Part Three

PREPARATION OF RECOMBINANT MODIFIED VACCINIA VIRUS ANKARA AND VACCINIA VIRUS WESTERN RESERVE STRAIN EXPRESSING A FULL-LENGTH RABIES VIRUS GLYCOPROTEIN GENE AND EVALUATION OF IMMUNOGENICITY IN A MOUSE MODEL

CHAPTER V

THE CONSTRUCTION AND ISOLATION OF RECOMBINANT MODIFIED VACCINIA VIRUS ANKARA AND VACCINIA VIRUS WESTERN RESERVE STRAIN EXPRESSING A FULL-LENGTH RABIES VIRUS GLYCOPROTEIN GENE

5.1 Introduction

Since its original use as a smallpox vaccine, vaccinia viruses, both parental and recombinant, have been used as tools in molecular biology studies. Not only are these viruses exploited for their immunogenic features but they are also useful for the investigation of molecular and cellular processes (Carroll and Moss, 1997b). Recombinant gene expression by a vaccinia virus was first achieved in 1982 (Mackett *et al*., 1982, Panicalli and Paoletti, 1982). In that study it was shown that stable recombinant vaccinia viruses could be generated by utilizing the process of *in vivo* homologous recombination between the parental virus genome and a plasmid encoding particular essential regions. This remains the most often used approach for the generation of these recombinants.

The generation and selection of recombinant vaccinia viruses, and other poxviruses, have since become standard laboratory procedure (Smith and Mackett, 1992). Nonetheless there are important differences in the generation of recombinants of Modified Vaccinia virus Ankara (MVA) and replication competent vaccinia viruses that should be considered. Firstly, the cell cultures that can be used for the procedures are different based on the host range specificities of the viruses. Vaccinia viruses, essentially, have a broad host range and can be cultured on many different cell cultures. In contrast, MVA only productively infect cell cultures of avian origin, such as chick embryo fibroblasts. Notably, although, the replication deficiency of MVA has been shown on many different cell cultures of mammalian and particularly human origin, MVA replicates on the cultures of the baby hamster kidney cell line, BHK-21 (Drexler *et al*., 1998). Secondly the regions for the insertion of expression cassettes also differ. Regions of deletion, most prominently the site of deletion III, are used as targets for

insertion in MVA (Sutter and Moss, 1992). Non-essential or truncated genes such as the hemagglutinin gene have also been targeted in MVA (Antoine *et al*., 1996). Most commonly recombinants of other vaccinia viruses are generated by insertion into non-essential genes such as the thymidine kinase gene. For generation of recombinant MVA the thymidine kinase locus can only be used in the presence of endogenous thymidine kinase activity (Scheiflinger *et al*., 1996). Another important consideration is that MVA theoretically can accommodate up to 50 000 base pair insertions, whereas insertion of 30 000 base pairs have been reported for vaccinia viruses (Sutter and Staib, 2003). Apart from generating recombinant through homologous recombination, poxvirus recombinants can also be generated through direct ligation, in other words molecular cloning, of the gene of interest (with relevant expression signals) into the virus genome and subsequent *in vivo* packaging of infectious virions (Pfleiderer *et al*., 1995; Merchilinksy and Moss, 1992; Scheiflinger *et al*., 1992). Various methods for the selection of recombinant viruses have been tested for the poxvirus system. Some of these are tabulated in table 5.1. Transient selection of recombinants is also feasible. This involves the removal of marker genes through intragenomic recombination events upon relief of selection pressure (Scheiflinger *et al*., 1998) (figure 5.1).

Table 5.1 Systems for selection of recombinant poxviruses (Smith and Mackett, 1992)

SELECTION METHOD	PRINCIPLE OF METHOD	REFERENCE
In situ hybridization	Labeled DNA probes are used to identify presence of foreign gene in recombinant virus plaques.	Panicalli and Paoletti, 1982
Chromatic selection through expression of enzymes such as β-galactosidase or β-glucoronidase	Recombinant viruses express the enzyme, which will in the presence of substrate, produce a chromogenic product that can be used to identify recombinant plaques.	Chakrabarti *et al*., 1987
Plaque morphology	Vaccinia virus genes such as the 14K structural protein allow normal plaque size, serpin K2L encodes antifusion factor which results in altered plaque phenotype. In other words, allows for visual selection of plaques.	Dallo *et al*., 1987 Law and Smith, 1992
Host range	Host range genes such as K1L and C7L affect virus growth on specific cell lines. Recombinant viruses encoding these genes can grow on these cell lines.	Perkus *et al*., 1990 Staib *et al*., 2000
Thymidine kinase selection	Can be used in two ways. The selection of TK negative recombinants due to metabolic bypass in the presence of 5-bromodeoxyurdine in a TK negative cell culture environment. TK positive recombinants can be selected if a TK negative parent virus was used, and in a TK negative cell culture environment in the presence of methotrexate.	Smith *et al*., 1983 Mackett *et al*., 1982
Resistance markers	The neomycin gene provides resistance against the antibiotic G-418 (abolished plaque formation in wild type virus). The guanine phosphoribosyl transferase gene (in the presence of xanthine and hypoxanthine) provides resistance against mycophenolic acid, an inhibitor of purine metabolism.	Franke *et al*., 1985 Boyle and Coupar, 1988

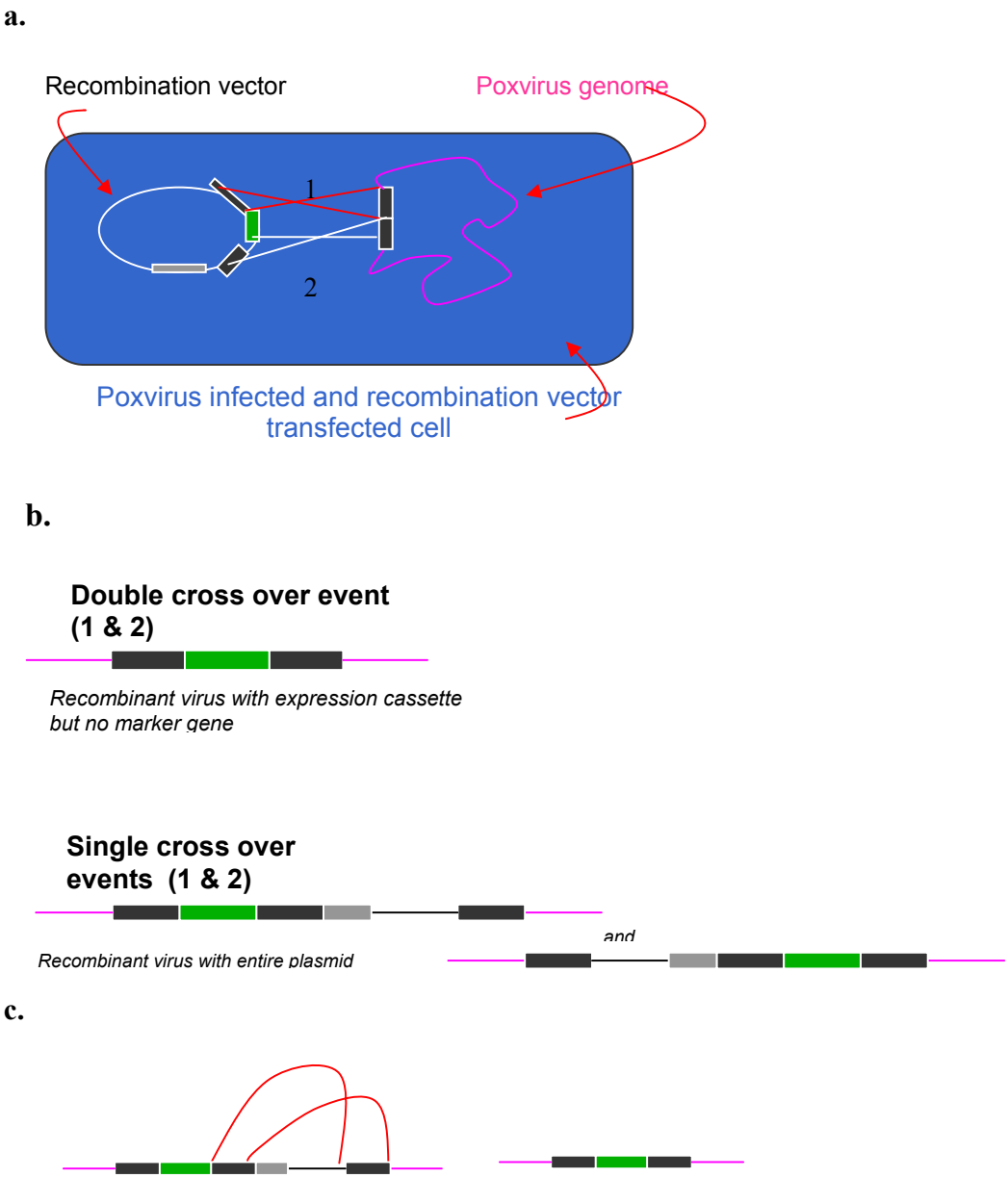


Figure 5.1: The principles of transient marker stabilization. Single or double cross over events may occur between the flanking regions (black blocks) on the transfer vector and the corresponding regions on the wild type virus genome (a). This will result in the generation of recombinant viruses with an incorporated expression cassette or entire plasmid (b).

Figure 5.1 (continued). Upon relief of selection, intragenomic recombination may occur, resulting in the generation of marker-free recombinant virus or a revertant wild type virus (c).

The aim of this section of the study was to generate and isolate homogenous recombinant MVA and vaccinia virus Western Reserve strain isolates, respectively, each expressing a full-length rabies virus glycoprotein gene. An additional goal of the study was the preparation of these recombinants and control viruses for immunization experiments.

5.2 Materials and methods

5.2.1 Viruses, plasmids and genes

A listing of the source of viruses, plasmids and genes used in this study is presented: The strain of MVA (isolate F6) used in this study was a generous gift from Dr. G. Sutter (Technical University of München, Germany) (Sutter and Moss, 1992). The MVA transfer vector, pIIIdHR-p7.5, was also provided by Dr. G. Sutter (Staib *et al*., 2000; Staib *et al*., 2003). Vaccinia virus Western Reserve strain and a vaccinia virus transfer vector, pGVRW1-gpt was obtained from Prof. G. J. Viljoen (Agricultural Research Council-Onderstepoort Veterinary Institute, South Africa). An experimental recombinant vaccinia virus Copenhagen strain expressing a rabies virus glycoprotein gene of the Evelyn-Rockitnicki-Abelseth strain was prepared as described elsewhere[11]. The gene was inserted into the hemagluttinin gene (HA) of the vaccinia virus genome and encoded a neomycin selection marker gene (Dvoracek and Shors, 2003). A rabies virus glycoprotein gene was obtained from a pFastBac1 clone, pFastBac1-RabG, which was provided by Prof. L.H. Nel (University of Pretoria, South Africa). The pFastBac1-RabG encodes the glycoprotein coding sequence of a South African yellow mongoose (*Cynictis penicillata*) isolate (ARC-OVI reference, isolate m710/90) cloned into the *Eco*R I site of the vector. A gene for enhanced green fluorescent protein (EGFP) of the jellyfish, *Aequorea victoria*, was obtained from a recombinant pGEM-T Easy® plasmid provided

[11] Refer to Part Four, Chapter VIII, sections 7.2.1 and 7.2.5.1

by Mrs. J. Mans (ARC-OVI, South Africa). The EGFP gene was under the regulation of the late vaccinia virus p11K promoter. Different *Escherichia coli* guaninephosphoribosyltransferase genes (*Eco*gpt), both under regulation of the vaccinia virus early-late p7.5K promoter, were obtained from the recombinant fowlpoxvirus transfer vector, pAF09, which was also provided by Mrs. J. Mans and a lumpy skin disease virus transfer vector, pLSEG, that was provided by Mr. D.B. Wallace (ARC-OVI, South Africa). The set of monoclonal antibodies used in rabies virus glycoprotein gene expression analysis, was a generous gift from Dr. A.I. Wandeler (Center of Expertise for Rabies, Canadian Food Inspection Agency, Canada). Embryonated chicken eggs for the primary cell culture were obtained from the division for Veterinary Tropical Diseases, Onderstepoort Campus, University of Pretoria, Pretoria, South Africa. Otherwise embryonated eggs were provided my Mrs. Henrietta Hall (CDC, USA).

