino acid sequence. The results provided evidence for at least three immunodominant epitopes which remained consistent between the two rMVA. The most immunogenic epitope was in the terminal region of the protein (amino acids 301-338) as has been reported previously for C57Bl/6 mice immunised with an Ag85A vectored by plasmid DNA (D'Souza *et al.*, 2003) and by Adenovirus (Radosevic *et al.*, 2007).

Responses to Vaccinia peptides were measured to investigate vector immunity and provide confirmation of the doses of MVA given. All of the samples were induced to release IFNγ in a pattern consistent with that reported previously for C57Bl/6 mice (Cottingham *et al.*, 2008; Tscharke *et al.*, 2005). The IFNγ release data confirmed that the rMVA expressing Ag85A had been delivered at two different doses and that the doses of the two rMVA had been equivalent to one another; no statistically significant differences between MVA-P7.5-Ag85A and MVA-mH5-Ag85A at each dose were observed. Responses for mice inoculated with 10^7 PFU were approximately 30% higher than those measured in mice inoculated with 10^6 PFU (this level of increase was also observed following restimulation with Ag85A peptides). The results provided evidence for the wild-type MVA group having been given a lower dose, closer to 10^6 PFU than the intended 10^7 PFU.

5.4.4 The impact of optimised expression on protective efficacy

Protective efficacy was investigated in C57Bl/6 mice after challenge with *M. tuberculosis*. All of the animals were infected as demonstrated by countable CFU in lung and spleen samples at four weeks post-infection. Data for the control groups were as expected; BCG vaccination led to a 1-2 $\log_{10}$ drop in the bacterial load relative to the unvaccinated group and this decrease was very highly significant in both the lung and the spleen ($P = 0.0002$

and P = 0.0003, respectively). Mice immunised with wild-type MVA were not expected to be protected from initial infection or dissemination of disease and no statistically significant differences in bacterial load were observed for either tissue relative to the unvaccinated group.

The rMVA test groups were assessed for their ability to lower the bacterial load in the organs relative to the unvaccinated control. Protective efficacy better than BCG was not expected as the vaccines are TB sub-units designed to boost BCG. As discussed in Section 1.4, BCG is a live, attenuated strain of *M. bovis*. It expresses multiple antigens and induces a relevant immune response for protection against TB, but requires improvement. Subunit vaccines are designed to be applied in the context of prior BCG vaccination, to boost responses to relevant antigens. Their efficacy can be evaluated in animal models in which BCG is used as the positive control, but it is often preferable to evaluate their effects relative to unvaccinated groups as BCG is very effective in small animal models making booting effects difficult to resolve.

Inoculation with MVA-P7.5-Ag85A at two doses of 10^6 PFU did not reduce the bacterial load in the lung relative to the unvaccinated control. However, the median $\log_{10}$ CFU was reduced (-0.38) after two doses of 10^7 PFU, and the difference was statistically significant (P = 0.0011). In keeping with the observation that MVA-mH5-Ag85A could induce equivalent immune responses at a 10 fold lower dose, equivalent protection was observed following immunisation with two doses of 10^6 PFU; the median $\log_{10}$ CFU was reduced (-0.40) and the difference was statistically significant (P = 0.0011). For both viruses there was a similar level of dissemination to the spleen which was not significantly different to the negative control. After immunisation with a higher dose, MVA-mH5-Ag85A was less protective and a possible explanation for this could be the strength of antigen delivery.

Successful vaccination is dependent upon the induction of immunological memory. As outlined in Section 1.1.2, immunisation leads to the expansion of an antigen-specific T cell

effector population (T_E) which can then differentiate to provide a reservoir of memory cells. Circulating effector memory (T_{EM}) cells provide immediate antigen recall, while central memory (T_{CM}) cells reside in lymphoid tissues to mount a response upon interaction with infected APC (Sallusto *et al.*, 2010). It is the T_{CM} population in particular that is critical for vaccine induced T cell immunity.

It has been shown that excessive antigen stimulation can drive strong effector responses with the result that T cells are terminally differentiated and memory cell populations depleted (Masopust *et al.*, 2006). Higher antigen doses and/or shorter intervals between priming and boosting immunisations can, therefore, reduce central memory and protective efficacy despite having induced stronger effector memory functions, such as those measured by IFNγ ELISpot assay. Thus, vaccination must strike a balance between the expansion of T_E cells and the retention of sufficient proliferative potential.

Excessive T cell differentiation may explain the lack of protection observed for the potently immunogenic MVA-mH5-Ag85A vaccine. The rMVA had been administered twice at two week intervals. In a clinical setting, a subunit vaccine would be given as a boost to BCG after a longer intervening period. A dose dependent reduction in protective efficacy has been reported for a novel TB vaccine tested in mice (Aagaard *et al.*, 2009). Increased doses reduced the quality of the immune response with the result that protective efficacy was lost. This may have happened in this study and could be investigated by the assessment of further immune parameters, in particular by cytokine profiling of antigen-specific T cell subsets.

5.5 Conclusions

Pre-clinical efficacy for a rMVA-based vaccine was first demonstrated in mice following delivery of influenza virus antigens (Sutter *et al.*, 1994). The study reported a correlation between the immunising dose of rMVA, levels of antigen-specific antibody subsequently

induced and the resulting survival rates against influenza challenge. Studies performed since have provided further evidence for a positive correlation between antigen expression and immunogenicity, but the extent to which optimised expression can increase immunological potency and in turn protective efficacy is not always apparent. Two limiting factors are inadequate investigation of effects over a range of doses and the absence of efficacy data.

The results presented here have examined the relationship between rMVA titre, antigen expression, immunogenicity and efficacy. They support the view that optimisation of expression is of vital importance as it has the capacity to reduce the inoculating doses of rMVA required to achieve protection. This has the potential to reduce subsequent anti-vector immune responses and may impact favourably on the cost per dose of the vaccine.

Chapter 6 Do antigen fusions have the potential to make the immune response to rMVA-vectored TB antigens more diverse?

6.1 Introduction

Recombinant MVA viruses deliver vaccine antigens in the form of nucleic acid. Post-immunisation, the antigen must be expressed before an immunological response can be evoked. In order to influence the way in which an MVA-vectored antigen is translated and processed, it is possible to genetically engineer a sorting signal to be fused to the amino (N) and/or carboxyl (C) terminus of the protein. This has the effect of influencing the manner in which the antigen is processed upon translation which in turn can influence the nature of the immune response that is stimulated. As summarised in Section 1.2.6, a number of antigen fusion sequences have been investigated for this purpose. The leader sequence most commonly used in TB vaccine research is the signal peptide from the human tissue plasminogen activator gene (TPA).

6.1.1 The TPA signal peptide

The first comparison of antigen delivery with and without a TPA leader was performed using plasmid DNA vaccines vectoring a malaria antigen (Haddad *et al.*, 1997). The authors had speculated that direction of the gene product to the secretory pathway would increase humoral responses. Whilst TPA-dependent secretion via the endoplasmic reticulum (ER) was observed, increased antibody responses, however, were not. Conversely, TB antigen studies have demonstrated that fusion to the TPA leader sequence can increase murine humoral and cell-mediated immune responses. Evidence for increased expression and enhanced murine immunogenicity has been demonstrated for plasmid DNA vectoring Ag85A (Montgomery *et al.*, 1997) and four other TB antigens (Li *et al.*, 1999), as well as for rVV delivering Ag85A, Ag85B or Ag85C (Malin *et al.*, 2000). Importantly, Li *et al.* also investigated protective efficacy. Following Mtb challenge, the bacterial load in the lung and

spleen of immunised mice was consistently reduced for groups receiving TPA-antigen fusions as compared to their non-TPA counterparts (Li *et al.*, 1999). The majority of recombinant TB vaccines researched since have employed a TPA leader sequence, including the clinical candidate MVA85A (McShane *et al.*, 2002) and more recent plasmid DNA (Mir *et al.*, 2009) and Adenovirus vaccines (Dicks *et al.*, 2015; Mu *et al.*, 2009).

6.1.2 Enhanced humoral immunity for novel TB vaccines

As discussed in Section 1.4.2, the induction of Th-1 immunity and IFNγ release is considered central to the development of an improved TB vaccine. However, recent reports have highlighted the potential for antibody responses to contribute to TB protection and, in particular, the prevention of initial infection (Achkar and Casadevall, 2013; Andersen and Woodworth, 2014). Cell surface expression of an antigen following delivery by rMVA can lead to the induction of higher antibody titres (Wyatt *et al.*, 2008a) and can be promoted by fusion of the antigen to membrane-anchoring sequences.

The Vesicular Stomatitis Virus (VSV) glycoprotein has been studied as a model integral plasma membrane protein. Initial reports of the protein's sequence suggested that a region at the C-terminus would be responsible for membrane anchoring - specifically, a 49 amino acid sequence comprising a hydrophobic and cytosolic domain (Rose *et al.*, 1980). This theory was supported by *in vitro* expression studies in which the C-terminus was removed; the glycoprotein was no longer observed to be membrane bound (Rose and Bergmann, 1982). Later, it was shown that the gene sequence for an otherwise secreted protein could be fused to the membrane-anchoring region with the result that the gene product would be cell surface expressed (Guan *et al.*, 1988). Srinivasan *et al.* used this approach to enhance the immunogenicity of an rVV-based human chorionic gonadotrophin β (βhCG) subunit vaccine. Briefly, two rVV expressing βhCG were generated - one with the VSV membrane-anchoring region (VSVg) and one without. High titres of anti-βhCG antibodies were measured in animals immunised with the VSVg construct, whilst no βhCG-

specific antibody was detected in animals immunised with the native form of βhCG (Srinivasan *et al.*, 1995).

As discussed in Section 1.4.2, current TB vaccine strategies are primarily concerned with enhancing anti-mycobacterial T cell responses. However, there is a growing body of evidence to suggest that humoral immunity may also contribute to protection against TB (Achkar and Casadevall, 2013). Fusion of a TB antigen to the VSV glycoprotein membrane anchoring region (VSVg) may be an appropriate means of achieving enhanced antibody responses. Further, bilateral targeting of the same antigen to multiple pathways may diversify the immune response induced. In the current study, TPA and the VSVg were selected for comparison of their relative and combined effects.

6.1.3 A model antigen for proof of principle

The model antigen ovalbumin (OVA) was selected to be the test antigen for this aspect of the current study. OVA is well characterised and has been the subject of previous vaccine-related studies investigating immuno-potentiating factors, including endogenously expressed antigen fusions (Diebold *et al.*, 2001), rMVA promoter selection (Baur *et al.*, 2010; Becker *et al.*, 2014), novel adjuvants (de Cassan *et al.*, 2011) and delivery systems (Huang *et al.*, 2010). As demonstrated through these, and earlier reports, the OVA antigen is known to induce IgG isotypes associated with Th-1 (IgG2a, IgG2b and IgG3) and Th-2 (IgG1) responses. The antigen also has restricted MHC Class I and MHC Class II epitopes through which to examine CD8+ and CD4+ T cell responses, respectively (Table 6.1).

Table 6.1 Murine-restricted OVA immunological determinants. Sections of the OVA 385-amino acid sequence contain MHC Class I (OVA I) and Class II (OVA II) epitopes. The exact peptide sequence is shown using the single-letter amino acid code. The MHC molecules binding these antigens are encoded by H-2 genes, residing in different loci and on different alleles (haplotype).

MHC H-2 restriction			Reference
Class	**Loci**	**Haplotype**	
OVA I: SIINFEKL ($OVA_{257-264}$)			
I	K	*b*	(Rotzschke *et al.*, 1991)
OVA II: ISQAVHAAHAEINEAGR ($OVA_{323-339}$)			
II	IA	*d*	(McFarland *et al.*, 1999)

The construction of rMVA expressing OVA using transfer plasmid methodology is described in Chapters 3 and 4. Transfer plasmids incorporating vaccinia promoter mH5 for regulation of antigen expression were selected to promote the induction of strong, measurable immune responses associated with different T cell subtypes. As discussed in Chapter 4, rMVA expressing a single copy of OVA were successfully isolated, but a double-recombinant expressing OVA fused to both TPA and VSVg, in separate insertion cassettes, could not be purified. The immunogenicity of the single rMVA was investigated to test the study hypothesis as far as possible and to inform the design of future studies.

6.2 Chapter Aims

The overall aim for this Chapter was to investigate the potential for diversifying the immune responses to an antigen through the use of multiple leader sequences, as per the study hypotheses outlined in Section 1.5. The immunogenicity of four rMVA viruses vectoring the Ovalbumin (OVA) gene was compared. Specific objectives were to:

- Investigate antibody-mediated responses to OVA by measuring antigen-specific IgG isotypes.
- Compare the cell-mediated response to OVA by measurement of antigen-recall cytokine release.

6.3 Results

6.3.1 rMVA for an investigation into leader sequence effects

Four rMVA viruses expressing OVA were purified as described in Chapter 4 (see Section 4.3.2). For each rMVA expressing OVA fused to a leader sequence there was a corresponding 'no-leader' control virus. A diagram of the expression product arising from each rMVA is presented in Figure 6.1. Detailed information regarding the sequence of each open reading frame is presented in Appendix III.

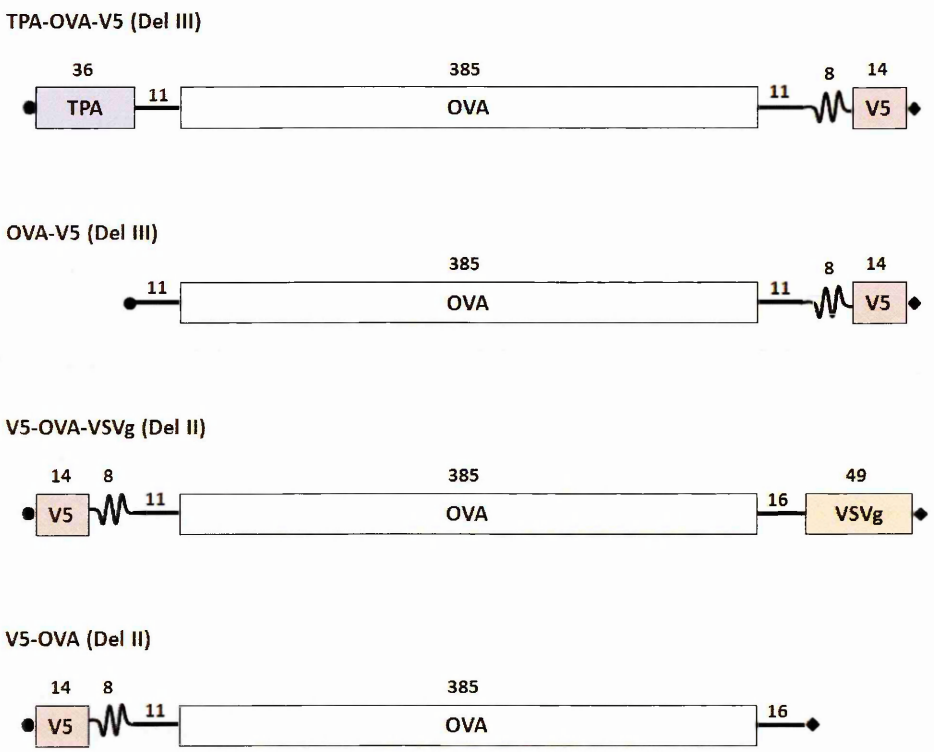


Figure 6.1 Schematic representation of rMVA-OVA expression products. Each rMVA expressed OVA fused to N and/or C terminal fusions. Either side of the OVA gene was a short sequence arising from the presence of the *att* recombination sites used in Gateway® cloning. The V5 tag was joined to a linker sequence (zig-zag line) to ensure adequate presentation of the B cell epitope. For each recombinant cassette incorporating a leader sequence, i.e. TPA-OVA-V5 and V5-OVA-VSVg, there was a no-leader control, identical in every regard, including the site of insertion. The start (•) and stop (♦) codons are shown. Numbers represent amino acid length.

The immunogenicity of the rMVA expressing OVA was investigated by immunising mice for subsequent evaluation of humoral and cell-mediated immune responses. In the first instance, an ELISA was developed for the detection of antigen-specific IgG isotypes.

6.3.2 Development of an ELISA for detection of mouse anti-OVA IgG isotypes

An indirect ELISA protocol was optimised for the detection and measurement of murine IgG isotypes. Biotinylated antibodies specific for murine IgG1, IgG2a, IgG2b and IgG3 were purchased (see Section 2.4.4) and investigated for their ability to accurately discriminate between each isotype and were optimised with regards to their concentration (described below). A suitable concentration of OVA protein was then selected for coating microtitre plates, so that OVA-specific murine IgG could be captured from serum samples and measured. In advance of describing the assay optimisation undertaken, summaries of the principal components of the assay are presented (Figure 6.2).

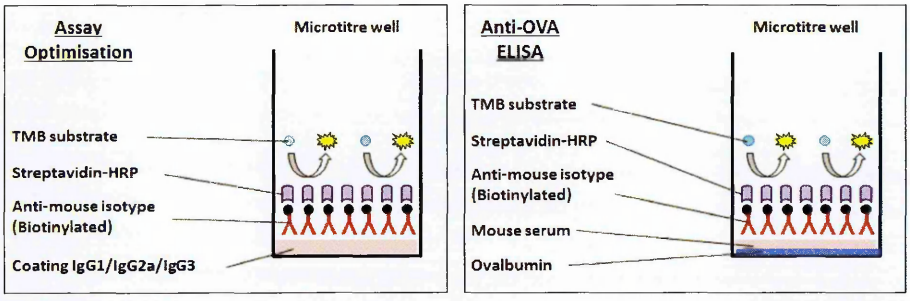


Figure 6.2 Indirect ELISA for detection of anti-OVA IgG isotypes. The main components of the assay are shown in one well of a 96-well microtitre plate. Plates were coated with commercially available murine IgG isotypes to optimise the protocol (left panel). Bound IgG was detected indirectly via the addition of biotinylated anti-mouse IgG followed by incubation with streptavidin - horse radish peroxidase (HRP) conjugate. The amount of IgG present was quantified by measuring HRP-mediated colour change of the substrate 3,3',5,5'-tetramethylbenzidine (TMB). Later, plates were coated with OVA protein and incubated with mouse serum to allow anti-OVA IgG to bind (right panel). The levels of anti-OVA were then measured as described above, using biotinylated anti-mouse IgG to detect specific isotypes.

Isotype-specific detection of IgG

The specificity of biotinylated antibodies raised against murine IgG1, IgG2a, IgG2b and IgG3 was investigated, to ensure that they would provide an accurate means of measuring IgG isotypes (protocols and reagents are described in detail in Section 2.4.4). Microtitre plates were coated with IgG1, IgG2a, IgG2b or IgG3 at a range of concentrations and then detection attempted with each biotinylated anti-mouse IgG (as described in Figure 6.3, left hand panel). The results confirmed that the biotinylated antibodies were isotype specific and that different concentrations of coating IgG would be detected in a dose dependent manner (Figure 6.3). In this experiment, each of the biotinylated anti-mouse IgG antibodies had been used at a dilution of 1 in 20,000. In the next round of experiments the concentration of anti-IgG would be optimised.

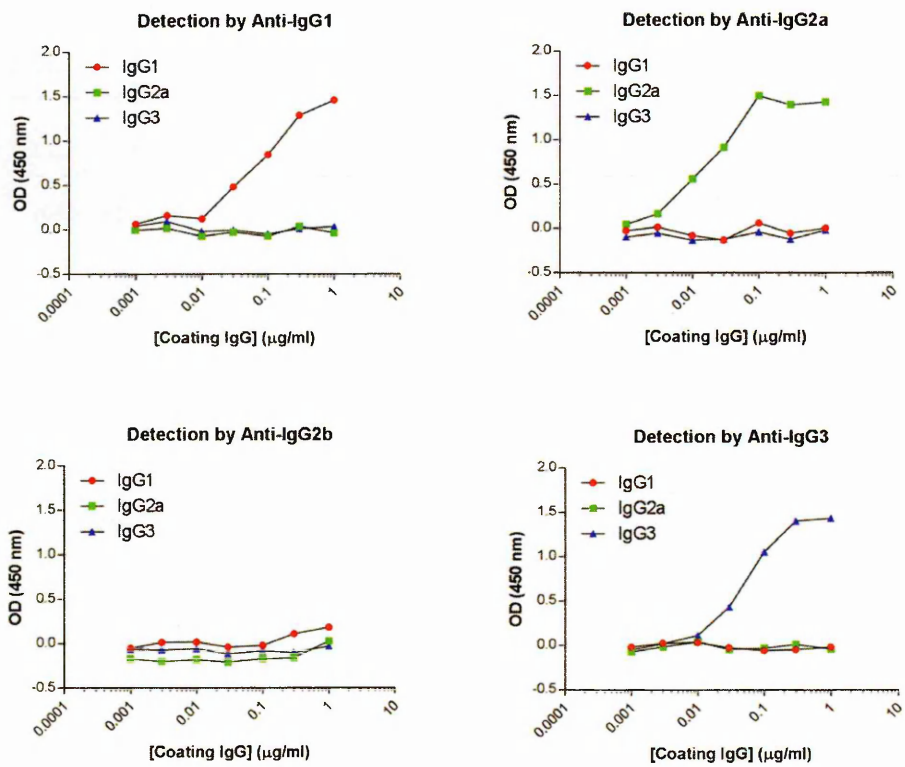


Figure 6.3 Isotype-specific detection of murine IgG. Plates were coated with IgG1, IgG2a, IgG2b (not shown) or IgG3, as indicated by the legend. Isotype-specific biotinylated anti-mouse IgG (raised in rabbit) was applied at a dilution of 1:20,000 to achieve indirect detection of the coating IgG using streptavidin-HRP and TMB reagents. Optical density (OD) was measured at 450 nm and was plotted after subtraction of values for non-specific binding (NSB). Data points represent the mean of duplicate wells.

Optimisation of secondary antibody concentration

The product information supplied with each of the four biotinylated anti-mouse IgG specified a range of dilutions at which they might be investigated for use in an ELISA. Four dilutions encompassing this range were selected for testing. Microtitre plates were coated with three concentrations of each IgG isotype (0.003, 0.03 and 0.3 µg/ml) and were then incubated with the different dilutions of biotinylated anti-mouse IgG. As before, the amount of anti-IgG binding to the plate was measured via the addition of Streptavidin-HRP followed by TMB substrate. The OD data are summarised in Figure 6.4.

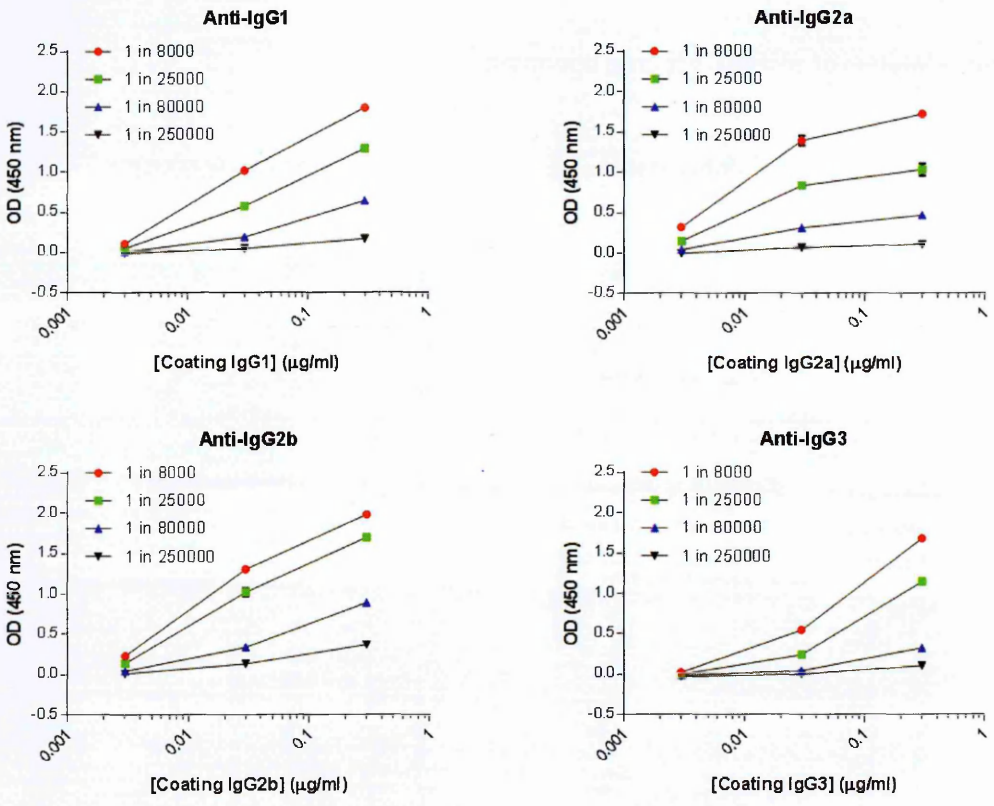


Figure 6.4 Optimisation of secondary antibody concentration. Microtitre plates were coated with one of four IgG istoypes. The corresponding isotype-specific secondary antibody was applied at four different dilutions to achieve indirect detection of the coating IgG using streptavidin-HRP and TMB reagents. Optical density (OD) for triplicate wells (mean +/- SEM) was plotted after subtraction of NSB readings.

The majority of the biotinylated anti-mouse IgG concentrations tested were able to detect the coating antibody and were able to do so dose dependently. However, the sensitivity of detection improved as the concentration of biotinylated anti-IgG antibody was increased. The discriminatory power to measure 10-fold changes in coating-IgG concentration was poorest at a dilution of 1 in 250,000 and at its most sensitive at 1 in 8,000. The lowest dilution (1:8,000) was, therefore, selected for use in subsequent assays.

Previously, the specificity of each of the biotinylated anti-IgG antibodies was investigated at a dilution of 1 in 20,000 (Figure 6.3). To ensure that non-specific binding would not manifest at a higher concentration, isotype specificity was re-examined at the revised dilution of 1 in 8,000. The results provided evidence for negligible cross-reactivity (Figure 6.5) and so each of the biotinylated anti-IgG antibodies was used at a dilution of 1 in 8,000 in subsequent assays.

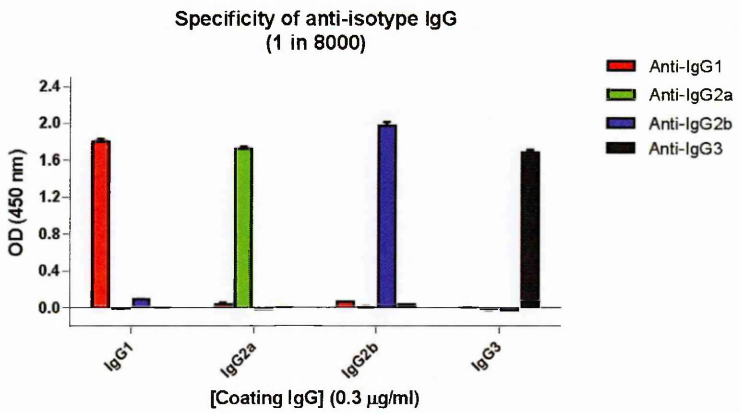


Figure 6.5 Specificity of secondary antibody at 1:8000. Microtitre wells were coated with one of four IgG isotypes at a single concentration (0.3 µg/ml). Detection of the bound IgG was then attempted using four isotype-specific biotinylated anti-mouse IgG, as indicated by the figure legend. For each isotype of coating IgG, detection was only achieved after addition of the corresponding isotype-specific anti-IgG. OD for triplicate wells (mean +/- SEM) was plotted after subtraction of non-specific binding (NSB).

Selection of an ovalbumin concentration for plate coating

As summarised in Figure 6.2, OVA-specific IgG antibodies in mouse serum were captured for detection using microtitre plates coated with OVA protein. In order to identify an optimal coating concentration of OVA, microtitre plates were coated with four different concentrations selected to fall within those reported for previous studies (Baur *et al.*, 2010; Bohnen *et al.*, 2013). The binding of antigen-specific IgG to the coated plates was investigated using a commercially sourced preparation of anti-OVA IgG. This was deemed preferable to using mouse serum from rMVA-OVA immunised mice, for which the anti-OVA IgG titre was unknown. The commercially sourced anti-OVA IgG was serially diluted and applied to the coated plates. Anti-OVA IgG binding curves were similar for all four coating concentrations of OVA (Figure 6.6). At a dilution of 1 in 100,000 (10^{-5}), anti-OVA IgG binding increased dose dependently with the coating concentration of OVA and revealed that binding was nearing its maximum at 5 µg/ml. The concentration above this (10 µg/ml) was selected for use in the anti-OVA ELISA protocol.

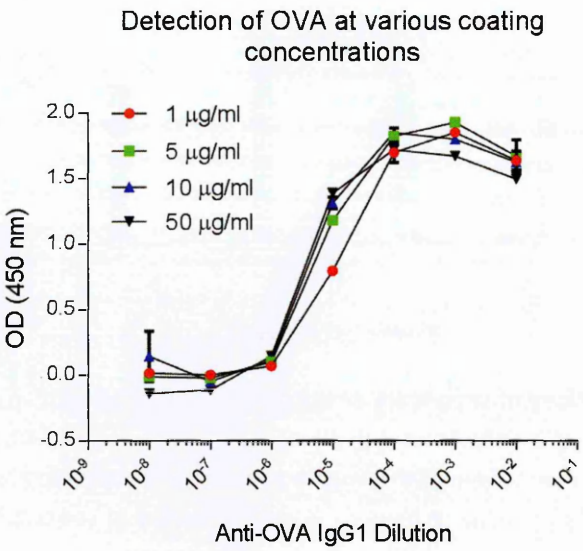


Figure 6.6 Selection of an OVA concentration for plate coating. Microtitre plates were coated with different concentrations of ovalbumin and then ten-fold dilutions of anti-OVA IgG were applied. Antibody binding was measured indirectly via the addition of biotinylated anti-IgG1. Optical density (OD) for triplicate wells (mean +/- SEM) was plotted after subtraction of NSB. At a dilution of 1 in 100,000, anti-OVA IgG binding correlated with the coating concentration of OVA.