5.2.2 General molecular biology and cell culture techniques

5.2.2.1 Molecular cloning techniques

The techniques and procedures described here are applicable to sections 5.2.3 and 5.2.4. The protocol collection in Sambrook and Russell, 2001 was used as a reference for the work described in this section. Plasmids were prepared by the alkaline lysis method as described by Birnboim and Doly, 1979. Clonable DNA fragments were prepared by restriction enzyme digestion. The manufacturers of the respective restriction endonucleases are indicated in brackets after the enzyme is mentioned in the text. The restriction reactions were preformed according to the particular manufacturer's prescribed conditions. DNA species were analyzed on 0.8% agarose gels using 1X TAE buffer (40 mM Tris-acetate, 1mM ethylenediaminetetraacetic acid). The DNA bands of interest were retrieved from agarose gels, when necessary, with the Wizard® DNA Clean-Up system (Promega) according to manufacturer's instructions. The purified products were resolved on 0.8% agarose gels and the concentrations of the products estimated and molecular size confirmed with the use of relevant molecular weight markers. Sticky-ended DNA fragments were ligated using T4 DNA ligase enzyme (Promega) in 1X T4 DNA ligase buffer (Promega) at 16 ºC for 20 hours. The ratio of

vector to insert fragments was calculated according to the molecular size of the respective fragments. Ligation mixes were transformed in transformation competent *Escherichia coli* cells (JM109 or DH5α strains) according to a heat shock protocol. Transformation transformation competent *E. coli* cultures were prepared using the modified calcium manganese based method (CCMB50) (Hanahan *et al*., 1991). The transformed cell cultures were plated out on Luria Bertani (LB) (1% tryptone, 1% sodium chloride, 0.5 % yeast; pH 7.4) agar supplemented with 100 µg/ml ampicillin. When required, blue-white selection through α-complementation was used for screening bacterial colonies, 40 µl of a 2 % 5-Bromo-4-chloro-3-indolyl-β-D-galactopyronoside (Boehringer-Mannheim) and 10 µl of 10 mM isopropyl β-D-1-thiogalactopyranoside (Boehringer-Mannheim), were plated out per LB agar plate (60 mm diameter, with 100 µg/ml ampicillin). The plates were incubated inverted, overnight at 37 ˚C. Colonies (for blue-white selection, white colonies) were picked with sterile toothpicks and inoculated in sterile LB broth with 100 µg/ml ampicillin. Inoculated cultures were incubated overnight at 37 ºC in a shake incubator shaking at 200 rotations per minute. Plasmids were screened by means of a rapid nucleic acid isolation technique from the overnight cultures as described in Beuken *et al*., 1998. Shortly, 200 µl of each of the overnight cultures was harvested by brief centrifugation in a benchtop microcentrifuge. The cell pellets were subsequently resuspended in 40 µl of agarose gel loading buffer (bromophenol blue in 40 % sucrose solution) and 14 µl phenol:chloroform:isoamylalcohol mixture (25:24:1). The suspensions were then centrifuged at 8 000 *g* in a benchtop microcentrifuge for 3 minutes. The aqueous top phase of each sample was then loaded onto an agarose gel and electrophoresed as before. Larger plasmids, observed as a band shift against parental plasmid DNA, were identified as possible recombinants. The plasmids from overnight cultures of the possible recombinants were prepared according to the alkaline lysis method, as before. Larger plasmids were analysed by restriction endonuclease analysis with appropriate enzymes to verify insertions. Sequencing reactions were prepared with Applied Biosystems BigDye® terminator kit (version 3.1) as prescribed by the manufacturer. The sequencing reactions were run at the DNA sequencing facility of the Faculty of Natural and Agricultural Sciences, University of Pretoria.

5.2.2.2 Cell culture techniques

Several different cell cultures were used in this study. The particulars of these cultures are tabulated in table 5.2. Cultures were grown in minimal essential medium (MEM) (Gibco, Invitrogen Life Technologies) supplemented with 4 mM glutamine, 2 X MEM vitamin solution (all from Gibco, Invitrogen Life Technologies) or Dulbecco's modified Eagle's medium supplemented with Ham's F12 (DMEM/F12) (Highveld Biologicals, South Africa). The medium was supplemented with 1 X antibiotics (100 µg/ml penicillin, 100 µg/ml streptomycin and 250 µg/ml amphotericin) (Gibco, Invitrogen Life Technologies) and fetal bovine serum (FBS) (Hyclone, USA). Cultures were cultured at 37 ˚C and at an atmosphere of 0.5 % CO_2. Confluent cultures were subcultured by removing spent media and washing the monolayers with phosphate buffered saline (PBS) (13.7 mM NaCl, 0.27 mM KCl, 0.43 mM $Na_2HPO_4.2H_2O$, 0.14 mM KH_2PO_4, pH 7.3) and then with 0.1% trypsin (using 40 µl per cm^2 of culture vessel). The dislodged cells were then resuspended in complete cell culture medium.

Table 5.2 Cell cultures used in this study**.**

CELL CULTURE (ATCC REFERENCE NUMBER OR PUBLICATION REFERENCE)	**TYPE OF CULTURE**	**PURPOSE IN THIS STUDY**
BHK-21 (CCL-10)	Baby hamster kidney	Growth and passage of MVA and vaccinia viruses
CEC-32 (Kaaden *et al*., 1982)	Chick embryo fibroblast	Transfection studies
CEF	Primary chick embryo fibroblast	Growth and passage of MVA
HeLa-229 (CCL-2.1)	Human cervix adenocarcinoma	Replication deficiency assay for MVA
Vero (CCL-81)	African green monkey kidney	Growth and passage of Vaccinia viruses Western Reserve and Copenhagen strain

Preparation of primary chick embryo fibroblasts

The primary cultures were prepared as described in Freshney, 1994 with some modifications.

9 to 11 day old special pathogen free embryonated chicken eggs were used to prepare the cultures. Briefly, the eggs were swabbed with 70 % ethanol or an equivalent disinfectant. All procedures were carried out with aseptic technique. The shell of the egg was then cut open at the blunt end and the chorioallantoic membranes removed. The embryo could then be removed from the egg using sterile forceps and placed in an erlen meyer flask with a magnetic stirrer bar. 10 ml of 0.05% trypsin was added per embryo and then stirred on a magnetic stirrer for 30 minutes at room temperature. The remaining masses of tissue (eyes et cetera) were removed from the soup followed by the addition of complete culture medium to neutralize the trypsin. The soup was centrifuged for 10 minutes at 200 *g* to collect the cells. The cell pellet was then resuspended in complete medium and filtered through sterile gauze to remove remaining masses of tissue. The cells were subsequently seeded on 75 cm^2 flasks at one egg per flask. Cultures were incubated at 37 ˚C and 5% CO_2 water-jacketed incubator. When cell cultures reached confluence the spent cell culture medium was removed, the monolayers washed with PBS and fed with cell culture medium with antibiotics but no added FBS. The cultures can be kept for up to one week at 37 ˚C with no CO_2. The cultures were subcultured as before.

5.2.3 Molecular cloning of a full-length rabies virus glycoprotein gene and an EGFP gene in a MVA transfer vector

5.2.3.1 Construction of a MVA transfer vector, pIIIp7.5-JW

Sequences flanking the site of deletion III in the MVA genome, and the early-late poxvirus promoter, p7.5K were obtained from the MVA transfer vector, pIIIdHR-p7.5 (figure 5.2). The left flanking, flank 1, and the p7.5K promoter sequences were digested from pIIIdHR-p7.5 with *Bam*H I (Promega) and *Spe* I (Promega) restriction endonucleases in a double digestion reaction setup in a compatible enzyme buffer system

(Promega). This fragment was then directionally inserted in to the *Bam*H I and *Xba* I (Promega) (compatible restriction endonuclease site with *Spe* I) sites of the cloning vector, pUC18 (figure 5.3) to generate pUC-delIIIflank1. The right flanking sequence, flank 2, was digested from pIIIdHR-p7.5 with *Kpn* I (New England Biolabs) and *Eco*R I (Promega) restriction enzymes in sequential digestions. The fragment was subsequently inserted into the *Kpn* I and *Eco*R I sites of pUC-delIIIflank1 to generate pUC-delIII. The *Eco*gpt resistance marker gene under control of the p7.5K promoter was retrieved from the lumpy skin disease virus transfer vector, pLSEG through digestion with *Eco*R I. The fragment including the marker was inserted into the *Eco*R I site of the pUC-delIII clone to obtain the MVA transfer vector, pIIIp7.5-JW (figure 5.4). Fragments of the expression cassette of the constructed vector were sequenced to verify integrity by using pUC/M13 universal forward and reverse primers (Promega).

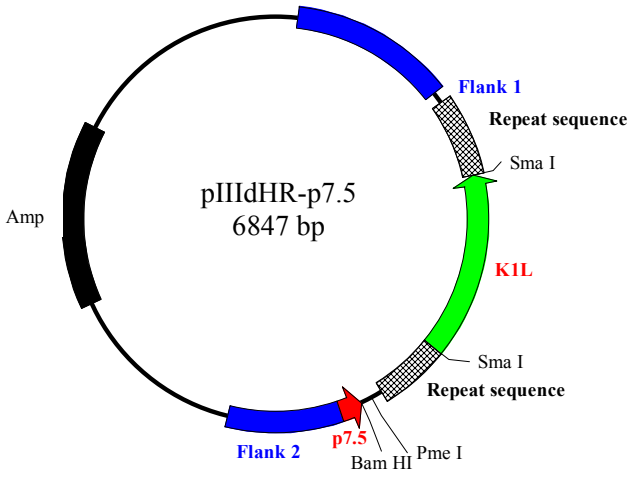


Figure 5.2: Plasmid map of MVA transfer vector, pIIIdHRp7.5 (adapted from Staib *et al.*, 2000)

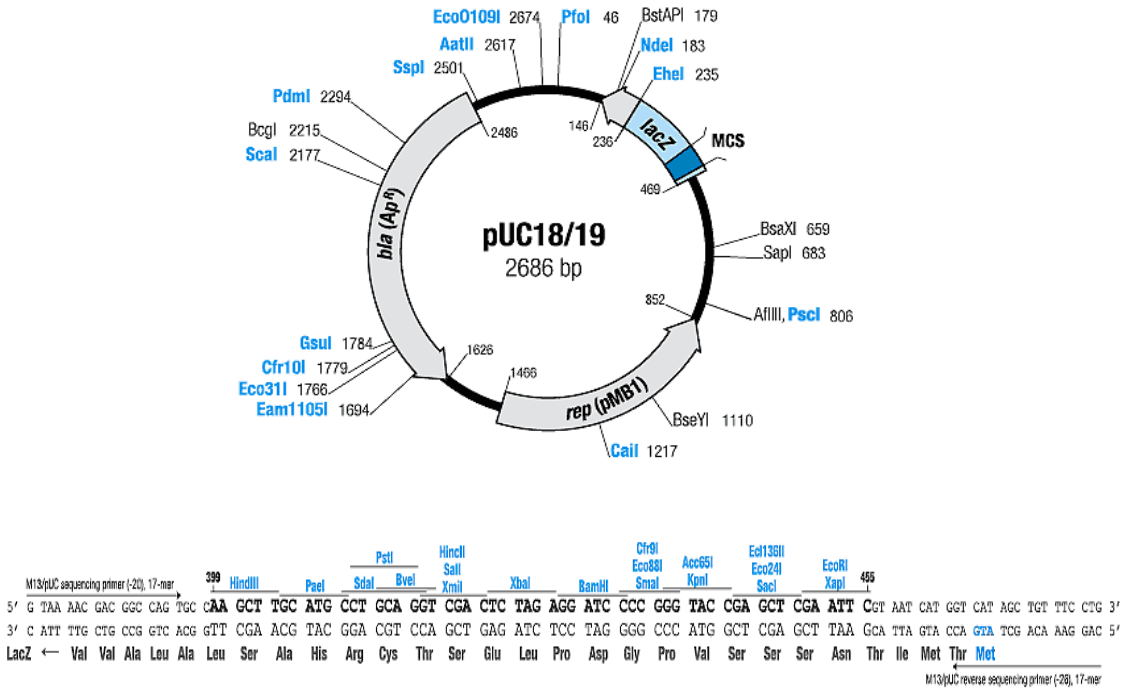


Figure 5.3: Plasmid map of the cloning plasmid, pUC18. Sequence information of the multiple cloning site is shown below the circular map (Internet reference 4).