6.3.3 A mouse study for investigating rMVA-OVA immunogenicity

Groups of CB6F1 mice (5 per group) were immunised intramuscularly with each of the four rMVA expressing OVA, twice, at two week intervals (see Section 2.4.1). The dose of rMVA administered on each occasion was 1×10^7 PFU.

In order to ensure consistency between the doses of each rMVA vaccine, the viral stocks used for immunisation were titrated in parallel to determine their concentration (see Section 5.4.1). The viral stocks were then diluted in PBS to achieve the specified dose.

Two weeks after the final immunisation, whole blood and spleens were harvested for evaluation of humoral and cell-mediated immune responses, respectively.

6.3.4 Measurement of anti-OVA IgG isotypes in rMVA-OVA immunised mice

Serum from immunised mice was separated from whole blood as described in Section 2.4.4, and tested for the presence of anti-OVA IgG isotypes, as described in Section 2.4.4 and above. Throughout, samples of test serum were evaluated in parallel with control serum to monitor for the specificity of any 'anti-OVA' IgG detected. The control serum was obtained from CB6F1 mice immunised with plasmid DNA vectoring various TB antigens (pooled stock from multiple studies). No cross-reactivity with OVA was expected and this was confirmed in preliminary studies in which control serum was serially diluted and incubated in OVA-coated plates; there was evidence for only limited binding at the highest serum concentrations. Representative data for control serum are presented in Figure 6.7 and are plotted as the mean plus three standard deviations. Test-serum OD values above this threshold were interpreted to represent positive responses.

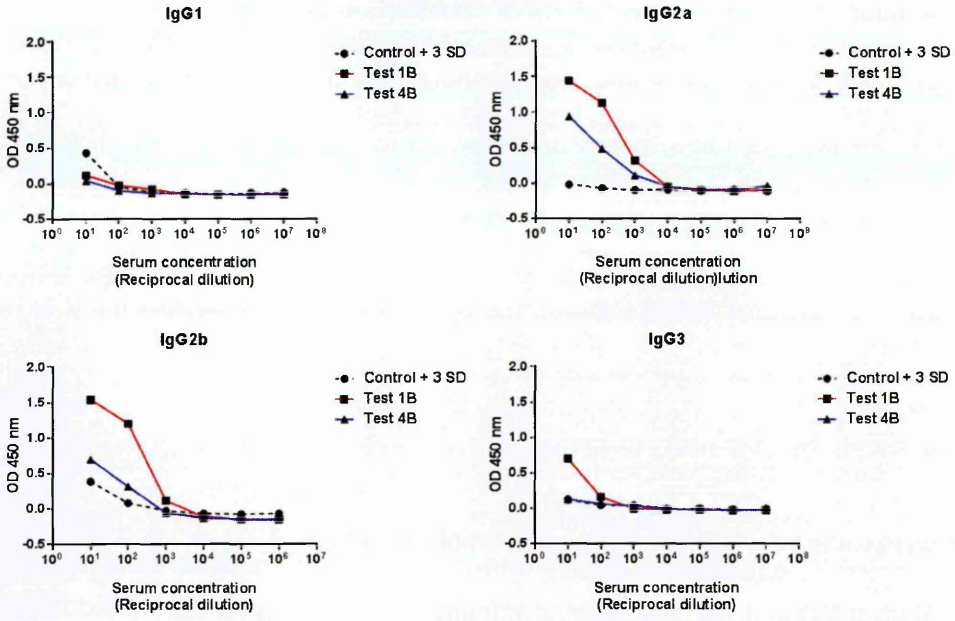


Figure 6.7 Measurement of anti-OVA IgG isotypes in rMVA immunised mice. Groups of CB6F1 mice (n=5) were immunised intramuscularly with rMVA expressing OVA (1 x 10^7 PFU), twice, at two week intervals. Two weeks after the final immunisation, serum was harvested for measurement of anti-OVA IgG. Two serum samples were selected for a preliminary assessment of their anti-OVA IgG titres by ELISA. Sample 1B was from a mouse immunised with rMVA-TPA-OVA-V5. Sample 4B was from a mouse immunised with rMVA-V5-OVA. Both were selected on account of having ample volume. Optical density (OD) was measured at 450 nm in duplicate wells and the mean value plotted after subtraction of NSB. Control serum was evaluated in parallel. Data were plotted after calculation of the mean plus 3 standard deviations.

Preliminary studies were performed to investigate the presence of anti-OVA IgG in serum derived from rMVA-OVA immunised mice. Two samples of the test serum were investigated over a dilution series and anti-IgG was detected in some of the samples in a dose-dependent manner. Representative data are shown in Figure 6.7. The results were interrogated for their ability to yield reliable endpoint titre values, as this is the method most commonly applied to the measurement of antibody titres in the absence of a positive control (Miura *et al.*, 2008). The data confirmed that for many samples there would be insufficient IgG present to implement this approach reliably. All of the test samples were subsequently investigated at a dilution of 1 in 100 as has been reported for other IgG-isotype studies involving mice (de Cassan *et al.*, 2011) and humans (Biswas *et al.*, 2014). All

of the serum samples were tested in parallel, with one another, and with control serum. The results are shown in Figure 6.8.

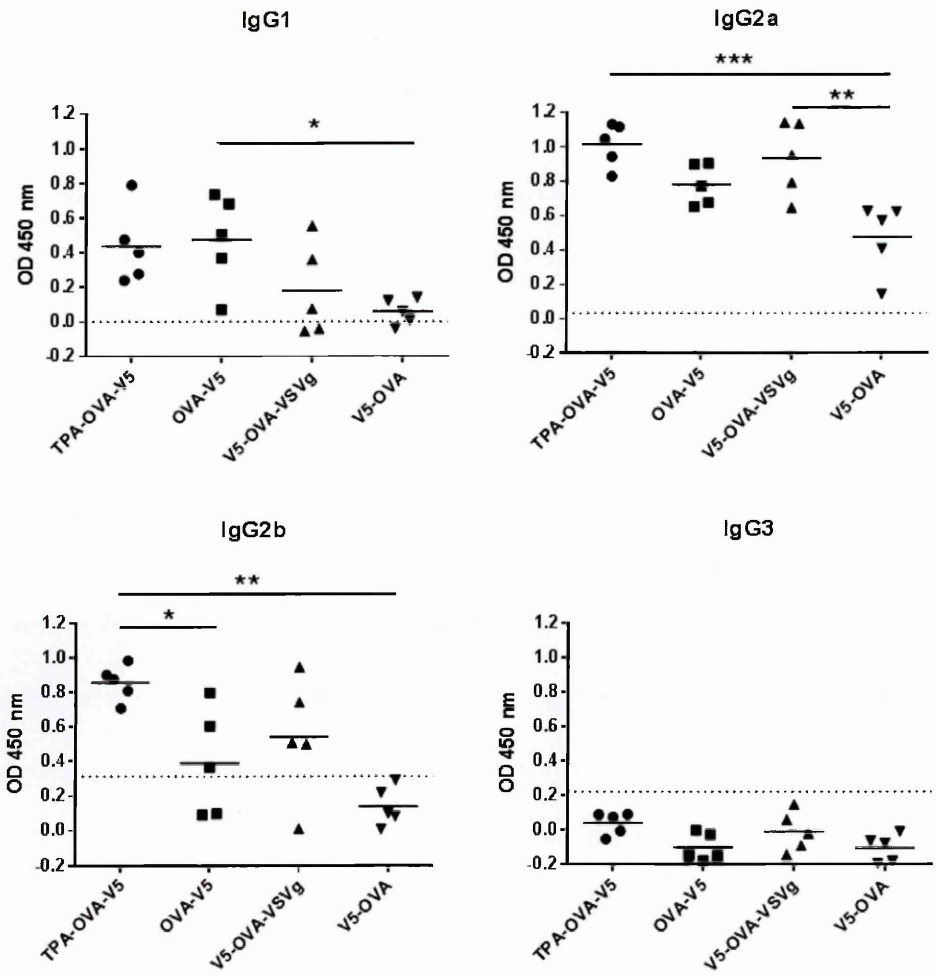


Figure 6.8 Anti-OVA IgG induced by rMVA immunisation. Groups of CB6F1 mice (n=5) were immunised intramuscularly with rMVA expressing OVA (1 x 10^7 PFU), twice, at two week intervals. Two weeks after the final immunisation, serum was harvested for measurement of anti-OVA IgG by ELISA. Serum samples were diluted 1 in 100 and added to duplicate wells. Control serum was diluted 1 in 100 and added to four wells divided between two microtitre plates. Mean OD values were calculated after subtraction of NSB. The rMVA vaccine groups are labelled according to the expression product delivered. Data are shown for individual mice (n=5) and the group mean. Dotted lines represent the threshold for positive responses and are equal to the control serum mean plus three standard deviations. For each IgG isotype the difference between groups was examined by one-way ANOVA and by post hoc Tukey's multiple comparison tests. Significant differences are highlighted (*P<0.05, **P<0.01, ***P<0.001).

For each IgG isotype, it was deemed important to compare test serum results with control serum data obtained within the same assay in order to control against inter-assay variability. Whilst the majority of the serum samples passed the threshold for positive responses, this was not the case for any of the IgG3 measurements and for many of the IgG2b readings. However, the control serum mean value may have been slightly higher for IgG2b and IgG3 than seen on other occasions (see Figure 6.7).

Only a few of the anti-OVA IgG1 measurements were below the threshold for a positive response and it was noted that this would not have been the case had the threshold been set to the mean plus two standard deviations. Statistical analysis was applied to all the data sets despite the failure of some samples to pass the threshold for a positive response, but later, the results would be interpreted with due reference to the relative magnitude of the IgG levels detected.

Analysis of the data by one-way ANOVA revealed statistically significant differences between the groups for each of the IgG isotypes (IgG1, $P = 0.0248$; IgG2a, $P = 0.0008$; IgG2b, $P = 0.0025$; IgG3, $P = 0.0482$). The difference between individual groups was further examined by post hoc Tukey's multiple comparison tests. Statistically significant differences are highlighted in Figure 6.8. The induction of anti-OVA responses in mice immunised with each of the 'no leader' control rMVA might have been expected to be similar to one another. However, rMVA expressing OVA-V5 inserted into Deletion site III generally induced higher IgG levels than rMVA expressing V5-OVA inserted into Deletion site II, and for anti-OVA IgG1, this difference was statistically significant ($P < 0.05$).

Relative to their corresponding 'no leader' controls, rMVA vectoring OVA fused to either TPA or VSVg evoked higher Th-1 associated IgG responses (IgG2a and IgG2b). These increases reached statistical significance for VSVg enhancement of anti-OVA IgG2a ($P < 0.01$) and TPA enhancement of anti-OVA IgG2b ($P < 0.05$). Relative to their respective

controls, there was no difference in the ability of the two leader-sequence constructs to modulate the levels of Th-2 associated IgG1.

The ratio of IgG2a to IgG1, based upon group means, was compared as an indicator of the balance between Th-1 and Th-2 immunity, as has been reported previously (de Cassan *et al.*, 2011). The results confirmed that all of the groups were skewed towards a Th-1 type response, but that rMVA employing insertion site Del III might have been less biased on account of their higher IgG1 levels (Figure 6.9A).

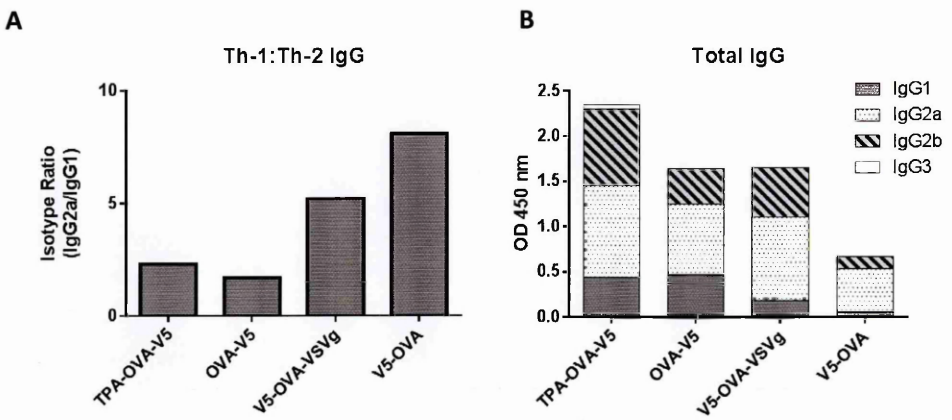


Figure 6.9 Anti-OVA IgG Isotype summary data. A) The Th-1:Th-2 balance of the IgG levels reported in Figure 6.8 were investigated by calculating the ratio of IgG2a: IgG1. The data are the ratio of group means. B) The total amounts of IgG induced were plotted according to all of the OD values. The contributing totals for each isotype are shown, as indicated by the figure legend.

Finally, the IgG data were summarised by plotting the sum of the responses (Figure 6.9B). Fusion of OVA to either TPA or VSVg increased the total levels of IgG induced relative to data for the corresponding 'no-leader' control. As already noted, the induction of anti-OVA humoral responses appeared to be higher for rMVA expressing OVA inserted into Deletion site III.

6.3.5 Measurement of OVA-specific IFNγ release

Splenocytes from immunised mice (see Section 6.3.3) were harvested for IFNγ ELISpot assay, as described in Section 2.4.2. Cells were incubated with whole OVA protein and non-OVA protein to investigate the induction of antigen-specific cytokine release. Tuberculin purified protein derivative (PPD) was added at a concentration routinely observed to induce cytokine release after BCG vaccination (10 μg/ml), but did not increase the number of IFNγ-releasing cells above background levels (Figure 6.10). Whole OVA protein, meanwhile, did evoke an increase in the number of responding cells enumerated for each group. Further, the splenocytes were incubated with two concentrations of whole OVA protein (10 and 50 μg/ml) to test whether the number of cells induced to release IFNγ would vary dose dependently. The results confirmed antigen-specific, dose-dependent stimulation.