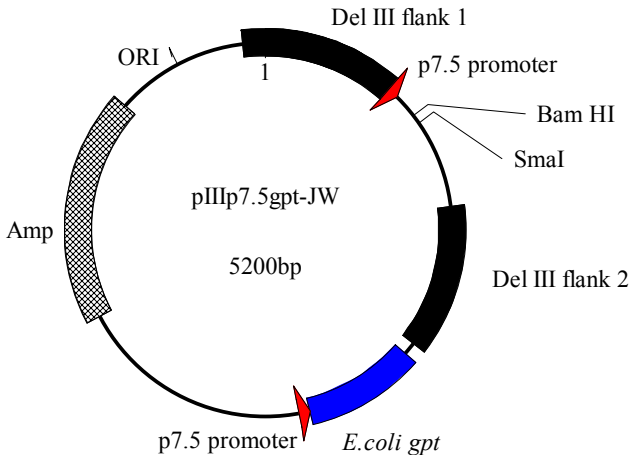


Figure 5.4: Plasmid map of the newly constructed MVA transfer vector, pIIIp7.5-JW.

5.2.3.2 Subcloning of a rabies virus glycoprotein gene in a cloning vector, pMTL-24

In order to obtain *Bam*H I restriction endonuclease sites, which were necessary for cloning into the MVA transfer vector, pIIIp7.5-JW, the gene was first subcloned into the cloning vector, pMTL-24 (figure 5.5) (Chambers *et al*., 1988). This vector has a unique restriction endonuclease cassette with a multiple cloning site flanked by a mirror-image of restriction endonuclease cut sites.

The pFastBac1-RabG plasmid was doubly digested in a compatible buffer system with *Hin d*III (Roche Molecular Biochemicals) and *Bam*H I (Promega) (figure 5.6). The pMTL-24 plasmid was doubly digested with *Hin d*III (Roche Molecular Biochemicals) and *Bgl* II (Roche Molecular Biochemicals) to yield the linearized plasmid. The restriction reaction was carried out in a compatible buffer system (Roche Molecular Biochemicals). The fragments were ligated and the products transformed and screened as described before[12]. To verify the insertion of the glycoprotein gene, *Bam*H I (Promega) restriction endonuclease digestion was carried out on plasmid preparations of possible recombinants. Recombinant plasmids were identified and dubbed pMTL-RabG.

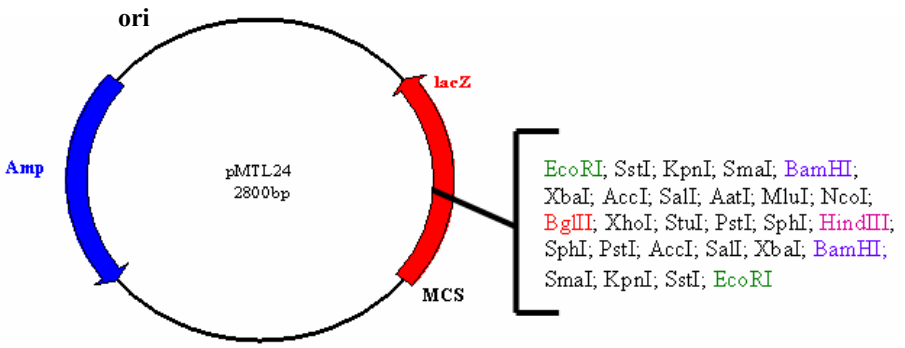


Figure 5.5: A diagrammatic representation of the pUC-based cloning vector, pMTL-24, indicating relevant features (Adapted from Chambers *et al*., 1988). Restriction endonuclease sites that were used during the described cloning procedure are indicated in colour.

[12] Refer to section 5.2.2.1

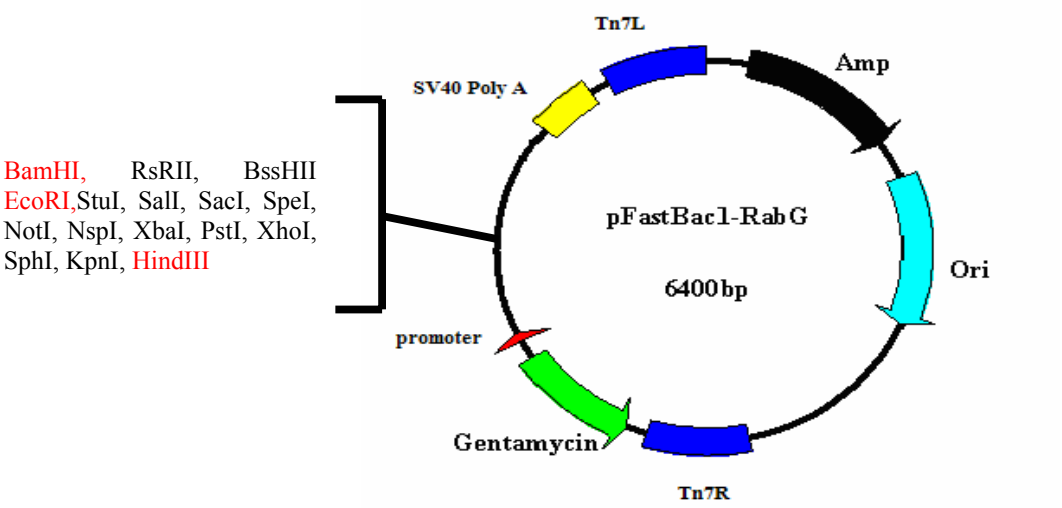


Figure 5.6: A plasmid map of the recombinant pFastBac1-RabG plasmid. A rabies glycoprotein gene was into the *Eco*R I site. Restriction endonuclease sites used during the described cloning are indicated in red.

5.2.3.3 Molecular cloning of the EGFP gene

The DNA fragment encoding the EGFP gene and p11K promoter sequence was excised from a recombinant pGEM-T Easy® clone with *Not* I enzyme (Amersham) (Figure 5.7). A pMTL-RabG clone was also digested with *Not* I (figure 5.8). The DNA fragment encoding the EGFP gene and p11K promoter sequences and the vector fragments were ligated in a 1:3 molecular weight ratio (vector DNA: insert DNA), respectively, as before. The ligation mixture was transformed and screened according to size, as previously described. Insertion of the gene and promoter sequence was verified by restriction with *Not* I, as before. The recombinants were given the name pMTL-RabG-EGFP.

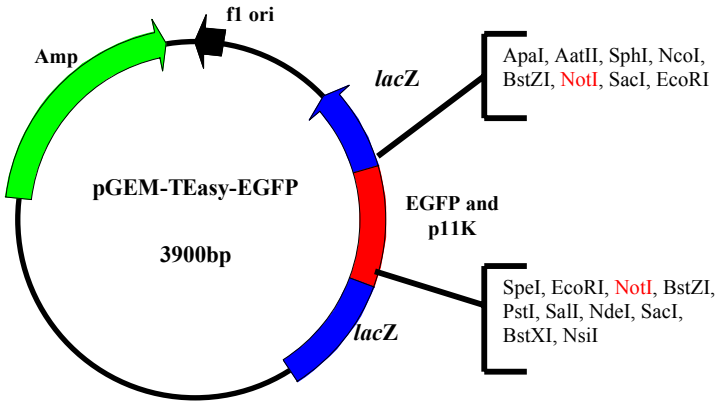


Figure 5.7: Plasmid map of the recombinant pGEM-T Easy vector containing an EGFP gene under regulation of the vaccinia virus p11K promoter sequence. The EGFP and promoter sequence insert is approximately 900 base pairs in size. The *Not* I site used during the described cloning procedure are indicated in red.

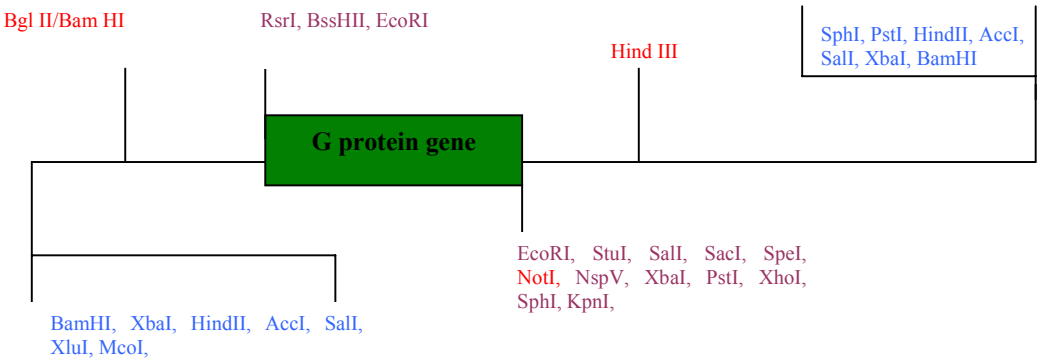


Figure 5.8: Diagrammatic representation of the multiple cloning site of the pMTL-RabG recombinant plasmid indicating the restriction endonuclease sites acquired during the described cloning steps. The sites given in blue are derived from the pMTL24 multiple cloning site. The sites in purple are derived from the pFastBac1 multiple cloning site. The sites used during different cloning steps are indicated in red. The *Bgl* II and *Bam*H I sites are not retrieved after ligation of the vector and insert fragments during the construction of pMTL-RabG.

5.2.3.4 Molecular cloning of the rabies virus glycoprotein and EGFP genes in pIIIp7.5-JW

The rabies virus glycoprotein and EGFP genes were excised from pMTL-RabG-EGFP with *Bam*H I (Promega). The fragment was inserted into the *Bam*H I site of pIIIp7.5-JW. The recombinants were prepared and screened for as before[13], and called pIIIp7.5-RabG-EGFP.

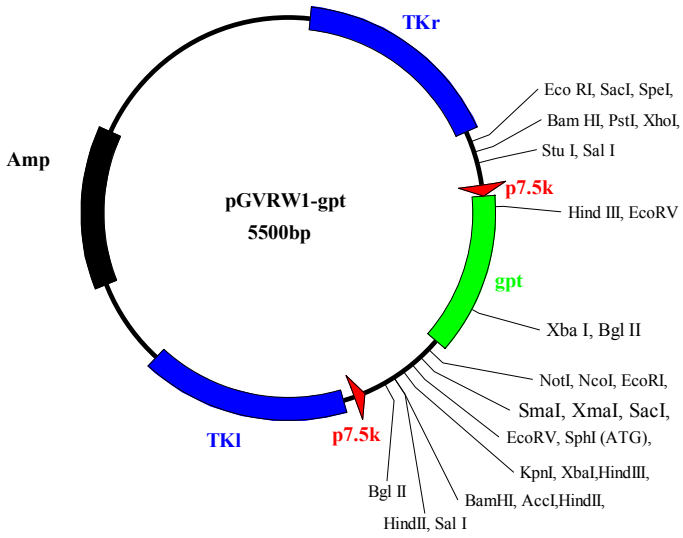
5.2.4 Molecular cloning of a full-length rabies virus glycoprotein gene in a vaccinia virus transfer vector, pGVWR1-gptNew

5.2.4.1 Modification of the vaccinia virus transfer vector, pGVWR1-gpt

The region between the p7.5K promoter, the multiple cloning site and the right thymidine kinase flank of the plasmid was removed by digestion with *Bam*H I and *Eco*R I restriction endonucleases (both Promega) (figure 5.9a). Since the composition of the buffer required for optimum activity of *Eco*R I had a comparatively higher salt concentration than that of the optimum buffer for *Bam*H I, the plasmid was firstly digested with *Bam*H I. The reaction was set up in 15 µl, as before[2]. A fraction of the restriction product was analysed electrophoretically to determine if the digestion was complete. The remainder of the digested product was then cut with *Eco*R I by increasing the volume of the reaction to 20 µl and adding the appropriate buffer. The product was self-ligated and the recombinant clones called pGVWR. An *Eco*gpt gene, under regulation of the p7.5K promoter, was obtained from the recombinant fowlpoxvirus transfer vector, pAF09 through digestion with *Eco*R I and *Bam*H I endonucleases, as before. The gene was then inserted into the *Eco*R I and *Bgl* II sites of pGVWR to obtain pGVWR-gptNew (figure 5.9b).

[13] Refer to section 5.2.2.1

a.



b.

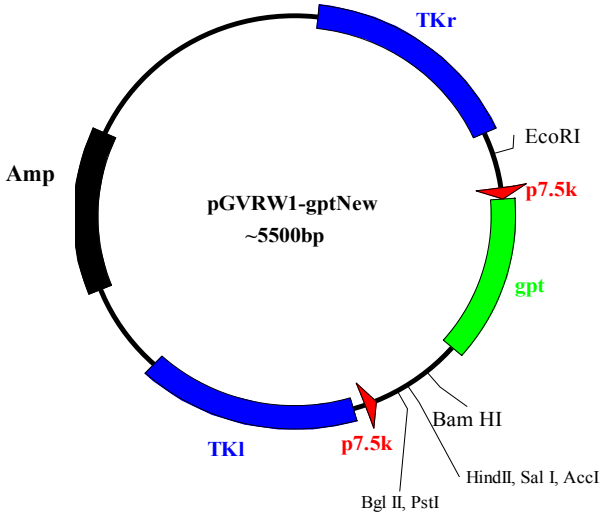


Figure 5.9: A diagrammatic map of the vaccinia virus transfer vectors, a) pGVWR1-gpt and b) pGVWR1-gptNew, indicating relevant features of the plasmids.

5.2.4.2 Cloning of a rabies virus glycoprotein and EGFP genes in pGVWR1-gptNew

The glycoprotein coding sequence together with the EGFP gene were digested from pMTL24-RabG-EGFP by using *Bam*H I (Promega) restriction endonuclease, employing conditions as recommended by the manufacturer[14]. The vaccinia transfer vector, pGWVR1-gptNew was digested with *Bam*H I in the same manner. The fragments were ligated, transformed and screened as described in section 5.2.2.1. To verify the insertion of the genes, *Bam*H I (Promega) restriction endonuclease digestion was carried out on the plasmid preparations of the possible recombinants. The orientation of the inserted gene was analyzed by digesting the recombinants with *Sma* I (Promega) and *Eco*R V (New England Biolabs) restriction endonuclease as pertained by the manufacturer. The recombinant plasmids were called pGVWR-RabG-EGFP.

5.2.5 Analysis of transient expression of the rabies virus glycoprotein and EGFP genes in infected cell culture

5.2.5.1 Transfection with recombinant transfer vectors in virus infected cell culture

The procedures were the same for transfection for the recombinant MVA and vaccinia virus transfer vectors, unless stated otherwise. Glass coverslips were sterilized in saturated Biocide D (Diversey Lever) solution, washed with sterile PBS then aseptically placed in the wells of a six well cluster cell culture plate (NUNC, Denmark). Approximately $1X10^5$ cells of a chicken embryo fibroblast cell line, CEC-32, were seeded per well of the prepared cell culture plate in DMEM/F12 with 5 % FBS. Following overnight incubation under standard growth conditions, the monolayers were infected with MVA or vaccinia virus Western Reserve strain at a multiplicity of infection (MOI) of 1. Additional wells were kept uninfected, to serve as a background controls in the immunofluoresence assay. The virus was allowed to absorb for 90 minutes at 37 °C and 5 % CO_2. During the incubation the transfection complexes were prepared with the recombinant plasmids, pIIIp7.5-RG-EGFP and pGVWR-RabG-EGFP. Plasmid DNA

[14] refer to section 5.2.3.2 and 5.2.3.3

was purified using spin columns (QIAquick® Gel extraction Kit, QIAGEN) and then spectrophotometrically analysed for purity and concentration. 1 µg of DNA with an A_{260}/A_{280} ratio of 1.8 were used for the preparation of the transfection complexes with the transfection reagent, FuGene® (Roche Molecular Biochemicals) according to the manufacturer's suggestions. In modification of the manufacturer's protocol, complex formation was allowed for 20 minutes. After the virus absorption period the medium was removed from the cells and the cells washed with sterile PBS to remove residual unabsorbed virus. The cells were fed with complete DMEM/F12 medium with 2 % FBS. The transfection complexes were then added to the cell cultures. Transfection complexes prepared with pIIIp7.5-RabG-EGFP was added to the MVA infected cultures, whereas the complexes prepared with pGVWR-RabG-EGFP was added to the Western Reserve strain infected cultures. The cell cultures were incubated under standard growth conditions for 3 hours, after which the inocula were removed, the monolayers washed with PBS and fed with complete medium. The plates were then incubated under standard growth conditions for another 48 hours.

5.2.5.2 Visualization of EGFP fluorescence and indirect immunofluoresence analysis

After 48 hours the wells were examined for EGFP fluorescence with an inverted fluorescence microscope (Orthoplan, Leitz Weitzler). Photographs were taken with an Olympus C-35 AD camera and Fujichrome Sensia ASA 400 slide film (FujiFilm, South Africa).

Hereafter the coverslips were removed from the six well plate using forceps and the cells fixed with ice-cold acetone for 10 minutes. In addition, as controls, coverslips with uninfected cells and untransfected, virus infected cells, were included in this assay. The coverslips were halved by cutting the glass with a diamond cutter, so that one half could be used in the assay and the other stored as a backup. The coverslips were then washed with PBS and could be stored in the PBS until required for the analysis. The fixed cultures were flooded with primary antibody diluted in a 1:2 ratio (pre-determined value, results not shown) in blocking solution (2% low fat milk powder (Elite, South

Africa) diluted in PBS) and incubated at 37 ºC for 30 minutes. The monoclonals M725 and M778 were used for the analysis of transient expression. Incubation was followed by four, 10 minute washes in PBS with gentle rocking. Fluorescein isothiocyanate (FITC) labeled, anti-mouse IgG (Sigma Aldrich, Cat. No. F5897) was diluted 1:64 (as suggested by the manufacturer) in blocking solution with 0.01% Evan's Blue and was transferred to the fixed cells and incubated for 30 minutes at 37 ºC. The incubation was followed by four times, 10 minute washes with PBS and a final ten-minute wash in distilled H_2O. The coverslips were then air-dried and mounted on glass microscope slides in non-permanent mounting fluid (50% glycerol, 50% PBS, pH 7.4) and visualized with a fluorescence microscope (Axioscope, Zeiss) and images captured with digital camera.

5.2.6 Generation of recombinant vaccinia viruses: transfection of infected cell culture with recombinant transfer vector

The protocols for the generation and isolation of recombinant vaccinia viruses were adapted from Byrd and Hruby, 2005 and Moss and Earl, 1998.

Monolayers of CEC-32 cultures where prepared in a six well cluster cell culture plate as before[15]. The following day the confluent cell monolayers were infected with MVA or vaccinia virus Western Reserve, respectively, at a MOI of 0.01. The virus was absorbed for 90 minutes at 37 °C and an atmosphere of 5 % CO_2. Transfection complexes were prepared with the recombinant plasmids that tested positive for transient expression of the antigen. The transfection reactions were setup and carried out as previously described[15].

5.2.7 Isolation of recombinant vaccinia viruses: positive, dominant selection for mycophenolic acid resistance

Recombinant MVA was passaged on monolayers of primary chick embryo fibroblasts or a baby hamster kidney cell line, BHK-21 (CCL-110), depending on

[15] Refer to section 5.2.5.1

availability of chicken embryos. Recombinant vaccinia Western Reserve strain was passaged on Vero cell cultures.

The transfected monolayers were harvested by three freeze thaw cycles. The cell culture soups were briefly centrifuged in a benchtop microcentrifuge to collect cellular debris. The resultant supernatants were then titrated in a tenfold serial dilution series (10^{-1} to 10^{-3}) onto six well plates with monolayers of the relevant cell cultures. The cell culture medium was substituted with complete DMEM/F12 with 2 % FBS and 25 µg/ml mycophenolic acid (MPA), 250 µg/ml xanthine and 15 µg/ml hypoxanthine (all from Sigma Aldrich). Infected cell cultures were incubated for 72 hours at 37 ˚C and 5 % CO_2. The infected monolayers were washed with PBS and single virus plaques (in the case of vaccinia virus Western Reserve strain) and foci (in the case of MVA) (from wells containing single plaques or the fewest number of plaques/foci) expressing EGFP were picked in 20 µl using a pipet. 80 µl of cell culture medium was added to each sample and the virus material then harvested by three freeze thaw cycles and a brief centrifugation in a benchtop microcentrifuge. The harvested supernatants were diluted in a tenfold serial dilution and titrated on relevant monolayers in 12 well cell culture cluster plates (NUNC, Denmark) under MPA selection, as before. The virus clones was processed and passaged under selection for a further 5 passages. The purification of isolates was monitored with PCR as described in the following section 5.2.7.1. Virus isolates that appeared to be homogenous after PCR analysis were passaged as before but without selection. The homogeneity of these samples was monitored in subsequent passages. The respective recombinants were named MVA-RG-EGFP and WR-RG-EGFP.

5.2.7.1 Analysis of homogeneity of recombinant vaccinia virus clones: PCR of the site of deletion III of the MVA genome and the TK region of the vaccinia virus Western Reserve strain genome

The progression of purification of the virus clones was monitored through PCR on extracts of virus DNA from subsequent passages. The PCR primers, MVA-III-5' (GAATGCACATACATAAGTACCGGCATCTCTAGCAGT) and MVA-III-3' (CACCAGCGTCTACATGACGAGCTTCCGAGTTCC) anneal in the virus genome at

the sites flanking the site of deletion III of the MVA genome (Staib *et al*., 2000). The primers, VacTKl (TCCCATCGAGTGCGGCTAC) and VacTKr (GTCCCATCGAGTGCGGCTAC) were designed and anneal in the virus genome at the sites flanking the thymidine kinase gene in the vaccinia virus genome.

Virus genomic material was extracted as described in Esposito *et al*., 1981. Briefly, up to a half the volume of the harvested lysates, were incubated with an equal volume poxvirus lysis buffer (1% sodiumdodecylsulphate, 20mM β-mercaptoethanol, 20mM ethylenediaminetetraacetic acid) and 100 µg/ml Proteinase K (Sigma Aldrich) for at least two hours at 56 °C. The genomic material was then separated from proteinacious material with phenol and chloroform and precipitated in ethanol (Sambrook and Russell, 2001). Shortly, an equal volume of phenol/chloroform/isoamylalcohol (25:24:1) was added to each sample and the mixture gently inverted to mix. The samples were then centrifuged at top speed in a benchtop centrifuge at 4 ˚C for five minutes. The top aqueous layer of the samples was then removed and placed in a nuclease free microcentrifuge tube. A half volume of chloroform:isoamylalcohol (24:1) was added to each sample, and centrifuged again for five minutes at in a benchtop centrifuge at 8 000 g and at 4 ˚C. Again, the top aqueous phase was removed and placed in a nuclease free microcentrifuge tube. The DNA was then precipitated in two volumes of ice-cold absolute ethanol with one tenth of the volume 3 M NaOAc (pH 5.3) added. Calf liver tRNA (Boehringer Mannheim) was added to each preparation to a final concentration of 100 µg/ml, to aid precipitation of the DNA. Precipitation was allowed overnight at –20 °C. The DNA was then pelleted by centrifugation in a benchtop microcentrifuge at 8 000 *g* and 4 ˚C for 15 minutes. The supernatant was discarded and the DNA pellets air-dried. The dried pellets were suspended in 20 - 50 µl of nuclease free H_2O. These samples were used as templates in PCR reactions to determine the homogeneity of the samples. The PCR reactions were setup by preparing a master mix of (indicated quantities per reaction):

2.5 µl *Ex Taq*™ Buffer (10X)*
2µl dNTP mixture (10 mM, 2.5 mM of each dNTP)*
10 pmol of forward and reverse primer each

0.5 U *Ex Taq*™ Polymerase*(5U/µl)
Nuclease-free H_2O to a final volume of 20 µl
(* All from TaKaRa Bio Inc, Amersham Biosciences)

5µl of the DNA extractions were used as templates per reaction. Positive control reactions were prepared with recombinant transfer plasmid, or parental genomic DNA, and a negative buffer control reaction was also included.

5.2.8 Analysis of stable expression of the rabies virus glycoprotein gene from recombinant vaccinia viruses: indirect immunofluoresence assay

Glass coverslips were sterilized in saturated Biocide D (Diversey Lever) solution, washed with sterile PBS and then aseptically placed in the wells of a six well cluster cell culture plate (NUNC). Approximately $1X10^5$ CEF or BHK-21 cells were seeded per well of a six well cluster plate. Following overnight incubation under standard growth conditions, the monolayers were infected with the respective recombinant virus clones or wild type virus (as control). An additional well was kept uninfected, to serve as a background control in the assay. After 48 hours the infected cell cultures were fixed and stained as previously described[16].