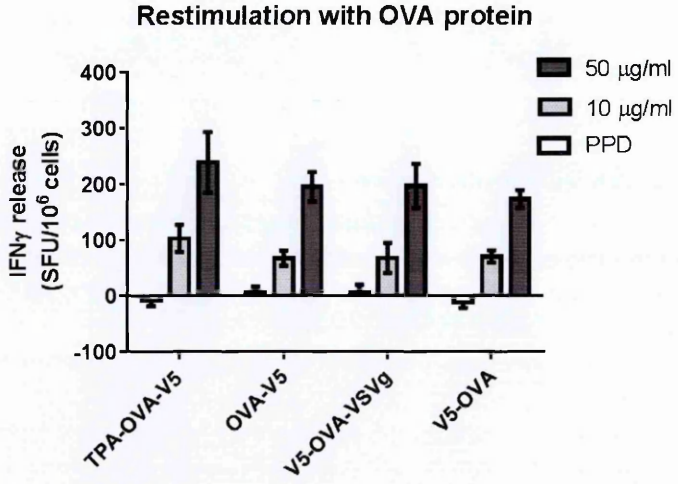


Figure 6.10 Whole OVA IFNγ responses. Groups of CB6F1 mice (n=5) were immunised intramuscularly with rMVA expressing OVA (1 x 10^7 PFU), twice, at two week intervals. Two weeks after the final immunisation, splenocytes were harvested for restimulation with whole OVA protein or tuberculin purified protein derivative (PPD). The number of cells induced to release IFNγ was measured by ELISpot assay. Results were plotted as the number of spot forming units (SFU) per million cells (mean +/- SEM) after subtraction of background, i.e. SFU/10^6 measured in the presence of cell culture medium only. No statistically significant differences between groups were observed (one-way ANOVA).

The number of cells induced to release IFNγ after restimulation with whole OVA protein was compared between the groups. No statistically significant differences were detected at either of the whole OVA concentrations tested (one-way ANOVA, P = 0.5429 and P = 0.6489, for 10 and 50 μg/ml, respectively).

Within the same assay, OVA-specific cellular responses were further dissected after incubation of the splenocytes with MHC Class I and MHC Class II OVA-restricted peptides (see Section 6.1). Peptides OVA I and OVA II stimulate CD8+ and CD4+ T cells, respectively, thereby enabling the number of anti-OVA responding cells to be enumerated for each subtype. Incubation of the splenocytes with either OVA I, or OVA II, led to an increase in the number of cytokine releasing cells. For each of the groups, there were more CD8+ responding T cells than there were CD4+ T cells (Figure 6.11).

The number of splenic T cells responding to stimulation with the OVA peptides was compared between the groups. There were no statistically significant differences between group means for the number of CD4+ cells induced to release IFNγ by peptide OVA II. The number of CD8+ responding cells, meanwhile, did appear to differ according to the rMVA used for immunisation (one-way ANOVA, P = 0.0118). Further examination of the group means by post hoc Tukey's multiple comparisons tests revealed that there were no differences in the number of CD8+ cells stimulated to release IFNγ following immunisation with each of the no-leader control viruses. This was in contrast to the data collected for humoral responses where rMVA vectoring OVA-V5 inserted into Del III had induced higher Th-1-associated IgG levels than rMVA vectoring V5-OVA inserted into Del II. In terms of CD8+ cytokine response, the results were slightly lower for OVA-V5 (Del III) than they were for V5-OVA (Del II), i.e. the opposite of what had occurred in terms of IgG induction

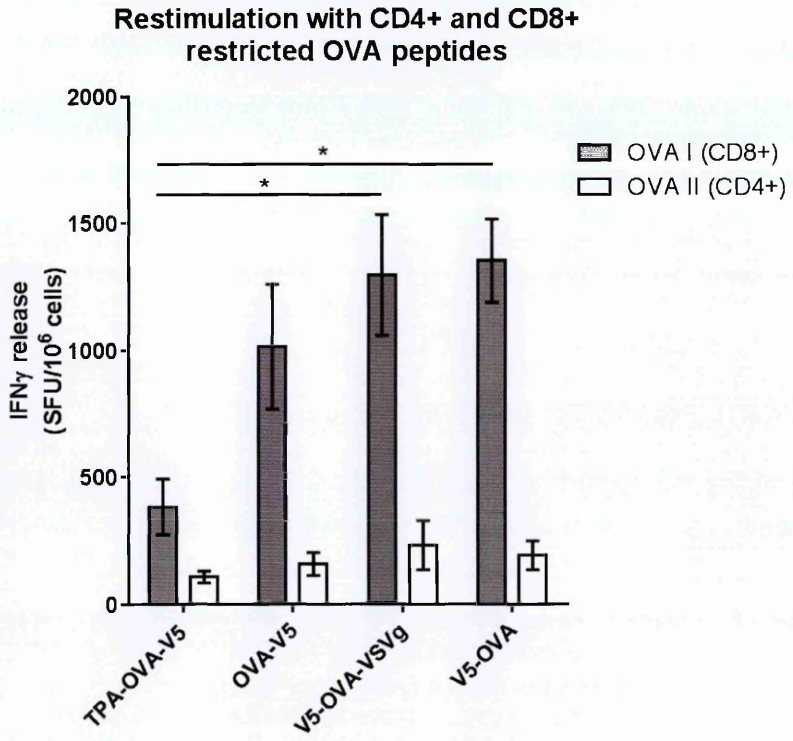


Figure 6.11 IFNγ responses to MHC Class I and MHC Class II restricted OVA peptides. Groups of CB6F1 mice (n=5) were immunised intramuscularly with rMVA expressing OVA (1 x 10^7 PFU), twice, at two week intervals. Two weeks after the final immunisation, splenocytes were harvested for restimulation with OVA I or OVA II. The number of cells induced to release IFNγ was measured by ELISpot assay. Results were plotted as the number of spot forming units (SFU) per million cells (mean +/- SEM) after subtraction of background, i.e. SFU/10^6 measured in the presence of cell culture medium only. For each OVA peptide the difference between groups was examined by one-way ANOVA. Differences between the responses to OVA I were statistically significant (P = 0.0118) while differences between OVA II responses were not (P = 0.5417). Post hoc evaluation by Tukey's multiple comparison tests revealed differences reaching statistical significance and these are highlighted (*P<0.05).

Relative to the corresponding no-leader control, there were no measurable differences in the number of CD4+ or CD8+ responding T cells induced following fusion of OVA to VSVg. The impact of fusing OVA to TPA, meanwhile, was to decrease the number of antigen-specific CD8+ IFNγ-releasing splenic T cells. The differences between the group mean for TPA-OVA-V5 and the group means for V5-OVA-VSVg and V5-OVA were statistically significant (P < 0.05). An outcome of the weaker CD8+ response for the TPA-OVA-V5 group

was that the ratio of CD8+ to CD4+ responding cells was more balanced than it had been for the other groups. Thus, fusion of the transgene to TPA appeared to reduce the CTL dominance of the anti-OVA immune response evoked by rMVA, as measured by IFNγ ELISpot assay.

6.3.6 Measurement of OVA specific IL-4 and IL-17 release

ELISpots for IL-4 and IL-17 release were conducted in parallel with assays for IFNγ. None of the restimulating proteins or peptides induced responses above background release. This remained the case upon repeat testing where the restimulation period was increased from overnight to two, and then five days (data not shown).

6.4 Discussion

The primary objective for this Chapter was to compare the immunogenicity of rMVA incorporating TPA and VSVg leader sequences, with a view to investigating the potential for leader sequence combinations to diversify the immune response to an MVA vectored antigen. Four rMVA expressing OVA were generated and evaluated for their immunogenicity in CB6F1 mice. Differences in humoral and cell-mediated immune responses were observed, confirming the potential for antigen fusions to modulate antigen-specific immunogenicity.

6.4.1 Measurement of anti-OVA IgG

An ELISA panel was developed for the detection of anti-OVA IgG isotypes. In the absence of a positive standard, it was not possible to create standard curves through which to interpret OD values. Instead, control serum was tested within each assay to monitor for positive responses. Experimental immunisation studies are commonly without a positive standard control and so quantitative analyses are performed on the basis of results for a negative control, typically by determination of reciprocal endpoint titre. Endpoint titre is determined from a full dilution curve of the test sample. It is equivalent to the highest dilution giving an OD reading two or three standard deviations above the negative control.

In instances where there are low antibody titres, dose curves are difficult to define and readings at a single dilution may be compared (Biswas *et al.*, 2014; de Cassan *et al.*, 2011). This was the approach applied in this study.

The limitations of assessing IgG levels at a single dilution must be noted. Chiefly, it is unclear where on the titration curve the OD value is taken from; the relative differences between readings are less meaningful if not from the linear region. However, the relative magnitude of the values remains informative.

6.4.2 Measurement of cytokine responses

Splenocytes were restimulated to measure antigen-specific cytokine responding cells via ELISpot assay. Th-1 immunity was investigated via enumeration of cells secreting IFNγ. ELISpot assays for IL-4 and IL-17 release were implemented to explore Th-2 and Th-17 responses, respectively.

The ELISpot assays for IL-4 and IL-17 were performed in parallel with the IFNγ tests and were implemented using the same methodology (MAbtech). Positive control wells were restimulated with PMA/I (as described in Section 2.4.2) and were induced to release IL-4 and IL-17. However, there were markedly lower SFU as compared to the IFNγ assay. Lack of sensitivity may, therefore, have been the root cause of the failure to detect measureable responses. Humoral responses for all of the vaccines had demonstrated skewing towards Th-1 immunity.

6.4.3. The design and evaluation of vaccine antigen fusions

Leader sequences have the potential to modulate the immunogenicity of virally vectored antigens. Complicating the selection of a sorting signal are conflicting reports about their expected impact on antigen immunogenicity and/or gaps in our understanding of their mechanism of action. For example, the MHC Class II-binding invariant chain (Ii) would be expected to target antigens towards CD4+ T cell stimulation (Holst *et al.*, 2008), but has

been found to enhance CD8+ responses to both rAd and rMVA vectored antigens (Mikkelsen *et al.*, 2011; Spencer *et al.*, 2014). The TPA signal sequence is expected to modify antigen immunogenicity by targeting the protein towards the ER for increased secretion (Haddad *et al.*, 1997). However, TB antigens fused to TPA are also more highly expressed (Li *et al.*, 1999; Malin *et al.*, 2000) and the relative contribution of expression to immune-potentiating effects, versus intracellular trafficking, has yet to be assessed.

Another consideration in the design of vaccine antigen fusions is whether to include the native sorting signal, should one be present. Both prokaryotic and eukaryotic genes can contain a signalling peptide at the N-terminus to direct the translated protein into the cell membrane or ER membrane, respectively, and both signals are recognised in eukaryotic cells (Hall *et al.*, 1990). The TB vaccine antigen, Ag85A, is a secreted protein and has a signal peptide at its N-terminus. The clinical candidate MVA85A expresses the full length, native protein fused TPA (McShane *et al.*, 2002). Previous studies have demonstrated that mature Ag85A, full length Ag85A and mature Ag85A fused to TPA become progressively more immunogenic (Huygen *et al.*, 1996; Montgomery *et al.*, 1997).

The OVA gene has an N-terminal signal peptide and this too was retained in the current study. This was important given that the VSVg membrane anchoring region would be dependent upon direction of the antigen to the ER in the first instance for co-translational membrane insertion (Lodish *et al.*, 2000). For consistency, the same full length OVA gene was included in each of the rMVA evaluated. The presence of two ER-targeting sorting signals in TPA-OVA-V5 is not anticipated to be detrimental and, coincidentally, mirrors the 'signalling content' of MVA85A.

Ultimately, for each novel antigen fusion it may be necessary for (a) the immunomodulatory effects conferred by a particular leader sequence to be empirically investigated and (b) that experiments testing the influence of the antigen fusion be robustly controlled. Consequently, in the current study, the two rMVA-OVA constructs

incorporating a cell sorting signal were matched with a corresponding no-leader control virus.

6.4.4. The impact of rMVA insertion site on the immunogenicity of the transgene

The panel of rMVA generated for the present study carried recombinant cassettes at one of two insertion sites – Deletion site II (Del II) or Deletion site III (Del III). The insertion site had not been anticipated to impact upon the immunogenicity of the transgene, OVA, and T cell responses for the no-leader control viruses were not significantly different. However, the levels of IgG induced by OVA-V5 (Del III) were generally higher than V5-OVA (Del II) and achieved statistical significance for IgG1 ($P < 0.05$).

When the rMVA were generated there was some evidence to suggest that Deletion site II may have conferred inferior stability (see Section 4.4.3). Future experiments should continue to monitor for insertion site effects and be extended to include characterisation of viral fitness and antigen expression (as performed for MVA expressing Ag85A in Chapter 5). Extended characterisation of the rMVA should also be complemented with repeat measurements of the immune responses induced; current observations have been made on the basis of one study. Measurement of anti-vaccinia responses should also feature to ensure successful and equivalent delivery of rMVA vaccines.

6.4.5 The impact of TPA and VSVg on cell mediated immunity

Fusion of OVA to VSVg was expected to lead to cell surface expression and the induction of higher antibody levels. The TPA signal sequence was expected to direct antigen to the ER and/or increase antigen expression with the result that antibody responses would be enhanced. Both VSVg and TPA increased anti-OVA IgG levels relative to their corresponding control viruses and by a similar order of magnitude.

Cytokine responses were measured after restimulation of splenocytes with either whole OVA protein or MHC-restricted OVA peptides. Whole protein is a less sensitive inducer of T

cell responses as it must first undergo internalisation and processing by APC in order to be presented to T cells. Peptides, meanwhile, can bind directly to MHC on the surface of APC in an ELISpot assay, leading to efficient and sensitive measurement of T cell responses (Schmittel *et al.*, 2001). A peptide panel of 15-20 mers offset by 1-5 amino acids is advised for detection of CD4+ and CD8+ responses based upon the average epitope length for MHC Class I and II molecules – 8 - 11 and 11 – 25 mers, respectively (Rodda, 2002). MHC Class I and MHC Class II restricted epitopes have been defined for OVA (summarised in Table 6.1) and were used in this study. Comparison of leader sequence effects was made on the basis of peptide-induced cytokine release. Cell-mediated antigen recall responses were investigated via IFNγ ELISpot assay; there were no measureable increases in the number of cells secreting IL-4 and IL-17.

In the current study, fusion of OVA to VSVg did not lead to detectable differences in the number of antigen-specific CD4+ or CD8+ IFNγ-secreting T cells. The effect of VSVg fusion on cell mediated immunity was previously unknown. The induction of T cell responses was not reported following delivery of rVV vectoring βhCG fused to VSVg (Srinivasan *et al.*, 1995). The immunogenicity of an rMVA virus promoting cell surface expression of an HIV antigen was reported to be enhanced, but was confined to increased humoral responses; the levels of splenic CD8+ cells induced to release cytokine upon restimulation with the vaccine antigen were investigated and found to be unchanged (Wyatt *et al.*, 2008a).

In contrast to the results obtained with V5-OVA-VSVg, immunisation with TPA-OVA-V5 induced the lowest number of splenic CD8+ and CD4+ IFNγ-releasing cells. In particular, the CD8+ response was reduced meaning that the skewing towards CTL-dominant immunity, observed for the other rMVA, was reduced. Novel TB vaccines are concerned with the induction of strong cellular immunity and so the expected effect of fusing OVA to TPA, a leader sequence commonly used in the TB vaccine field, might have been to enhance T cell responses. In the limited number of reports describing head-to-head comparison of TB

antigen delivery, with and without an N-terminal TPA leader sequence, murine splenic cytokine release studies have been performed. However, these reports, which suggest T cell responses might have been unchanged (Li *et al.*, 1999), or moderately enhanced (Malin *et al.*, 2000), are difficult to interpret as short-term cytokine release was investigated using purified whole mycobacterial proteins. As demonstrated here, this may have compromised the ability to accurately investigate the impact of TPA fusion on cell-mediated immunity.