5.2.9 Large scale growth and semi-purification of the parental and recombinant vaccinia viruses from single, plaque isolates

MVA, parental and recombinant viruses, were cultured as described by Staib *et al*., 2004, with some modifications. These viruses were cultured on BHK-21 or CEF monolayers, depending on availability of embryonated chicken eggs. Recombinant and parental Western Reserve, and recombinant and parental Vaccinia Copenhagen[17], were cultured as described in Moss and Earl, 1998, and on Vero cell cultures. Single, plaques isolates were harvested by freeze-thawing three times and a brief centrifugation at 200 *g* in a benchtop centrifuge to collect cellular debris. The supernatants were then inoculated

[16] Refer to section 5.2.5.2
[17] Refer to section 5.2.1

on a 25 cm^2 cell culture flask. The infected cultures were harvested as before, after 2-3 days of incubation, until 90-100% cytopathic effect (CPE) become apparent. The medium on the flask was reduced and the infected cells harvested as before[18]. The harvested virus lysates were then used as inoculum on a 75 cm^2 cell culture flask. The infected cultures were once again incubated for 2-3 days or until 90-100% CPE was observed. This culture was harvested and used as inoculum on 10, 150 cm^2 cell culture flasks. The infected flasks were then incubated for 2-3 days and harvested as before.

The material was transferred to 50 ml centrifuge tubes and centrifuged for 5 minutes at 1800 *g* and 4 °C. The resultant supernatants were collected and the pellets were resuspended in cell culture medium, vortexed briefly and centrifuged as before. After the second centrifugation, the supernatants were pooled and transferred to polycarbonate Oakridge tubes. Sterile, 36 % sucrose (36 % sucrose in 1mM Tris, pH 9.0) cushions were injected unto the bottom of the loaded Oakridge tubes. The tubes were centrifuged for 2 hours at 19 000 *g* and 4 °C. The supernatants were discarded and the pelleted virus material resuspended in 1 ml of MEM with 1X antibiotics but no FBS. The virus material was stored in aliquots at -80 °C.

5.2.10 Infectivity assays

5.2.10.1 MVA immunostaining

The protocol is described in Staib *et al*., 2004. The semi-purified virus material was thawed and briefly sonicated in water bath sonicator (Sonorex TK 52, Bandelin, Germany). The procedures were carried out in duplicate. The virus was diluted in a tenfold serial dilution in cell culture medium and plated out on monolayers of BHK-21 (in a total volume of 1 ml) prepared in 6 well cell culture cluster plates as before. The infections were incubated at 37 °C and 5 % CO_2 for 90 minutes. The inoculum was then replaced with DMEM/F12 with 2% FBS and incubated for a further 72 hours. After 72 hours, the medium of the infected cell cultures were removed and the monolayers fixed in 2ml/well of a 1:1 preparation of acetone: methanol. The cells were fixed for 5 minutes

[18] Refer to section 5.2.7

and subsequently blocked with PBS supplemented with 3% FBS. Anti-vaccinia virus primary antibody (polyclonal rabbit anti-vaccinia IgG, Biogenesis Limited, Poole, England, catalogue number 9503-2057) was diluted 1:1000 in PBS with 3% FBS. The blocking solution was replaced with 1ml of primary antibody solution per well followed by incubation at 37 °C for 1 hour. After incubation, the cells were washed twice for 2 minutes with PBS with 3% FBS. The cells were then flooded with 1ml/well peroxidase conjugate (horseradish peroxidase conjugated polyclonal goat anti-rabbit IgG, Dianova, Hamburg, Germany, catalogue number 111-035-114) diluted 1:500 in PBS with 3% FBS and incubated for 45 minutes at room temperature while gently rocking. The conjugate was washed off in two 2 minute washes with PBS. The conjugate substrate was prepared by making a saturated solution of σ-dianisidine (Sigma Aldrich) in absolute ethanol. 200 µl of the solution and 10 µl of H_2O_2 (>30%) was added to 10 ml of PBS. 2ml of this solution was added per well followed by 5-30 minute incubation (until reaction develops) at room temperature. The number of foci was counted and the focus forming units per ml calculated.

5.2.10.2 Vaccinia virus Western Reserve and Copenhagen strains: Plaque assay

The virus stock was thawed and sonicated for 30 seconds. A tenfold serial dilution of the sonicated virus material was prepared in the range 10^{-2} to 10^{-5}, and in duplicate. The dilutions were then pippeted unto confluent Vero cells, which were seeded the previous day on six well plates. The infected cells were then incubated under standard growth conditions for three to four days, or until CPE became apparent. Plaques were stained with 30 µg/ml Neutral Red (Gibco). The stained plaques were then counted under an inverted microscope. The titer was subsequently estimated in plaque forming units per milliliter (pfu/ml).

5.2.11 Characterization of growth characteristics of the recombinant vaccinia viruses

5.2.11.1 MVA replication deficiency assay

The protocol is described in Staib *et al*., 2004. Confluent monolayers of HeLa-229 on six well cell culture cluster plates were infected with MVA, recombinant MVA-RG, or Western Reserve strain (as replication competent control) at a MOI of 0.01 in a total volume of 1 ml cell culture medium. Uninfected cell controls were also included in the test. The viruses were absorbed for 1 hour at 37 °C and 5 % CO_2. Inoculums were removed and the infected monolayers washed twice. The monolayers for time point of 0 hours were harvested, while the remaining cultures were incubated for 72 hours under the previous conditions. These cultures were harvested after the 72 hour incubation period. The harvested cultures were titrated on monolayers of BHK-21 as before, and the replication efficiency of the viruses calculated and compared.

5.2.11.2 One step growth assays

One step growth assays were conducted with MVA, MVA-RG-EGFP, Western Reserve, WR-RG-EGFP, Vaccinia Copenhagen and an experimental vaccinia virus glycoprotein[19]. Monolayers of BHK-21 were prepared in 75 cm^2 cell culture flasks. The cell cultures were infected at a high multiplicity of infection of 3 IU/cell. The viruses were absorbed for 1 hour at 37 °C and 5 % CO_2. Inoculums were removed and the infected monolayers washed twice with PBS. The cultures were subsequently fed with 10 ml of cell culture medium with 2 % FBS. The monolayers for a time point of 0 hours were harvested immediately, while the remaining cultures were incubated for 24 hours under the previous conditions. Infected cultures were harvested at time points 6, 12, 18 and 24 hours. Data was then plotted to obtain one step growth curves.

[19] Refer to section 5.2.1

5.3 Results

5.3.1 Construction of a MVA transfer vector, pIIIp7.5-JW, and molecular cloning and transient expression of rabies virus glycoprotein and EGFP genes

The critical regions of the MVA transfer vector were cloned into a pUC18 plasmid backbone. These included the regions homologous to the areas flanking the site of deletion III on the MVA genome and an expression cassette encoding a p7.5K promoter and multiple cloning sites for foreign gene insertion. The multiple cloning locus include unique *Bam*H I and *Sma* I restriction sites. An *Eco*gpt marker gene was cloned adjacent to flank 2 in pIIIp7.5-JW following the principles of transient marker stabilization (Falkner and Moss, 1990). The plasmid map of pIIIp7.5-JW is depicted in figure 5.4.

The rabies virus glycoprotein gene was subcloned into the cloning vector, pMTL-24 (results not shown). The rabies glycoprotein gene was digested from an existing clone, pFastBac1-RabG with *Hin d*III and *Bam*H I, and a DNA band of about 1 600 base pairs inserted into the *Hin d*III and *Bgl* II sites of pMTL-24. The EGFP gene (about 900 base pairs) was inserted into the *Not* I site of the pMTL clone. Thereafter the glycoprotein gene and EGFP gene (with a p11K promoter) were inserted into the *Bam*H I site of pIIIp7.5-JW and the direction of inserted genes determined by digestion with *Sma* I (figure 5.10).

The recombinant plasmids were transfected into infected cell culture and transient expression of the cloned genes assessed. Transient expression of the EGFP was observed with a fluorescence microscope (results not shown), whilst transient expression of the rabies virus glycoprotein gene from the newly constructed vector was confirmed with an indirect immunofluoresence assay (results not shown).

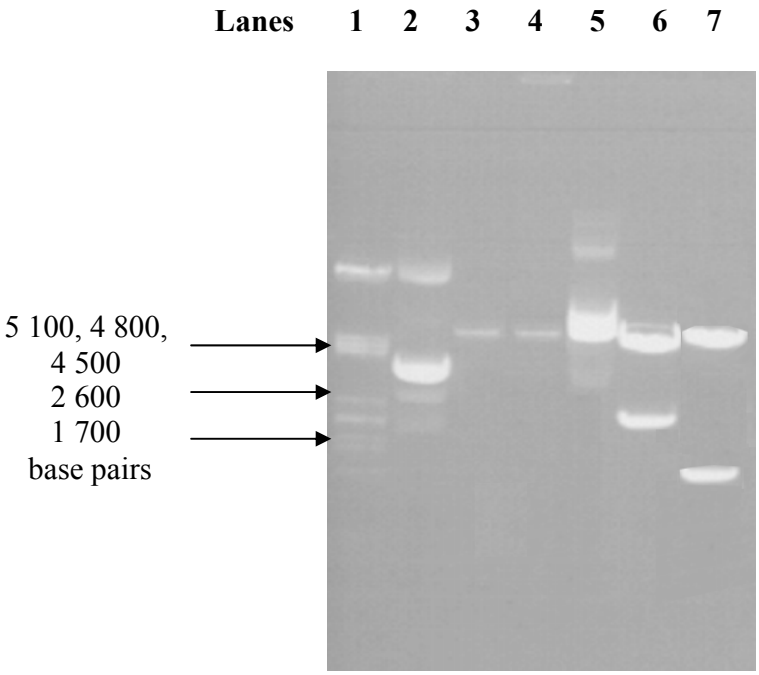


Figure 5.10: Molecular cloning of the rabies glycoprotein and EGFP genes in the MVA transfer vector, pIIIp7.5-JW. In lane (1), molecular weight marker (lambda DNA Pst *I*), (2) undigested pIIIp7.5-JW , pIIIp7.5-JW digested with *Bam*H I (3) and *Sma* I (4), (5) undigested pIIIp7.5-RG-EGFP, and pIIIp7.5RG-EGFP digested with *Bam*H I (6) and *Sma* I (7).

5.3.2 Construction of the vaccinia virus transfer vector, pGVWR1-gptNew and molecular cloning and transient expression of rabies glycoprotein and EGFP genes

Due to a lack of suitable unique restriction endonuclease sites in the multiple cloning site of the pGVRW1-gpt plasmid, the vector was modified to include a multiple cloning site with unique sites for foreign gene insertion. The transfer vector, pGVWR1-gptNew was hence constructed (figure 5.9b). A DNA fragment encoding a rabies virus glycoprotein and EGFP gene (approximately 2 500 base pairs band), were digested from a pMTL clone with *Bam*H I, and subsequently inserted into the *Bam*H I site of pGVWR1-gptNew vector (figure 5.11). Transient expression of the cloned genes was determined to establish the integrity of the modified expression plasmid. Transient expression of EGFP

was observed with a fluorescence microscope (results not shown) and transient expression of rabies glycoprotein was determined by an indirect immunofluoresence assay (results not shown).

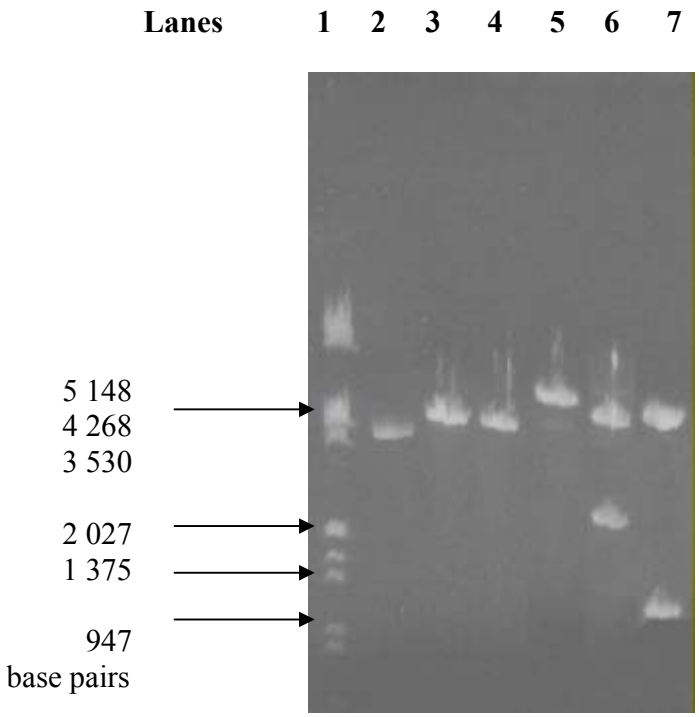


Figure 5.11: Cloning of a rabies glycoprotein and EGFP genes in pGVWR1-gptNew. In lane (1) DNA molecular weight marker (Lambda DNA *Hin* dIII/*Eco*R I), (2) undigested pGVWR1-gptNew, (3) pGVWR1-gptNew digested with *Bam*H I, (4) pGVWR1-gptNew digested with *Sma* I and *Eco*R V, (5) undigested pGVWR-RG-EGFP, (6) pGVWR-RG-EGFP digested with *Bam*H I and (7) pGVWR-RG-EGFP digested with *Sma* I and *EcoR* V.

5.3.3 Generation and isolation of recombinant vaccinia viruses

Recombinant MVA and recombinant vaccinia virus Western Reserve strain were generated upon transfection of virus infected CEC-32 cell culture with the respective transfer plasmids. Transfection efficiency on CEC-32 proved superior to primary CEF

and BHK-21 cell cultures (results not shown). Recombinant foci and plaques where identified by visualization of EGFP expression and passaged under mycophenolic acid selection for 6 passages. The purification of the recombinant virus isolates from wild type viruses were monitored by PCR. A primer pair that binds in the regions flanking the site of deletion III on the MVA genome was used for amplification of MVA virus isolates. The recombinant viruses yielded a band of about 3 500 base pair, whereas the wild type MVA genomic DNA yielded a 700 base pair band (figure 5.12). For the MVA recombinant, recombinants that undergone a single cross over event was selected, since the *Eco*gpt marker is carried outside of the flanking region (for principles, refer to figure 5.1). Once the isolates were purified from parental virus background, as shown with PCR, selection pressure was lifted. Passage in the absence of selection led to the loss of the plasmid backbone and *Eco*gpt marker through intragenomic homologous recombination between the double flanking regions in the recombinant virus, thus yielding marker free recombinant virus. These events were confirmed by PCR, indicated by the difference in size of the amplicons yielded with the MVA primer pair (figure 5.12)

Likewise a primer pair that targets the TK region of vaccinia virus was used to determine the homogeneity of recombinant vaccinia viruses clones. The primer pair yielded a band of about 4 500 base pair for the recombinant viruses and 400 base pairs for the wild type genome (figure 5.13).

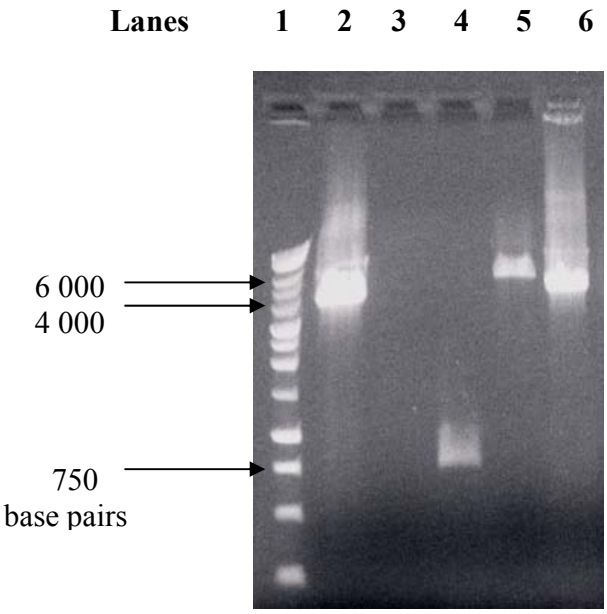


Figure 5.12: PCR amplification with primers binding in the flanking areas of the site of deletion III in the MVA genome. In lane (1), molecular weight marker (1 Kb ladder, Promega); and PCR with (2) positive plasmid control; (3) negative buffer control; (4) wild type MVA genomic DNA; (5) MVA-RG-EGFP genomic DNA and (6) marker free MVA-RG-EGFP genomic DNA as templates.

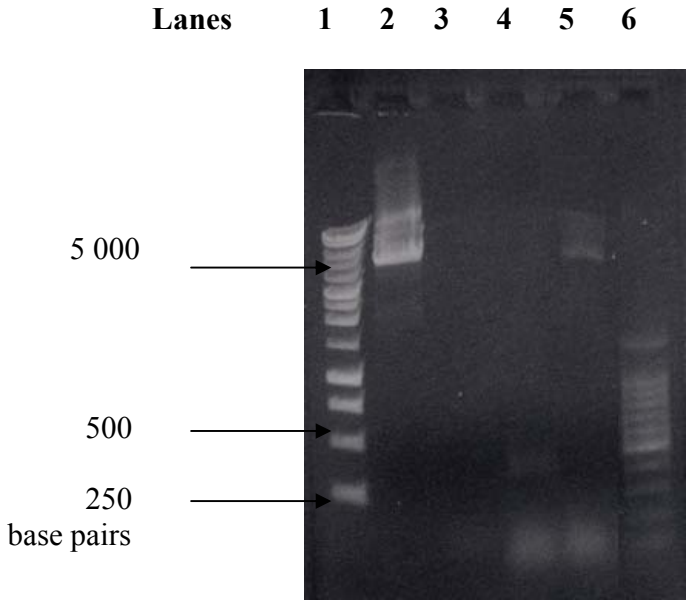


Figure 5.13: PCR amplification with primers binding in the flanking regions of the thymidine kinase locus of vaccinia virus. In lane (1; 6) molecular weight marker (1 Kb ladder and 100 bp ladder, Promega); and PCR with (2) plasmid positive control, (3) negative, buffer control, (4) wild type vaccinia Western Reserve genomic DNA and (5) WR-RG-EGFP genomic DNA as template.

5.3.4 Stable expression of rabies virus glycoprotein and EGFP genes from the recombinant MVA and vaccinia viruses

Stable expression of EGFP was determined from the recombinants as visualized with a fluorescence microscope (figure 5.14). Stable expression of rabies virus glycoprotein from the recombinant viruses where confirmed by indirect immunofluoresence analysis. Monoclonals directed to the important antigenic sites II and III on the rabies glycoprotein were used as primary antibodies. In the case of recombinant MVA and recombinant vaccinia viruses strong, specific signals were observed, confirming expression of the protein from these recombinants. In contrast very low, unspecific signals were observed from background control. Results with both the monoclonals were similar, and the results obtained with monoclonal M725 are depicted in figure 5.15.

a. **b.**

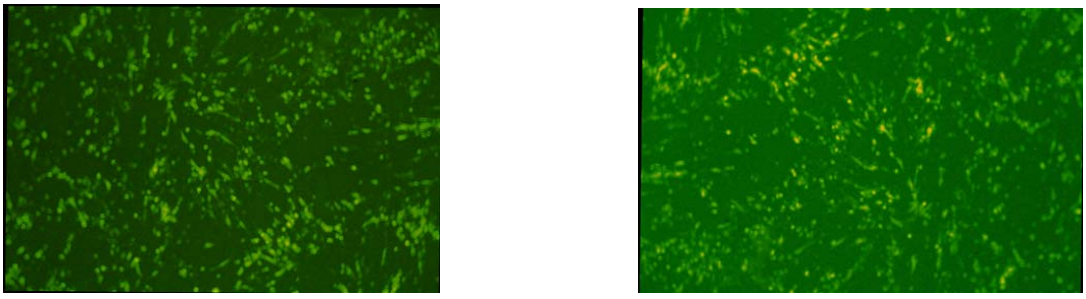
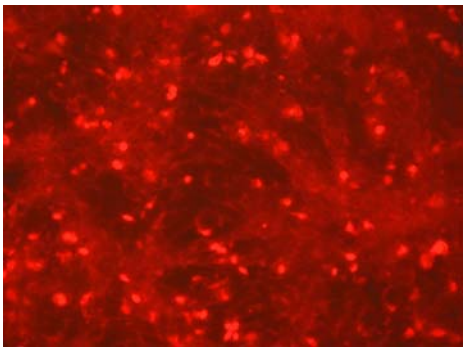
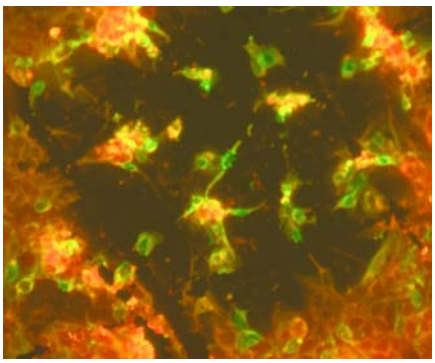


Figure 5.14: Stable expression of EGFP in (a) MVA-RG-EGFP and (b) WR-RG-EGFP infected BHK-21 cell culture. EGFP fluoresces upon excitation under a fluorescence microscope. Control monolayers appeared only as a black background and are not shown here. (Magnification: 10 X objective and 10 times for the microscope's eye piece and an additional 1.2 times for the magnification of the camera lens, totals at 120 times magnification).

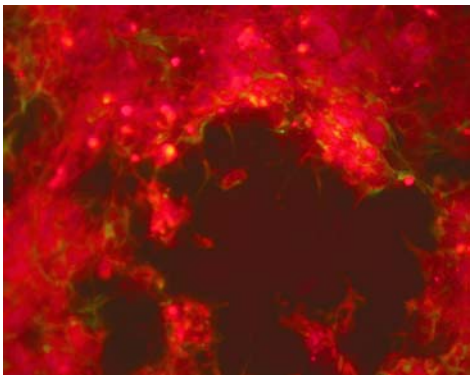
a.



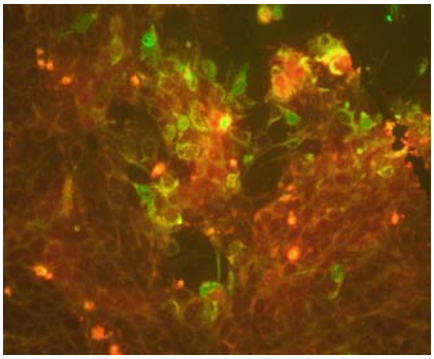
d.



b.



e.



c.

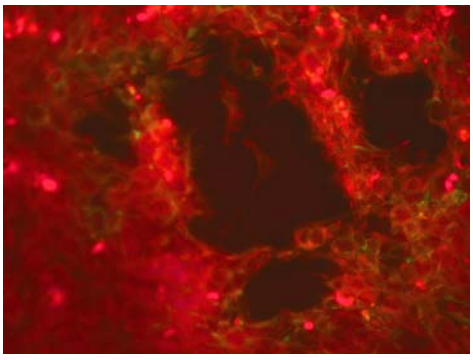


Figure 5.15: Indirect immunofluoresence assay of rabies glycoprotein in BHK-21 cell culture. Here cultures were probed with M725 directed to antigenic site II on the glycoprotein. In (a) uninfected, (b) Vaccinia Western Reserve infected, (c) MVA infected, (d) WR-RG-EGFP infected and (e) MVA-RG-EGFP infected BHK-21 monolayers. (Magnification: 20 X objective and 10 times for the microscope's eye piece and an additional 1.2 times for the magnification of the camera lens, totals at 240 times magnification).

5.3.5 Growth characteristics of recombinant and parental vaccinia viruses

5.3.5.1 Replication deficiency assay for MVA

The replication deficiency of recombinant MVA was compared with that of parental MVA and Vaccinia Western Reserve Strain. The relative deficiencies are indicated in table 5.3. Even though the recombinant virus replicated more readily on the non-permissive HeLa cell culture, and yielded nearly a logarithm more virus under the same culture conditions, it remained markedly less replication competent than Western Reserve strain on HeLa cultures and MVA on permissive CEF cultures. The recombinant MVA maintained a replication deficient character.