In the current study, fusion to either TPA or VSVg enhanced the levels of IgG2a and IgG2b in particular, indicating that a Th-1 biased response had been induced, and all of the rMVA were biased towards the induction of CD8+ T cell responses. Both of these observations are consistent with viral vector delivery of the antigen.

6.5 Conclusions

The impact of TPA and VSVg leader sequences on OVA humoral and cell-mediated immune responses was investigated. The results have confirmed the potential for antigen fusions to modulate the immune response evoked by the antigen to which they are fused.

The original aim of this study had been to compare single rMVA expressing OVA with a double-recombinant containing both the TPA-OVA-V5 and V5-OVA-VSVg cassettes. This was not achieved on account of the instability encountered with the double-recombinant virus (see Section 4.3.3). The combined effects of multiple leader sequence on a single antigen are still worthy of investigation and insertion of recombinant cassettes into alternative insertion sites, including intergenic regions (see Section 1.2.7), may support improved stability.

The capacity for sorting signals to direct different antigens to different pathways to avoid competition and/or promote the induction of favourable antigen-specific immune responses may also form part of future investigations. Recombinant MVA expressing multiple antigens in the same insertion site could be expediently created for these studies.

The current study has developed tools with which to expedite future work. Transfer plasmids were generated and are available for assessment of further antigens, which can be inserted by Gateway® cloning. Should the transfer plasmids need to be modified, they have been rendered amenable to alteration by restriction enzyme digest (see Section 3.4.3). An in-house panel of ELISA assays was developed in preference to purchasing commercially available ELISA kits. The protocol developed was less expensive to implement and remains available for other immunisation studies investigating TB antigens; only the plate-coating protein would need to be replaced. Other parameters would remain unchanged and the specificity of isotype detection would have already been confirmed.

Viral vectors are a promising delivery strategy as they induce strong cellular immunity comprising CD8+ as well as CD4+ responses (Rollier *et al.*, 2011). Hence, they are being developed for vaccination against important diseases requiring T cell immunity such as HIV, malaria and TB. The manner in which TPA reduced OVA-specific T cell response was not expected. Mice immunised with MVA vectoring TPA-fused Ag85A are reported to yield a balanced T-helper/CTL response, i.e. the numbers of Ag85A-specific CD4+ and CD8+ cytokine releasing T cells are similar (Spencer *et al.*, 2012), but the extent to which TPA might be limiting the induction of T cell immunity has not been previously tested.

Protection against Mtb infection is believed to require both CD4+ and CD8+ T cell immunity (Andersen and Woodworth, 2014). Should the role of TPA be to balance the relative magnitude of these two arms of defence then an ability to induce fewer CD8+ reactive T cells may be advantageous, but this should be explored more fully and then either purposefully implemented or otherwise, further optimised. The potential for leader sequences to improve vaccines required to induce T cell immunity was more recently exemplified by fusion of a malaria antigen to the MHC-II binding invariant chain (Spencer *et al.*, 2014). Similar studies are required in the TB field of viral vaccine vector development.

Chapter 7 General Discussion

Recombinant MVA is a promising strategy for the development of novel vaccines; there are many clinical trials ongoing for viral, bacterial and parasitic diseases, and for cancer immunotherapies. The attributes of MVA include an extensive safety profile, large capacity for foreign DNA and the ability to induce potent humoral and cell-mediated immune responses. There are many factors to consider when constructing rMVA vaccines (described in full in Section 1.2), but therein lies an opportunity to apply rational optimisation for improved vaccine antigen delivery. The current study investigated the potential for vaccinia promoter selection and antigen fusions, or 'leader sequences', to enhance the immunological potency of rMVA delivering TB antigens.

7.1 Construction and characterisation of novel rMVA

7.1.1. Transfer plasmid methodology

As discussed in Section 1.3, there are two main approaches to rMVA construction - transfer plasmid and BAC methodology. Transfer plasmid methodology was applied in the current study because of the ease of set up; it is less technically demanding to implement and there were existing transfer plasmids available for use.

Transfer plasmids serve as a shuttle-vector for insertion of recombinant cassettes into the MVA genome. As demonstrated in Chapter 3 (see Section 3.3), they can be modified using standard cloning techniques to achieve any recombinant design. This has the potential to be an efficient process but, where more complex alterations are required, custom synthesis of plasmids and/or DNA fragments may be preferable. During the course of this study, the preparation of custom synthesised DNA has become more advanced and affordable and would be put to greater use in future work, for example, by custom synthesising recombinant cassettes in their entirety, for insertion into the plasmid DNA vector. Establishing the BAC system would not have negated the requirement to create the

required recombinant cassettes by conventional cloning, or by custom synthesis, but might have expedited rMVA isolation.

7.1.2 Generation and isolation of rMVA

The rMVA viruses evaluated in the current study were isolated by plaque purification. Generally, this was an expedient process and rMVA were purified away from the parent virus in less than five passages (see Sections 4.3.1 and 4.3.2). However, genetically unstable or otherwise unwanted recombinant populations could lengthen the process. This was found to occur after the insertion of recombinant cassettes designed for transient GFP expression; a population containing the selection marker alone could be created and had to be distinguished from the desired recombinant viral population (see Section 4.3.2).

The application of BAC technology might have circumvented the issue of unwanted recombination events, but would not have resolved issues associated with genetic instability. In support of the plaque purification process, examination of MVA gDNA by analytical PCR provided a practical and effective means of achieving routine interrogation of the viral samples. Thus, future studies would continue to utilise transfer plasmid methodology and an extended panel of tools has been made available for this purpose by the current project.

7.1.3 An improved rMVA vaccine for TB

Until recently, the most advanced clinical candidate under development for use against TB was based on MVA vectoring a single TB antigen – Ag85A (see Section 1.4.3). MVA85A became the first TB vaccine to enter an efficacy trial since BCG studies performed over 40 years prior, but was unable to improve upon the effects observed for the placebo control in BCG vaccinated infants (Tameris *et al.*, 2013). MVA85A has since been evaluated in clinical studies investigating aerosol delivery (Satti *et al.*, 2014), Adenovirus boosting (Sheehan *et al.*, 2015) and fusion of the TPA-Ag85A to a C-terminal oligomerisation domain (Minhinnick *et al.*, 2016). It has been concluded that additional vaccine antigens and immune-

enhancing strategies are still required. The current study undertook to assess how the underlying rMVA design might be improved, in advance of applying additional measures.

During the period of this study, pre-clinical evaluation of another novel rMVA targeting TB was reported. Candidate MVATG18377 is based on rMVA vectoring 14 TB antigens for prevention or treatment of active, latent and reactivating TB disease (Leung-Theung-Long *et al.*, 2015). The vaccine incorporates three antigen strings under the transcriptional regulation of three different vaccinia promoters. One string has an N-terminal signal peptide derived from measles virus F protein; another has an N-terminal signal peptide and a C-terminal transmembrane anchoring domain, both of which were derived from rabies virus, while the third string has no antigen fusions. The design was reportedly optimised on the basis of bioinformatics and a review of the biochemical properties of each antigen. Thus far, the antigen-specific immune response to each gene vectored by MVATG18377 is variable, in mice and non-human primates (NHP). Vaccine candidate Rv0111 has been included in the construct and is expressed under the control of vaccinia promoter P7.5, along with three other antigens. As demonstrated in the current study, this may not confer optimal expression and immunogenicity and could explain the weak responses induced against these antigens following delivery in MVATG18377.

7.2 The study hypotheses

7.2.1 Vaccinia promoter selection

The current study sought to address whether vaccinia promoter selection could influence the immunogenicity and protective efficacy of TB vaccine antigens vectored by MVA. It was shown that increased expression could improve immunological potency and efficacy. This improvement was to an extent that would be relevant to the conservation of vaccine dose. Products based on rMVA can induce anti-vector immune responses and so reduced doses are preferable. Lower doses are also beneficial from the perspective of vaccine manufacture and product cost; viral vectored vaccines are propagated on live cells and

must be produced according to stringent regulatory (GMP) conditions. As stated in Section 1.1.4, there are established processes for rMVA production, but strategies for improving viral yields and lowering vaccine cost should always be pursued. An optimised vaccine offering equivalent protection for one tenth of the dose would serve to increase product yields, in terms of the number of doses per manufacturing batch, and would reduce vaccine cost, in terms of cost per dose. This is particularly pertinent for vaccines sponsored by non-profit organisations for use in the developing world.

7.2.2 Leader sequence immunomodulatory effects

It was hypothesised that the type of immune response evoked by an MVA-vectored antigen could be altered by the addition of particular leader sequences, and that by targeting the antigen to multiple pathways the immune response could be made more diverse. Leader sequence effects were demonstrated for a single model antigen, OVA, and highlighted the potential for antigen-fusions to optimise transgene immunogenicity. An unexpected outcome was that the specific leader sequence used in several recombinant TB vaccines, TPA, served to reduce antigen-specific CD8+ T cell responses. This is a significant finding as there is evidence to support the view that novel TB vaccine strategies should be optimised for the induction of both CD4+ and CD8+ mediated anti-mycobacterial responses (Behar *et al.*, 2007; Ryan *et al.*, 2009).

7.3 Future work

7.3.1 Efficient construction of rMVA

In the course of generating markerless rMVA (see Section 4.3.2) it was apparent that an unwanted population, expressing the GFP marker alone, was liable to be selected. It was also speculated that an antigen-only population could arise. The frequency of this occurrence could be investigated to gauge the impact on the efficiency of rMVA production, and to explore measures that might reduce it. An expedient means of achieving this would be to perform the infection-transfection (I/T) step after replacing the

gene of interest with a second marker gene, e.g. mCherry, which encodes a red fluorescent protein. Upon plating of the I/T harvest, viral plaques containing the selection marker (GFP) only, the antigen (mCherry) only, or both could then be enumerated according to the presence of green, red or red & green fluorescent protein.

7.3.2 Vaccinia promoter optimisation

Vaccinia promoter selection was able to enhance the immunological potency and efficacy of MVA-mH5-Ag85A, but at a higher dose the protective effect was lost. This was speculated to have occurred following immunisation with an excessive antigen dose, leading to terminal differentiation of effector T cell subsets with the loss of memory cell populations. This phenomenon was possibly confounded by the short time interval between the prime and boost immunisations applied in the experimental setting of the current study (described in full in Section 5.4.4). Detailed immunological analyses should be undertaken to determine if this was indeed due to a reduction in the quality of the immune response. In particular, T cell subsets including those associated with memory should be studied for antigen-specific induction of cytokine release. Multi-parameter flow cytometry would be a suitable approach (Aagaard *et al.*, 2009) and could be performed on murine peripheral blood mononuclear cells (PBMCs) over a time course following immunisation. In the first instance, a panel of cellular markers and antibodies for intracellular cytokine staining (ICS) would need to be established to distinguish effector T cell and memory T cell subsets. Detailed immunological studies should also investigate alternative vaccination schedules, to examine how the benefits conferred by an rMVA optimised for expression might be harnessed in a clinical setting, for example, by testing single doses of rMVA administered after a priming immunisation with BCG.

7.3.3 The influence of TPA on TB antigen immunogenicity

Studies comparing TB antigen delivery with and without a TPA signal sequence have been reported, but the influence on T cell immunity after *ex vivo* restimulation with overlapping

peptides as opposed to whole protein should be reassessed (see Section 6.4.5). In the current study, TPA served to significantly lower the number of antigen-specific CD8+ responding T cells. MVA85A has a TPA leader sequence and is reported to primarily induce potent and enduring antigen-specific CD4+ T cell responses (Tameris *et al.*, 2014). The impact of TPA on the immunogenicity of TB antigens, including Ag85A, should be examined in a properly controlled study, i.e. after antigen delivery in rMVA that are identical in every regard except for the presence of the leader sequence.

7.4 Conclusions

The advent and evolution of vaccinology research is captured within the history of Vaccinia virus. In 1796, cowpox lesion matter, a close relative of Vaccinia virus, was administered in the first vaccination study ever to be undertaken. In the 1970's the virus was attenuated by serial passage to create a safer, live vaccine and today, MVA is subject to development as a recombinant viral vaccine vector using the latest molecular biology, vaccine evaluation and product manufacturing techniques.

There are many aspects of rMVA design to consider when generating novel rMVA-based vaccines. This presents an opportunity to undertake rational optimisation for the induction of potent and long-lasting immune responses that are appropriate for the target disease. As such, it is insufficient to report that a given MVA-antigen combination is or is not effective. As demonstrated in the current study, efficacy is intrinsically linked to the precise nature of the rMVA vaccines' construction. A recent human efficacy study for MVA85A was unsuccessful, but factors such as vaccinia promoter selection and leader sequence optimisation may provide a means of improving future constructs which will need to deliver several antigens in order to target multiple stages of disease.

The value of vaccinia promoter selection may be to reduce the dose and thereby the cost and anti-vector effects of rMVA vaccines. Leader sequences represent an opportunity to further explore and enhance TB antigen immunogenicity. The rational selection of an

appropriate leader sequence may result in a more diverse immune response to an antigen, or help to explore changes in the quality of the immune response and their impact on protective efficacy. Vaccinia promoter selection and leader sequence optimisation should be a feature of future rMVA TB vaccine development.

Appendix I

Transfer plasmids designed to insert OVA into the MVA genome were sequenced across the open reading frame to obtain continuous data for one or both strands. The sequenced region is indicated by a green arrow. The position of restriction enzyme recognition sites utilised in the cloning process is also highlighted. The plasmids maps were generated in SeqBuilder DNA Lasergene 11.