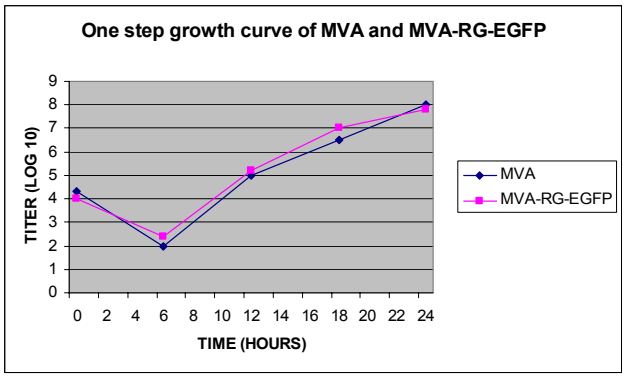
Table 5.3: Replication deficiency of recombinant MVA on non-permissive cell culture

VIRUS	REPLICATION DEFICIENCY RATIO (T_{72}/ T_0)
MVA on HeLa	0.60
MVA-RG-EGFP on HeLa	9.50
MVA on CEF	19 598.00
Western Reserve strain on HeLa	22 059.00

5.3.5.2 One step growth assays

Viruses were cultured and tittered as described. The data was then plotted on one step growth curves (figure 5.16). The growth characteristics of the respective viruses remained largely the same.

a.



b.

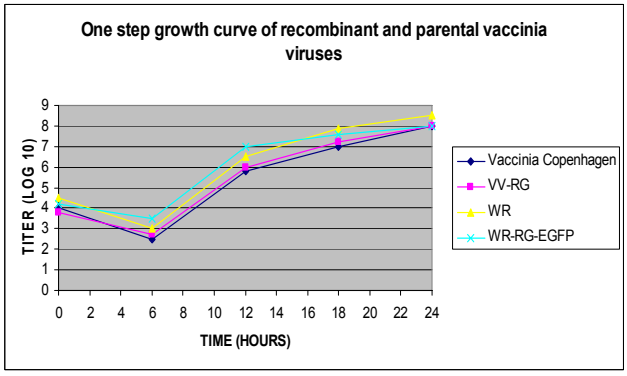


Figure 5.16: One step growth curve for a) MVA and MVA-RG-EGFP and the other (b) parent and recombinant vaccinia viruses used in this study, infected at a high MOI in BHK-21 cell cultures.

5.3.6 Preparation of vaccine and parental viruses for immunization of animals

Viruses were cultured in cell monolayers and semi-purified by centrifugation through a sucrose cushion. The titers of the viruses were determined by an immunoassay for MVA and the MVA recombinants and a plaque assay for the other recombinant and non-recombinant vaccinia viruses. The yields obtained under the described culture conditions are tabulated in table 5.4.

Table 5.4: Titers of virus cultures obtained after a semi-purification procedure.

VIRUS	TITER (IN FFU OR PFU/ML)
MVA-RG-EGFP	1×10^9
MVA	1×10^9
Experimental V-RG	5×10^8
Vaccinia Copenhagen	5×10^8
rWR-RG-EGFP	1×10^9
Western Reserve	1×10^{10}

5.4 Discussion

A variety of different methods are available for the construction and isolation of recombinant vaccinia viruses[20]. Off late, the importance of constructing and using marker free recombinants, especially resistance markers, has been realized (Traavik, 2002). This becomes particularly important when these constructs are to be used as vaccines. Therefore selection methods such as host range selection markers have become more popular. In addition the implementation of principles for transient marker stabilization has also become useful (Scheiflinger *et al*., 1998; Falkner and Moss, 1990).

In this study the principle of transient marker stabilization of mycophenolic acid resistance was applied for the generation of recombinant MVA viruses. A transfer vector was constructed with an *Eco*gpt marker gene cloned adjacent to the expression cassette of the vector. During the first set of plaque purification rounds, recombinant viruses that underwent single cross over events and therefore incorporated the entire transfer plasmid were selected. The recombinant viruses were purified under selection, and once homogenous isolations were collected, selection was lifted. After several passages in the absence of selection, recombinant isolates were collected that did not include the marker

[20] Refer to section 5.1

gene. To our knowledge this is the first description of the application of transient expression of an *Eco*gpt gene for the selection of recombinant MVA (Sutter and Staib, 2003). Transient marker stabilization of the host range selection marker, K1L for the selection of recombinant MVA has been described before (Staib *et al*., 2003; Tscharke and Smith, 2002; Staib *et al*., 2000). The chromogenic marker β-galactosidase has also been expressed in this fashion in MVA (Drexler *et al*., 1999).

EGFP was used as a visible marker to facilitate the isolation of recombinant MVA and vaccinia virus Western Reserve strain. The use of EGFP omitted the use of chromogenic markers such as β-galactosidase and β-glucoronidase, which requires additional processing time for application of substrates and other reagents. The utility of EGFP as a visual marker in the isolation of poxvirus recombinants have been outlined in previous studies (Wallace and Viljoen, 2005; Staib *et al*., 2000; Chen *et al*., 1996).

Transient expression of the cloned genes was tested to determine the intactness of the newly cloned MVA transfer vector and improved vaccinia virus transfer vector. In both cases, the EGFP gene was expressed and green fluorescent protein could be visualized under a fluorescence microscope. The expression of rabies virus glycoprotein was confirmed with an immunofluoresence assay using monoclonal antibody directed against important epitopes on the glycoprotein. Stable expression of these genes from the homogenous recombinants was also shown using the same approaches.

The growth characteristics of the recombinant and parental viruses were investigated on permissive cell culture. It appeared that the recombinant viruses did not have particularly different growth characteristics compared to the parental viruses, as shown in high MOI, one-step growth curves analysis. This was true for all the recombinants used in this study. The replication comtency of the recombinant MVA was also compared to that of parental virus and replication competent controls. The recombinant MVA virus exhibited improved growth on HeLa cell cultures opposed to the parental and control viruses. It could be argued that the passage of the virus on mammalian cell culture BHK-21 during the construction and purification of the recombinant viruses could have resulted in improved growth characteristics in mammalian cells.

In summary, recombinant vaccinia viruses were generated using the dominant, positive selectable marker for mycophenolic acid resistance. The final recombinant MVA constructs were marker free and was constructed based on the principles of transient marker stabilization. The replication competency of the recombinant was compared with that of parental viruses and found not to be substantially different.

CHAPTER VI

IMMUNE RESPONSES TO HOST-RESTRICTED AND REPLICATION COMPETENT RECOMBINANT VACCINIA VIRUSES EXPRESSING FULL-LENGTH RABIES VIRUS GLYCOPROTEIN GENES

6.1 Introduction

A vaccinia virus recombinant expressing a rabies virus glycoprotein gene, commonly known as V-RG, has been extensively used in oral vaccination programs in the in some European countries and the USA. This vaccine is applied for the control and prevention of rabies in wildlife species and reports on its use have been mainly favourable (Pastoret *et al*., 1992). The wide scale use and efficacy of this recombinant vaccine is without a doubt a milestone in the history of vaccination. The vaccine offers protective immunogenicity and is also relatively heat stable. A very desirable attribute for vaccines since it therefore does not require cold chain maintenance and enables the release of the vaccine in the form of oral baits in nature.

Safety concerns, however, plague the use of this vaccine elsewhere in the world, particularly in developing countries (Traavik, 2002). The use of this vaccine is mostly required for the control of rabies in dogs in settings that would lead to close contact with humans. This is problematic since the vaccinia virus-based vaccine remains potent and replication competent. Vaccinia virus also exhibits a wide host range and this combination of factors provides the seed for the fear that the vaccine virus could spread to non-target recipients with harmful effects. The latter is especially true when the non-target recipients are immunocompromised as shown in a fatal case of vaccinia in an patient with AIDS (Redfield *et al*., 1987). Scientific evidence to substantiate these fears has not yet been found, and studies have failed to show the spread of the vaccine virus to unintended recipients in oral vaccination settings (Hanlon *et al*., 1997, personal communication M. Niezgoda). An exposure of, and adverse reactions suffered by a pregnant woman to V-RG vaccine baits, however, reiterated the possible dangers of this vaccine (Rupprecht *et al*., 2001). An alternative vaccine that offers the same or enhanced efficacy but improved safety would be advantageous. In addition the vaccine should also

offer the other advantages of use that a poxvirus vectored vaccine offers, such as relative heat stability.

It was in the premise of this study to investigate the efficacy of a recombinant highly attenuated vaccinia virus, the modified vaccinia virus Ankara (MVA) in comparison with a replication competent counterpart such as V-RG. MVA is a severely attenuated strain of vaccinia virus that, through adaptation of growth on avian cell cultures, lost its ability to productively infect cells of mammalian origin. The nature of the attenuation is the manifestation of a phenotype due to the loss (truncation or deletion) of host range genes and genes involved in the regulation of host immune responses (Antoine *et al*., 1998). A MVA based vaccine should offer all the advantages of other poxvirus based vaccines, but with improved safety. Therefore in this study the immunogenicity of recombinant MVA expressing a rabies virus glycoprotein gene was investigated in different animal models through different routes of administration, including the oral route. The MVA vaccine was compared with replication competent counterparts, an experimental V-RG and recombinants prepared from the neurovirulent Western Reserve strain of vaccinia virus. The effects of attenuation on the vaccine efficacy of MVA recombinants are discussed.

6.2 Materials and methods

6.2.1 Recombinant vaccine and challenge viruses and cell cultures

A recombinant MVA and a recombinant vaccinia virus Western Reserve strain expressing a rabies virus glycoprotein gene was constructed and prepared for immunization of animals as previously described[21]. A recombinant vaccinia virus Copenhagen strain expressing the glycoprotein of the Evelyn-Rockitniki-Abelseth strain of rabies virus (ERA) was obtained from Dr. C.E. Rupprecht (Centers for Disease Control and Prevention, United States of America) and prepared as described elsewhere[22]. The origin and preparation of the parent vaccinia viruses have also been previously discussed[21]. Mouse neuroblastoma cell culture and Challenge Virus Standard

[21] Refer to Part Three, chapter V
[22] Refer to Part Three, chapter V and Part Four, chapter VII

(CVS-11) as challenge virus for the serology assays, was obtained from Michael Niezgoda at the CDC (USA).

Lyophilized cultures of rabies virus isolates were obtained from Dr. C.T. Sabeta (Agricultural Research Council-Onderstepoort Veterinary Institute, South Africa). A southern African rabies virus isolate from a yellow mongoose (*Cynictis penicillata*) (ARC-OVI reference number: m710/90) and an isolated made from a dog (*Canis familiaris*) in KwaZulu-Natal (ARC-OVI reference number: ND77) were used in this study.

6.2.2 Experimental animals

BALB/c, ICR mice and pregnant mice (for suckling mice) were obtained from Harlan Sprague Daly (USA). Previously vaccinated and challenge surviving raccoons (*Procyon lotor*) and beagles (*Canis familiaris*) were housed at the Lawrenceville Animal Facility, CDC (USA) (Lodmell *et al*., 2006). Animals were housed and handled according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Centers for Disease Control and Prevention.

6.2.3 Preparation of challenge viruses

6.2.3.1 Preparation of suckling mouse brain homogenates

Lyophilized rabies virus isolates, m710/90 and ND77, was reconstituted in phosphate buffered saline (PBS) (13.7 mM NaCl, 0.27 mM KCl, 0.43 mM $Na_2HPO_4.2H_2O$, 0.14 mM KH_2PO_4, pH 7.3) with 2 % horse serum (Hyclone). Two to three day old suckling mice received 30 µl of the reconstituted material intracranially with a 0.5cc tuberculin syringe and 8 mm, 31 gauge needle (Becton Dickinson and Company, USA). Animals were monitored for up to 30 days after inoculation and collected upon death. The brain material of the dead animals was removed aseptically. The direct fluorescent antibody test (dFA) was used for postmortem diagnosis of rabies

infection in the animals[23]. The brain material that tested positive by dFA was pooled and homogenized to prepare 20 % (w/v) suspensions. Briefly, the material was weighed and subsequently ground in sterile PBS with 2% horse serum with a chilled glass mortar and pestle. The macerated suspension was then collected in 50 ml centrifuge tubes and centrifuged at 200 *g* for 10 minutes at 4 ˚C. The supernatant was collected and stored in aliquots at -80 ˚C.