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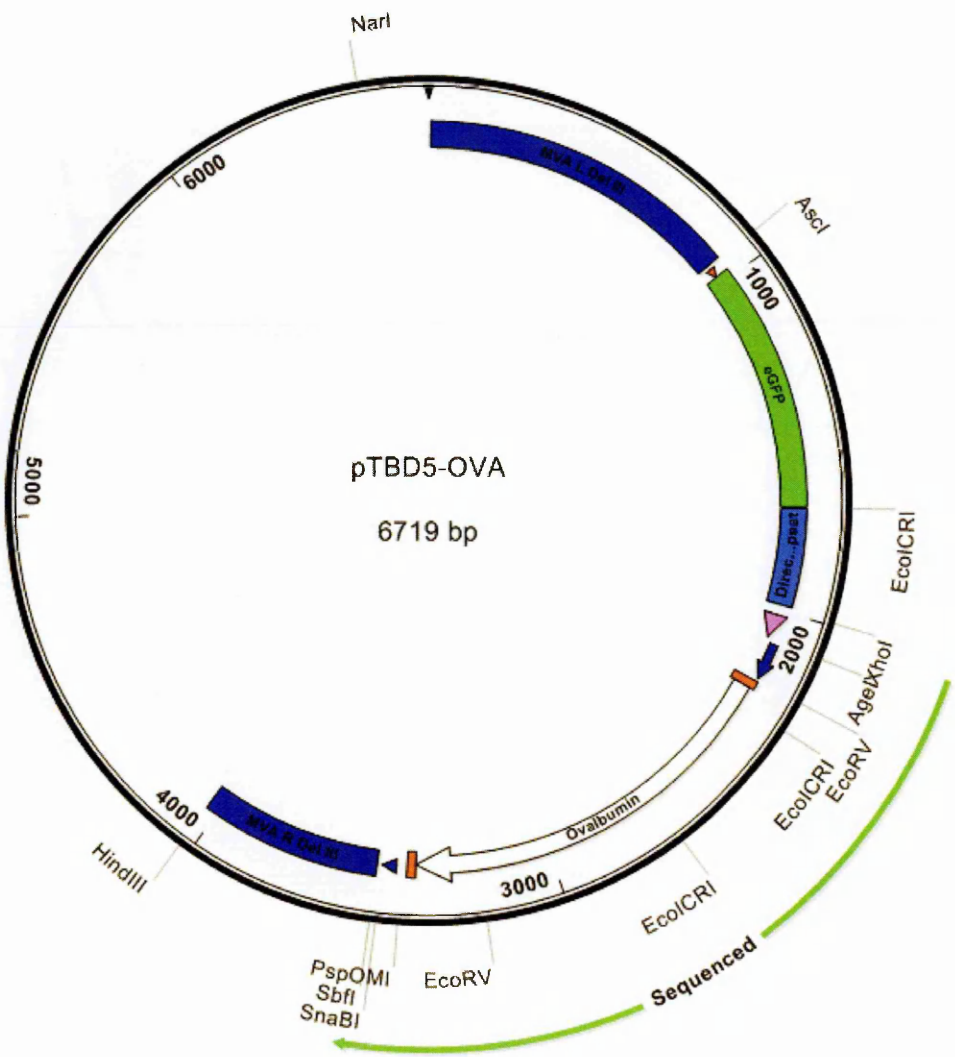
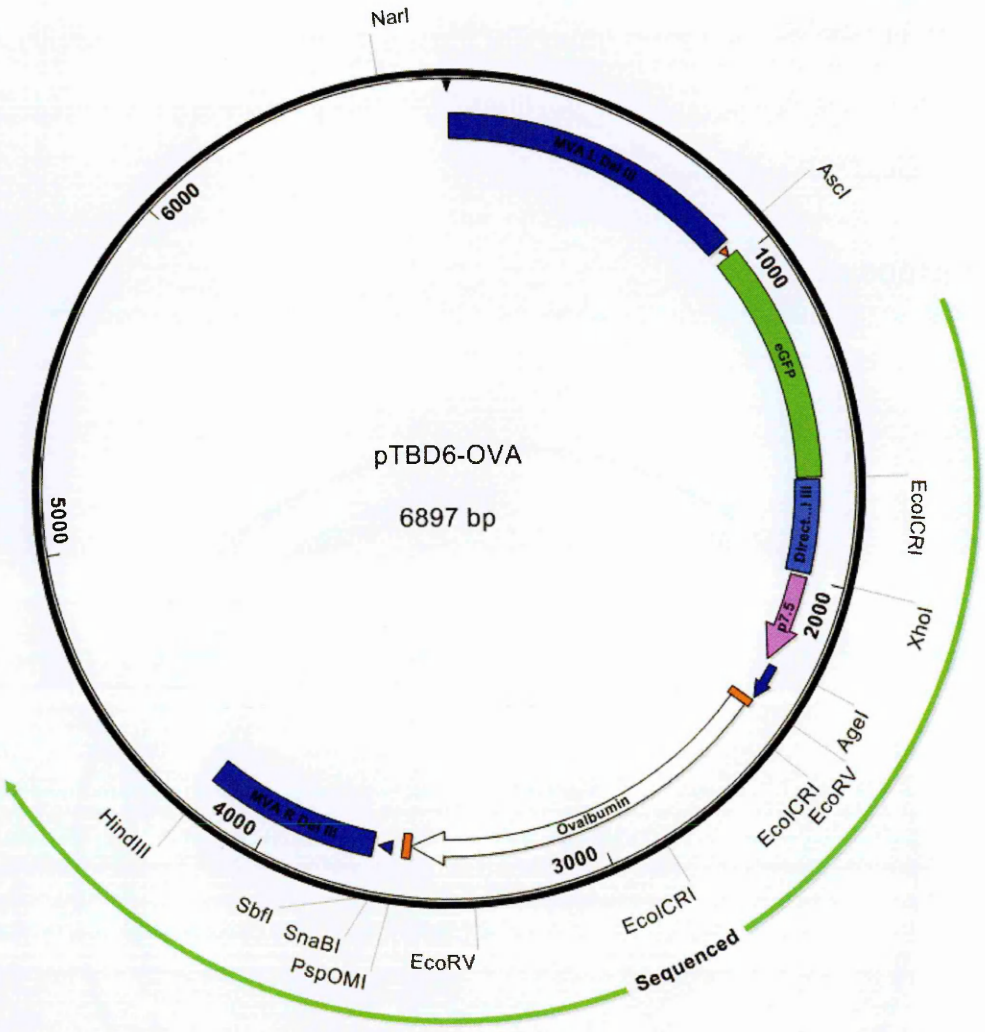


ii. pTBD6-OVA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AscI
EcoICRI
XhoI
AgeI
EcoICRI
EcoRV
EcoICRI
EcoRV
SnaBI
PspOMI
SbfI
HindIII
pTBD6-OVA
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4000
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eGFP
p7.5
Ovalbumin
Sequenced

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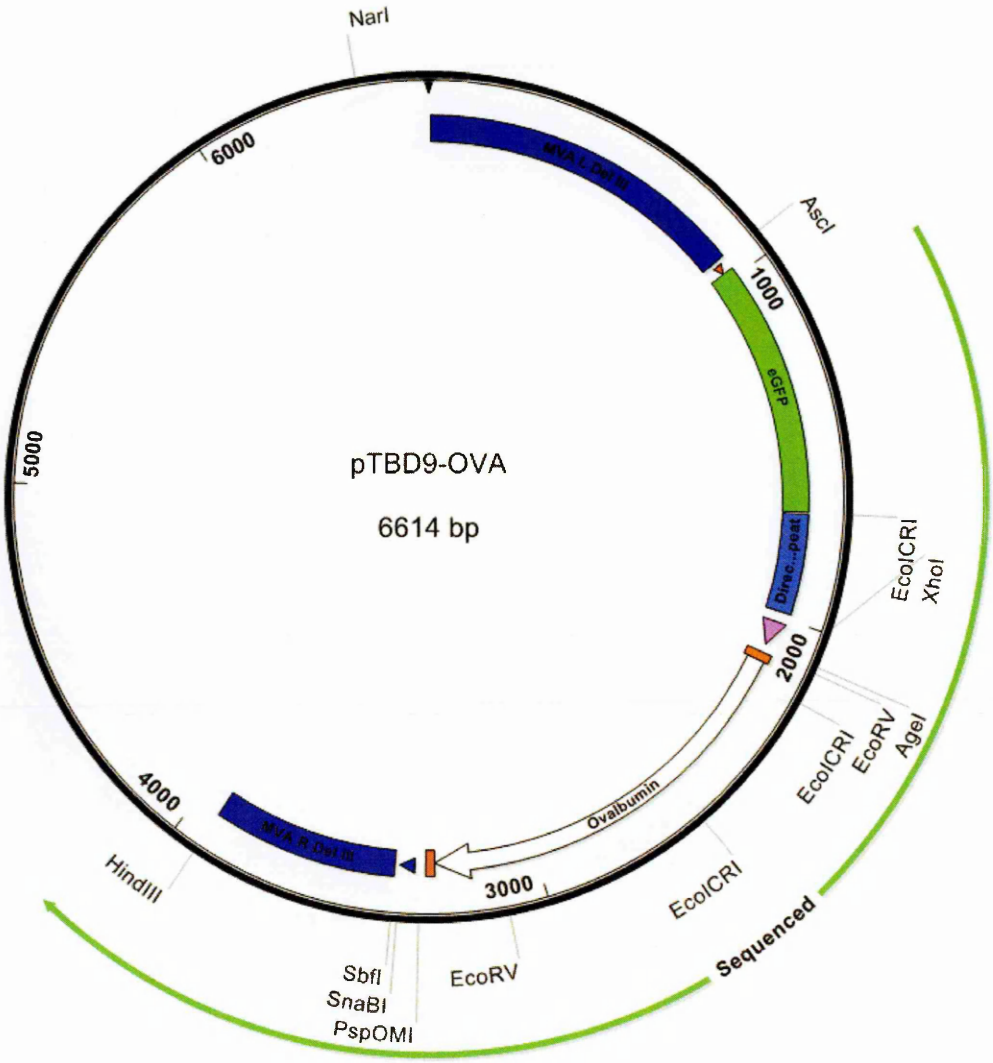

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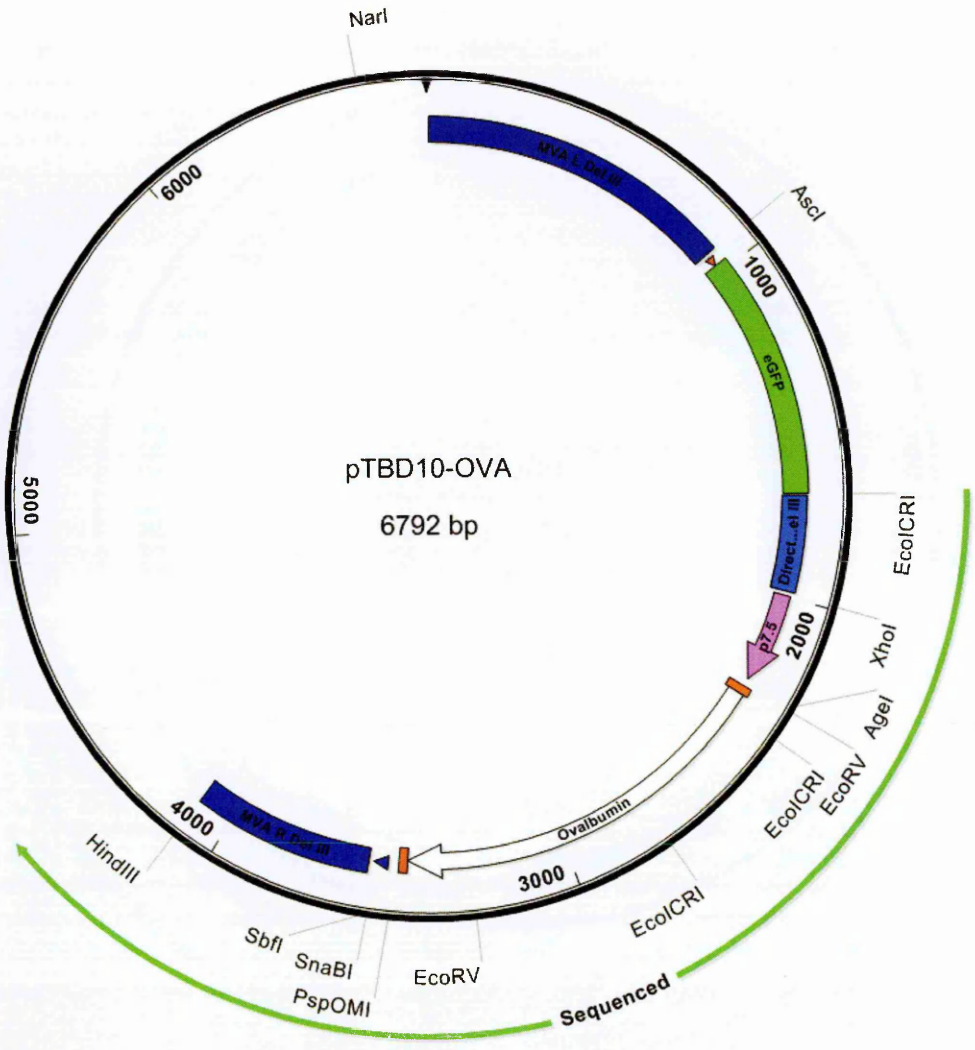

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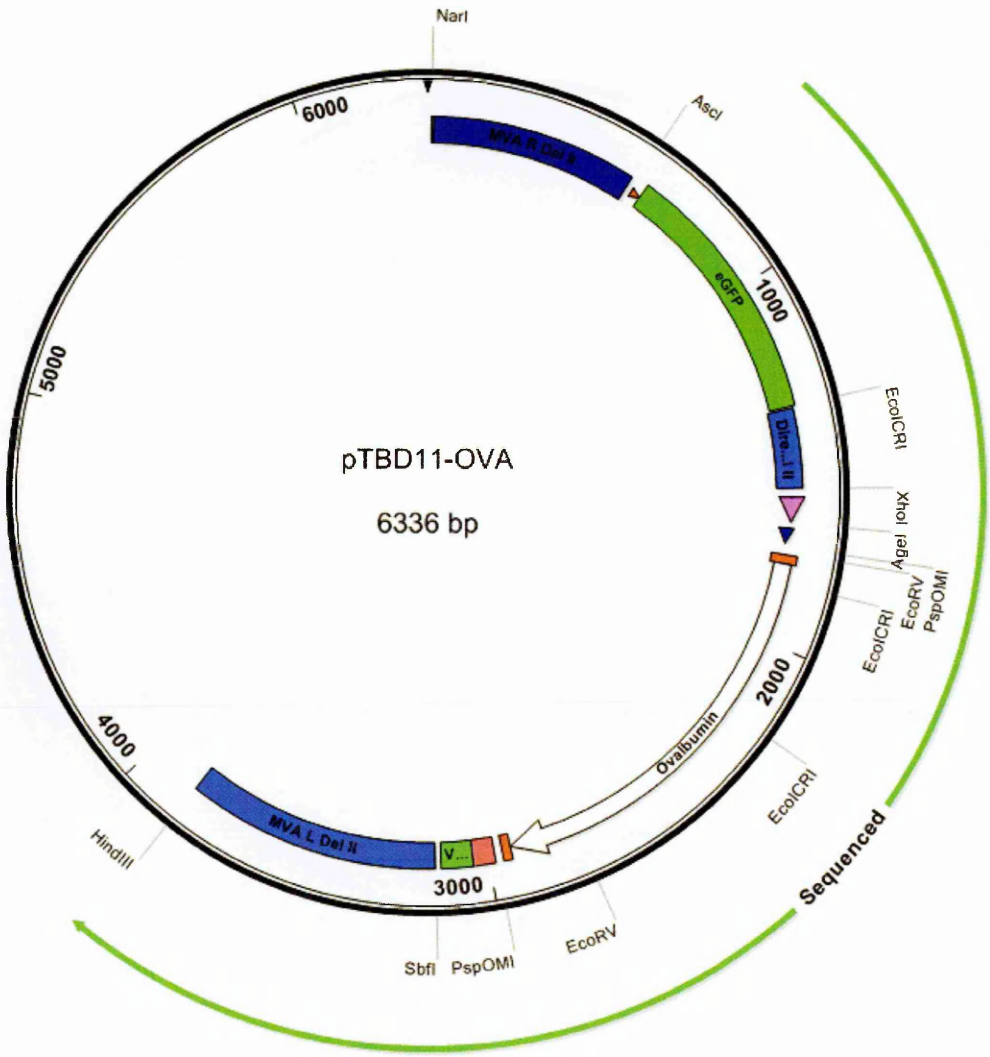
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AscI
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1000
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XhoI
AgeI
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EcoICRI
2000
Ovalbumin
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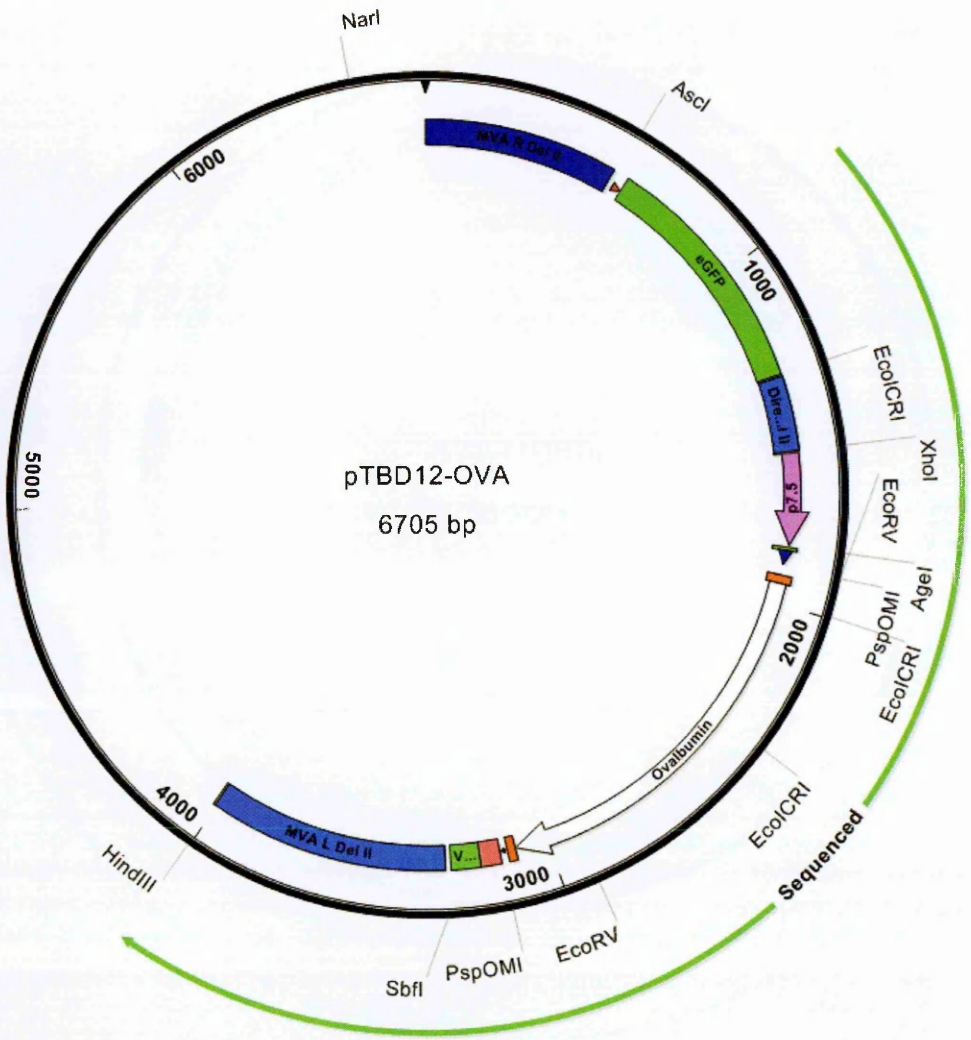
HindIII
MVA L Del II
3000
SbfI
PspOMI
EcoRV
Sequenced

vi. pTBD12-OVA



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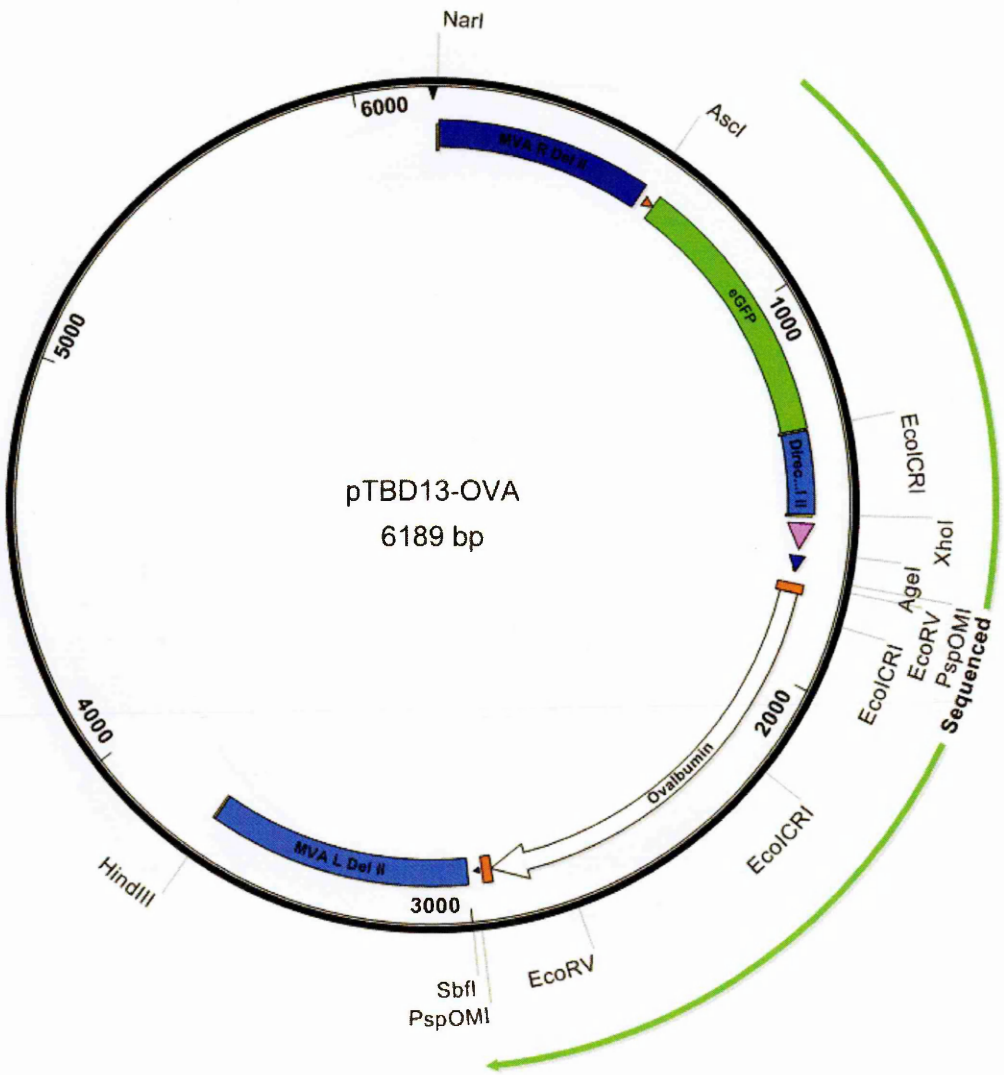
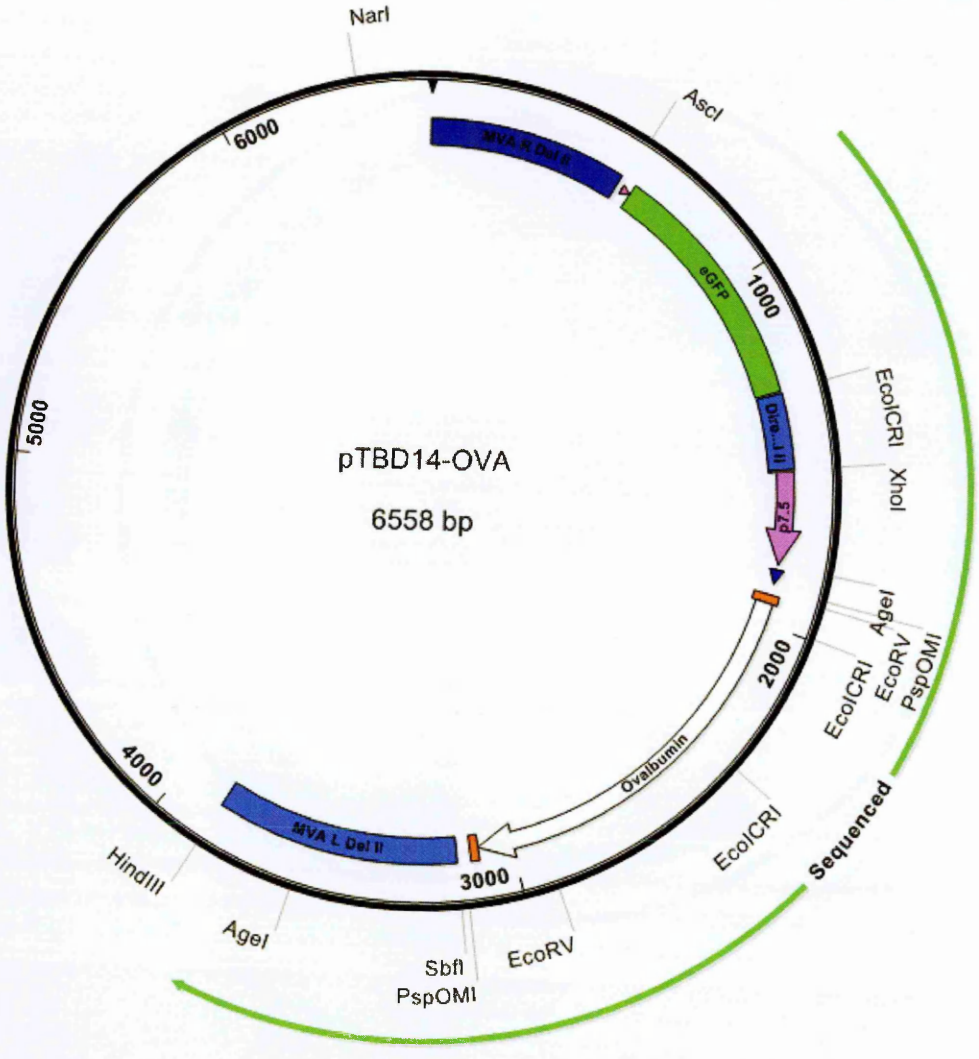


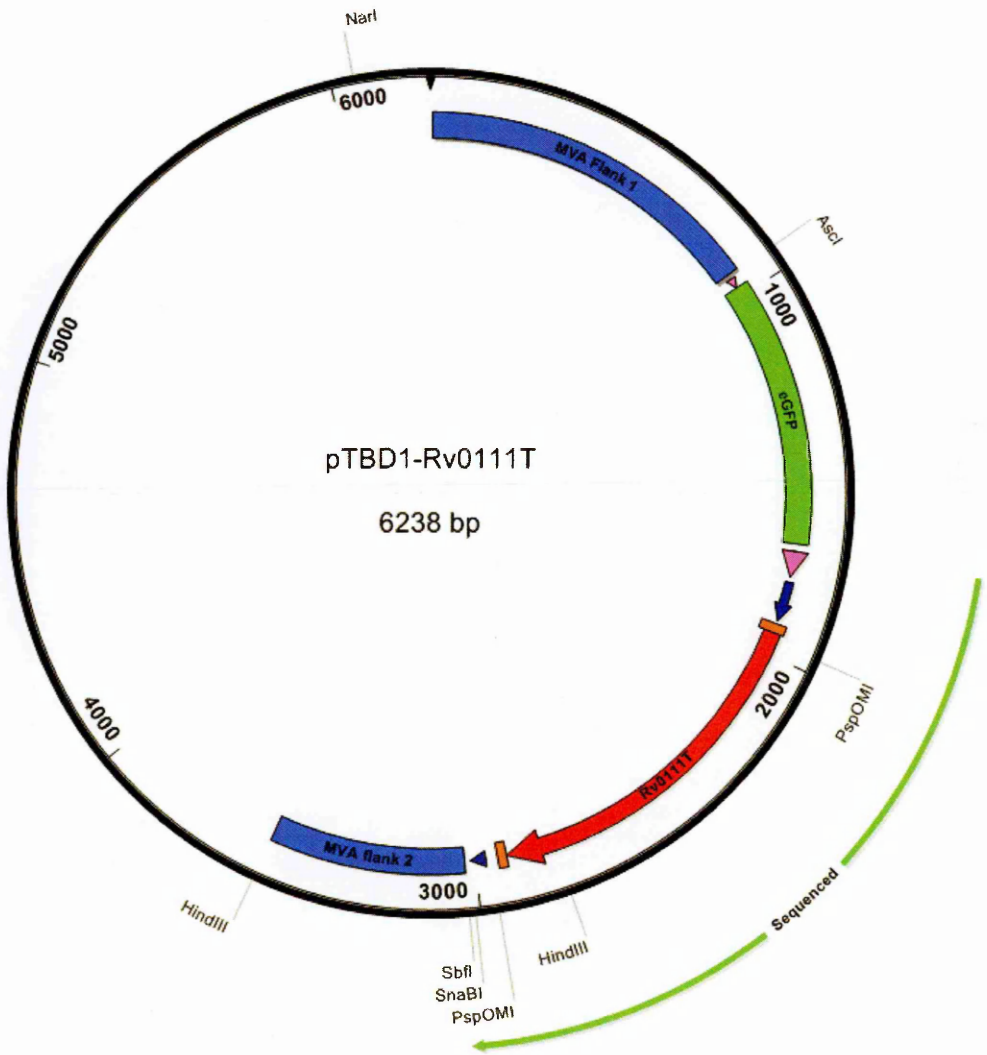
viii. pTBD14-OVA



Appendix II

A transfer plasmid designed to insert a truncated version of Rv0111 (Rv0111T) into the MVA genome was sequenced across the open reading frame to obtain continuous data for one or both strands. The sequenced region is indicated by a green arrow.

i. pTBD1-Rv0111T



Appendix III

The pTBD plasmid series are *E. coli* cloning vectors incorporating an Ampicillin resistance gene (see Section 2.1.4). The sequence of each recombinant cassette is as follows:

i. pTBD1-Rv0111T

Feature	Plasmid bases	GenBank Accession No./Sequence
MVA Del III Left flank	7 - 933	U94848.1; 148413 – 149339
Promoter P11	948 - 978	TTTCATTTTGTTTTTTCTATGCTATAAATG
GFP	976 -1695	JQ693016.1
Promoter mH5	1717 - 1786	AAAAATTGAAAATAAATACAAAGGTTCTTGA GGGTTGTGTTAAATTGAAAGCGAGAAATAAT CATAAATA
Optimised Kozak	1795 - 1804	GCCACCATGG
TPA	1801 - 1908	X13097.1; 109 – 216
RE site/Gateway attB1	1909 - 1941	CCCATCAAACAAGTTTGTACAAAAAAGCAGG CT
Rv0111T	1942 - 2913	CP003248.2; 135031 - 136002
Gateway attB2/RE site	2914 - 2946	ACCCAGCTTTCTTGTACAAAGTGGTTCGATGG G
Linker	2947 - 2970	GATCTAGAGGGCCCGCGGTTCGAA
V5 tag/Stop	2971 - 3012	GGTAAGCCTATCCCTAACCCTCTCCTCGGTCT CGATTCTACGTAA
MVA Del III Right flank	3028 - 3549	U94848.1; 149345 – 149866

ii. pTBD2-Rv0111

Feature	Plasmid bases	GenBank Accession No./Sequence
MVA Del III Left flank	7 - 933	U94848.1; 148413 – 149339
Promoter P11	948 - 978	TTTCATTTTGTTTTTTTCTATGCTATAAATG
GFP	976 -1695	JQ693016.1
Promoter 7.5	1704 - 1975	AY243312.1; 190028 - 190295
Optimised Kozak	1978 -1987	GCCACCATGG
TPA	1984 - 2091	X13097.1; 109 – 216
RE site/Gateway attB1	2092 - 2124	CCCATCAAACAAGTTTGTACAAAAAAGCAGG CT
Rv0111	2125 - 4176	CP003248.2; 133951 - 136002
Gateway attB2/RE site	4177 - 4209	ACCCAGCTTTCTTGTACAAAGTGGTTCGATGG G
Linker	4210 - 4233	GATCTAGAGGGCCCGCGGTTCGAA
V5 tag/Stop	4234 - 4278	GGTAAGCCTATCCCTAACCCTCTCCTCGGTCT CGATTCTACGTAA
MVA Del III Right flank	4291 - 4812	U94848.1; 149345 – 149866

iii. pTBD1-Ag85A

Feature	Plasmid bases	GenBank Accession No./Sequence
MVA Del III Left flank	7 - 933	U94848.1; 148413 – 149339
Promoter P11	948 - 978	TTTCATTTTGTTTTTTCTATGCTATAAATG
GFP	976 -1695	JQ693016.1
Promoter mH5	1717 - 1786	AAAAATTGAAAATAAATACAAAGGTTCTTGA GGGTTGTGTTAAATTGAAAGCGAGAAATAAT CATAAATA
Optimised Kozak	1795 - 1804	GCCACCATGG
TPA	1801 - 1908	X13097.1; 109 – 216
RE site/Gateway attB1	1909 - 1941	CCCATCAAACAAGTTTGTACAAAAAAGCAGG CT
Ag85A (Rv3804c)	1942 - 2955	CP003248.2; 4266836 - 4265823
Gateway attB2/RE site	2956 - 2988	ACCCAGCTTTCTTGTACAAAGTGGTTCGATGG G
Linker	2989 - 3012	GATCTAGAGGGCCCGCGGTTCGAA
V5 tag/Stop	3013 - 3057	GGTAAGCCTATCCCTAACCCTCTCCTCGGTCT CGATTCTACGTAA
MVA Del III Right flank	3070 - 3591	U94848.1; 149345 – 149866

iv. pTBD2-Ag85A

Feature	Plasmid bases	GenBank Accession No./Sequence
MVA Del III Left flank	7 - 933	U94848.1; 148413 – 149339
Promoter P11	948 - 978	TTTCATTTTGTTTTTTTCTATGCTATAAATG
GFP	976 -1695	JQ693016.1
Promoter 7.5	1704 - 1975	AY243312.1; 190028 - 190295
Optimised Kozak	1978 -1987	GCCACCATGG
TPA	1984 - 2091	X13097.1; 109 – 216
RE site/Gateway attB1	2092 - 2124	CCCATCAAACAAGTTTGTACAAAAAAGCAGG CT
Ag85A (Rv3804c)	2125 - 3138	CP003248.2; 4266836 - 4265823
Gateway attB2/RE site	3139 - 3171	ACCCAGCTTTCTTGTACAAAGTGGTTCGATGG G
Linker	3172 - 3195	GATCTAGAGGGCCCGCGGTTCGAA
V5 tag/Stop	3196 - 3240	GGTAAGCCTATCCCTAACCCTCTCCTCGGTCT CGATTCTACGTAA
MVA Del III Right flank	3253 - 3774	U94848.1; 149345 – 149866

v. pTBD5-OVA

Feature	Plasmid bases	GenBank Accession No./Sequence
MVA Del III Left flank	7 - 933	U94848.1; 148413 – 149339
Promoter P11	948 - 978	TTTCATTTTGTTTTTTCTATGCTATAAATG
GFP	976 -1695	JQ693016.1
MVA Del III Direct repeat	1702 - 1983	U94848.1; 149058 - 149339
Promoter mH5	2015 - 2084	AAAAATTGAAAATAAATACAAAGGTTCTTGAG GGTTGTGTTAAATTGAAAGCGAGAAATAATCA TAAATA
Optimised Kozak	2093 - 2102	GCCACCATGG
TPA	2099 - 2206	X13097.1; 109 – 216
RE site/Gateway attB1	2207 - 2239	GATATCAAACAAGTTTGTACAAAAAAGCAGGC T
Ovalbumin	2240 - 3394	NM_205152.1; 68 - 1222
Gateway attB2/RE site	3395 - 3427	ACCCAGCTTTCTTGTACAAAGTGGTTCGATGGG
Linker	3428 - 3451	GATCTAGAGGGCCCGCGGTTCGAA
V5 tag/Stop	3452 - 3496	GGTAAGCCTATCCCTAACCCTCTCCTCGGTCTC GATTCTACGTAA
MVA Del III Right flank	3509 - 4030	U94848.1; 149345 – 149866

vi. pTBD6-OVA

Feature	Plasmid bases	GenBank Accession No./Sequence
MVA Del III Left flank	7 - 933	U94848.1; 148413 – 149339
Promoter P11	948 - 978	TTTCATTTTGTTTTTTTCTATGCTATAAATG
GFP	976 -1695	JQ693016.1
MVA Del III Direct repeat	1702 - 1983	U94848.1; 149058 - 149339
Promoter 7.5	1997 - 2264	AY243312.1; 190028 - 190295
Optimised Kozak	2271 - 2280	GCCACCATGG
TPA	2277 - 2384	X13097.1; 109 – 216
RE site/Gateway attB1	2385 - 2417	GATATCAAACAAGTTTGTACAAAAAAGCAGG CT
Ovalbumin	2418 - 3572	NM_205152.1; 68 - 1222
Gateway attB2/RE site	3573 - 3605	ACCCAGCTTTCTTGTACAAAGTGGTTCGATGG G
Linker	3606 -3629	GATCTAGAGGGCCCGCGGTTCGAA
V5 tag/Stop	3630 – 3674	GGTAAGCCTATCCCTAACCCTCTCCTCGGTCT CGATTCTACGTAA
MVA Del III Right flank	3687 - 4208	U94848.1; 149345 – 149866

vii. pTBD9-OVA

Feature	Plasmid bases	GenBank Accession No./Sequence
MVA Del III Left flank	7 - 933	U94848.1; 148413 – 149339
Promoter P11	948 - 978	TTTCATTTTGTTTTTTTCTATGCTATAAATG
GFP	976 -1695	JQ693016.1
MVA Del III Direct repeat	1702 - 1983	U94848.1; 149058 - 149339
Promoter mH5	2015 - 2084	AAAAATTGAAAATAAATACAAAGGTTCTTGA GGGTTGTGTTAAATTGAAAGCGAGAAATAAT CATAAATA
Optimised Kozak	2093 - 2102	GCCACCATGG
RE site/Gateway attB1	2099 - 2134	ATGGATATCAAACAAGTTTGTACAAAAAAGC AGGCT
Ovalbumin	2135 - 3289	NM_205152.1; 68 - 1222
Gateway attB2/RE site	3290 - 3322	ACCCAGCTTTCTTGTACAAAGTGGTTCGATGG G
Linker	3323 - 3346	GATCTAGAGGGCCCGCGGTTCGAA
V5 tag/Stop	3347 - 3391	GGTAAGCCTATCCCTAACCCTCTCCTCGGTCT CGATTCTACGTAA
MVA Del III Right flank	3404 - 3925	U94848.1; 149345 – 149866

viii. pTBD10-OVA

Feature	Plasmid bases	GenBank Accession No./Sequence
MVA Del III Left flank	7 - 933	U94848.1; 148413 – 149339
Promoter P11	948 - 978	TTTCATTTTGTTTTTTTCTATGCTATAAATG
GFP	976 -1695	JQ693016.1
MVA Del III Direct repeat	1702 - 1983	U94848.1; 149058 - 149339
Promoter 7.5	1997 - 2264	AY243312.1; 190028 - 190295
Optimised Kozak	2271 - 2280	GCCACCATGG
RE site/Gateway attB1	2277 -2312	ATGGATATCAAACAAGTTTGTACAAAAAAGC AGGCT
Ovalbumin	2313 - 3467	NM_205152.1; 68 - 1222
Gateway attB2/RE site	3468 -3500	ACCCAGCTTTCTTGTACAAAGTGGTTCGATGG G
Linker	3501 -3524	GATCTAGAGGGCCCGCGGTTCGAA
V5 tag/Stop	3525 -3569	GGTAAGCCTATCCCTAACCCTCTCCTCGGTCT CGATTCTACGTAA
MVA Del III Right flank	3582 - 4103	U94848.1; 149345 – 149866

ix. pTBD11-OVA

Feature	Plasmid bases	GenBank Accession No./Sequence
MVA Del II Right flank	17 - 583	U94848.1; 21284 - 20719
Promoter P11	601 - 631	TTTCATTTTGTTTTTTTCTATGCTATAAATG
GFP	629 - 1348	JQ693016.1
MVA Del II Direct repeat	1355 - 1571	U94848.1; 20935 - 20719
Promoter mH5	1596 - 1665	AAAAATTGAAAATAAATACAAAGGTTCTTGA GGGTTGTGTTAAATTGAAAGCGAGAAATAAT CATAAATA
Optimised Kozak	1674 - 1683	GCCACCATGG
V5 tag	1683 - 1724	GGTAAGCCTATCCCTAACCCTCTCCTCGGTCT CGATTCTACG
Linker	1725 -1748	GATCTAGAGGGCCCGCGGTTCGAA
RE site/Gateway attB1	1749 - 1781	GATATCAAACAAGTTTGTACAAAAAAGCAGG CT
Ovalbumin	1782 -2936	NM_205152.1; 68 - 1222
Gateway attB2/RE site	2937 – 2984	ACCCAGCTTTCTTGTACAAAGTGGTTCGATGG GGATCTAGAGGGCCCG
VSVg/Stop	2985 - 3134	GU177825.1; 1387 - 1536
MVA Del II Left flank	3148 - 3842	U94848.1; 20666 - 19972

x. pTBD12-OVA

Feature	Plasmid bases	GenBank Accession No./Sequence
MVA Del II Right flank	1 - 567	U94848.1; 21284 - 20719
Promoter P11	585 - 615	TTTCATTTTGTTTTTTTCTATGCTATAAATG
GFP	613 - 1332	JQ693016.1
MVA Del II Direct repeat	1339 - 1555	U94848.1; 20935 - 20719
Promoter 7.5	1562 - 1829	AY243312.1; 190028 - 190295
Optimised Kozak	1836 - 1845	GCCACCATGG
V5 tag	1845 - 1886	GGTAAGCCTATCCCTAACCCTCTCCTCGGTCT CGATTCTACG
Linker	1887 - 1910	GATCTAGAGGGCCCGCGGTTCGAA
RE site/Gateway attB1	1911 - 1943	GATATCAAACAAGTTTGTACAAAAAAGCAGG CT
Ovalbumin	1944 - 3098	NM_205152.1; 68 - 1222
Gateway attB2/RE site	3099 - 3146	ACCCAGCTTTCTTGTACAAAGTGGTTCGATGG GGATCTAGAGGGCCCG
VSVg/Stop	3147 - 3296	GU177825.1; 1387 - 1536
MVA Del II Left flank	3310 - 4004	U94848.1; 20666 - 19972

xi. pTBD13-OVA

Feature	Plasmid bases	GenBank Accession No./Sequence
MVA Del II Right flank	17 - 583	U94848.1; 21284 - 20719
Promoter P11	601 - 631	TTTCATTTTGTTTTTTTCTATGCTATAAATG
GFP	629 - 1348	JQ693016.1
MVA Del II Direct repeat	1355 - 1571	U94848.1; 20935 - 20719
Promoter mH5	1596 - 1665	AAAAATTGAAAATAAATACAAAGGTTCTTGA GGGTTGTGTTAAATTGAAAGCGAGAAATAAT CATAAATA
Optimised Kozak	1674 - 1683	GCCACCATGG
V5 tag	1683 - 1724	GGTAAGCCTATCCCTAACCCTCTCCTCGGTCT CGATTCTACG
Linker	1725 -1748	GATCTAGAGGGCCCGCGGTTCGAA
RE site/Gateway attB1	1749 - 1781	GATATCAAACAAGTTTGTACAAAAAAGCAGG CT
Ovalbumin	1782 -2936	NM_205152.1; 68 - 1222
Gateway attB2/RE site/Stop	2937 – 2987	ACCCAGCTTTCTTGTACAAAGTGGTTCGATGG GGATCTAGAGGGCCCGTAA
MVA Del II Left flank	3001 - 3695	U94848.1; 20666 - 19972

xii. pTBD14-OVA

Feature	Plasmid bases	GenBank Accession No./Sequence
MVA Del II Right flank	1 - 567	U94848.1; 21284 - 20719
Promoter P11	585 - 615	TTTCATTTTGTTTTTTTCTATGCTATAAATG
GFP	613 - 1332	JQ693016.1
MVA Del II Direct repeat	1339 - 1555	U94848.1; 20935 - 20719
Promoter 7.5	1562 - 1829	AY243312.1; 190028 - 190295
Optimised Kozak	1836 - 1845	GCCACCATGG
V5 tag	1845 - 1886	GGTAAGCCTATCCCTAACCCTCTCCTCGGTCT CGATTCTACG
Linker	1887 - 1910	GATCTAGAGGGCCCGCGGTTCGAA
RE site/Gateway attB1	1911 - 1943	GATATCAAACAAGTTTGTACAAAAAAGCAGG CT
Ovalbumin	1944 - 3098	NM_205152.1; 68 - 1222
Gateway attB2/RE site/stop	3099 - 3149	ACCCAGCTTTCTTGTACAAAGTGGTTCGATGG GGATCTAGAGGGCCCGTAA
VSVg/Stop	3147 - 3296	GU177825.1; 1387 - 1536
MVA Del II Left flank	3310 - 4004	U94848.1; 20666 - 19972

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