6.2.3.2 Titration of suckling mouse brain homogenates

The protocol was adapted from Koprowski, 1994. Titrations were carried out in 3-4 week old BALB/c or ICR mice. The virus homogenates were diluted in a ten fold serial dilution in the range of 10^0 to 10^{-8} in PBS with 2% horse serum. Animals received 50 µl of homogenate intramuscularly in the right quadriceps muscle (lower dilutions of the series) or 30 µl intracranially (higher dilutions of the series) with a 0.5cc tuberculin syringe and 8 mm, 31 gauge needle (Becton Dickinson and Company, USA). Animals were observed for up to 30 days after inoculation and collected upon death or humanely sacrificed as determined by the IACUC of the CDC. The dFA was used for postmortem diagnosis of rabies infection in the animals[23]. The mouse intracranial lethal dose 50 ($MICLD_{50}$) titers were subsequently calculated from the data according to Reed and Muench (1938).

6.2.3.3 Postmortem diagnosis of rabies infection in animals: Direct Fluorescent Antibody test

Standard operation procedure for the direct fluorescent antibody test can be found at www.cdc.gov/ncidod/dvrd/rabies/Professional/publications/DFA_diagnosis (internet reference 5).

The brains of the mice were surgically and aseptically removed and stored in ointment tins at -20 ˚C. Smears of brain material (hippocampus or cerebellum) were prepared by collecting a piece of brain (any section of the brain can be used to confirm a

[23] Refer to section 6.2.3.3

positive finding), blotting the material on tissue paper and pressing against a Teflon coated microscope slide (3-4 smears per slide). Control slides were prepared from known positive or negative brain material, in the same way as described above. Impressions were left to dry at room temperature and then fixed in ice-cold acetone for 60 minutes. Slides were allowed to air dry at room temperature. The smears were then flooded with anti-rabies fluorescein isothiocyanate conjugate (Centocor, Inc. or Fujirebio Diagnostics, Inc., USA) through a syringe with a 0.45 µm low protein binding filter. The slides were stained in the order, positive control and test slides followed by the negative control slides. The slides were then incubated at 37 ˚C in a high humidity chamber. The conjugate was then drained from the slides and the slides washed two times for 5 minutes in PBS. The slides were blotted to remove excess PBS and briefly air dried at room temperature. The slides were subsequently mounted in wet mounting fluid (20 % glycerol, Tris buffered saline pH 9.0) and cover slipped. The slides were examined under a fluorescence microscope as suggested in the standard protocol.

6.2.4 Immunization studies in animals

6.2.4.1 Immunization of mice

Six week old, female BALB/c or ICR mice were grouped in groups of 10 mice each. The groups received 10^7, 10^8 or 10^9 focus forming units (ffu) of recombinant MVA or 10^7 ffu of the parent virus, MVA, intramuscularly in 50 µl minimal essential medium (MEM) supplemented with 1 X antibiotic mixture (100 µg/ml streptomycin, 100 µg/ml penicillin and 250 µg/ml amphotericin) (Gibco, Invitrogen Life Technologies). Other groups received 10^7 plaque forming units (pfu) of the recombinant vaccinia virus Copenhagen or Western Reserve strain or the parent viruses, intramuscularly. The intramuscular administrations were made in the right quadriceps muscle using a 0.5cc tuberculin syringe with an 8mm, 31-gauge needle (Becton Dickinson and Company, USA). The remaining groups received 10^7 ffu or pfu *per os* of all the different viruses. Animals were fed vaccine *via* a filter tip and pipet for the *per os* administrations. Half of the groups received booster immunizations of 10^5 ffu or pfu through the same route as the

primary inoculation and two weeks thereafter. The immunization schedule is summarized in table 6.1.

Blood was collected *via* the retro-orbital route on day 0, 7 and 21. Blood was collected with heparinized microhematocrit capillary tubes (Becton Dickinson and Company, USA) or heparinized Natalson blood collecting capillaries (Fisher Scientific, United States of America). Sera were separated in Microtainer® serum separation tubes with SST™ (Becton Dickinson and Company, USA) as suggested by the manufacturer, and stored in aliquots at – 20 ˚C until analysis.

Animals received lethal homologous or heterologous rabies virus challenge intracranially on day 28. Animals were monitored for signs of rabies for the following 30 days and collected upon death or killed humanely by euthanisia when required and as determined by the IACUC of the CDC.

The sera were tested for rabies virus neutralizing antibodies per rapid fluorescence focus inhibition test (RFFIT) as described elsewhere[24].

[24] Refer to Part Two, Chapter IV, section 4.2.3

Table 6.1: Details of the schedule for the immunization of mice with recombinant vaccinia viruses. Ten animals were allocated to each group.

GROUP	INOCULUM AND DOSES	ROUTE OF ADMINISTRATION	DAY OF SCHEDULE
1	rMVA-RG, 10^7 ffu	Intramuscularly	1
2	10^7 ffu and 10^5 ffu		1 and 14
3	rMVA-RG, 10^8 ffu	Intramuscularly	1
4	10^8 ffu and 10^5 ffu		1 and 14
5	rMVA-RG, 10^9 ffu	Intramuscularly	1
6	10^9 ffu		1 and 14
7	rMVA-RG, 10^7 ffu	*per os*	1
8	10^7 ffu and 10^5 ffu		1 and 14
9	MVA, 10^7 ffu	Intramuscularly	1
10	10^7 ffu and 10^5 ffu		1 and 14
11	MVA, 10^7 ffu	*per os*	1
12	10^7 ffu and 10^5 ffu		1 and 14
13	V-RG 10^7 ffu	Intramuscularly	1
14	10^7 ffu and 10^5 ffu		1 and 14
15	V-RG 10^7 ffu	*per os*	1
16	10^7 ffu and 10^5 ffu		1 and 14
17	Vaccinia Copenhagen, 10^7 pfu	Intramuscularly	1
18	10^7 pfu and 10^5 pfu		1 and 14
19	Vaccinia Copenhagen, 10^7 pfu	*per os*	1
20	10^7 pfu and 10^5 pfu		1 and 14
21	Recombinant Western Reserve, 10^7 pfu	Intramuscularly	1
22	10^7 and 10^5 pfu		1 and 14
23	Recombinant Western Reserve, 10^7 pfu	*per os*	1
24	10^7 and 10^5 pfu		1 and 14
25	Western Reserve strain 10^7 pfu	Intramuscularly	1
26	10^7 and 10^5 pfu		1 and 14
27	Western Reserve strain 10^7 pfu	*per os*	1
28	10^7 and 10^5 pfu		1 and 14
29	MEM	Intramuscularly	1
30			1 and 14
31	MEM	*per os*	1
32			1 and 14

6.2.4.2 Immunization of dogs and raccoons

Dogs were previously vaccinated with recombinant DNA vaccines and survived street rabies virus challenge, whilst the raccoons were vaccinated with recombinant rabies vaccines and survived street rabies virus challenge (Lodmell *et al*., 2006). The animals were sedated with Telazol® (Tiletamine HCL and Zolazepam HCL) anesthetic prior to the procedures according to the manufacturer's suggestions (Fort Dodge, USA). Raccoons and dogs received one booster dose of 1 X 10^8 ffu of MVA or recombinant MVA in 1 ml MEM *per os*, or 500 µl intramuscularly on day 1. Some animals also received mock vaccinations consisting only of MEM. The vaccine was fed to the animals with a needleless syringe when required. The intramuscular administrations were given in the right deltoid muscle with 23-gauge needle and 1 ml syringes (Becton Dickinson and Company, USA). Blood was collected on day 0, before administration of the vaccines, and again 7 days thereafter. With the raccoons, blood was collected through puncture of the neck vein, and with the dogs, the animals where bled through a vein in the forearm. The blood was collected in Vacutainer® serum separation tubes (Becton Dickinson and Company, USA). The sera was collected and stored in aliquots at -20 ˚C until it could be analyzed. The titer of rabies virus neutralizing antibodies in the collected sera was determined by RFFIT as described before[25].

6.3 Results

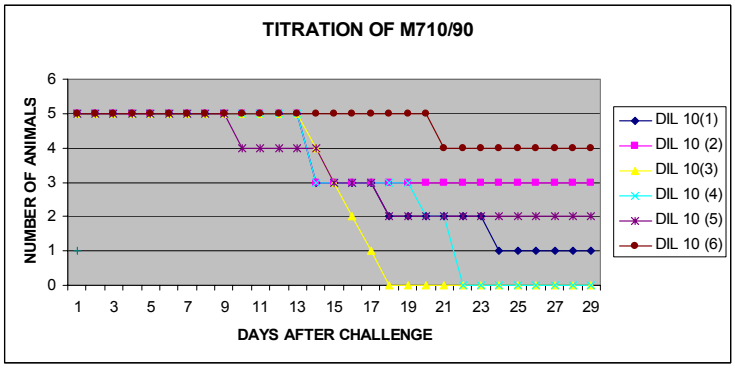
6.3.1 Preparation of challenge viruses

Animals succumbed after peripheral or intracranial challenge with the suckling mouse brain homogenates (figure 6.1). Animals that displayed clinical signs of rabies and were killed by euthanasia, or animals that were collected upon death, were all tested positive for the presence of rabies virus antigen in the brain by using the dFA test (results not shown). The $MICLD_{50}$ of the preparations were determined using the Reed and Muench approach. The m710/90 isolate had a titer of $10^{5.25}$ $MICLD_{50}$ per 30 µl and the ND77 isolate had a titer of $10^{4.4}$ $MICLD_{50}$ per 30 µl (calculations not shown). The first

[25] Refer to Part Two, Chapter IV, section 4.2.3

deaths after inoculation with the mongoose rabies virus isolate, m710/90 started occurring at day 9, in comparison to day 13 for the dog rabies virus isolate, ND77. This may be attributed to the relatively higher titer of virus in the suckling mouse brain homogenates of the m710/90 isolate.

a.



b.

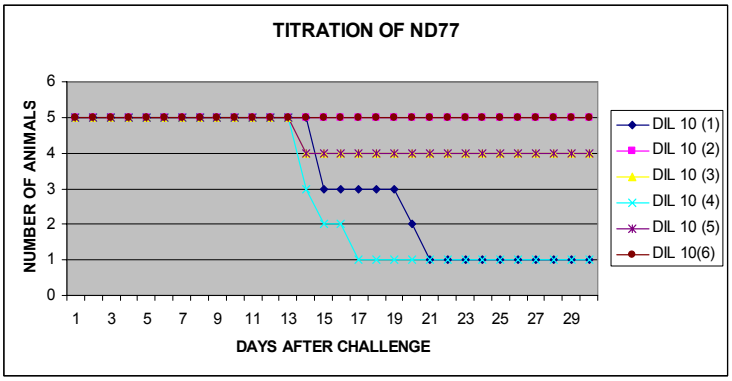


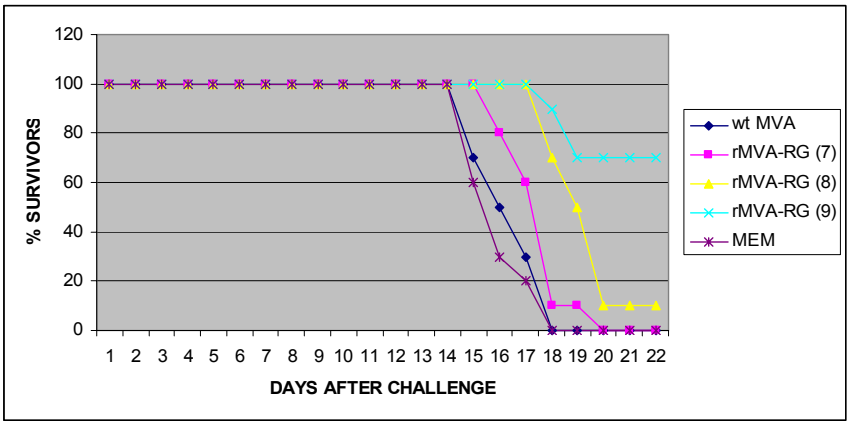
Figure 6.1: Deaths of mice after challenge with rabies virus isolates, (a) m710/90 and (b) ND77. Dilutions 10^{-1} and 10^{-2} (DIL 10(1) and DIL 10(2)) were administered peripherally whilst dilutions 10^{-3} to 10^{-6} (DIL 10(3) TO DIL 10(6)) were administered intracranially.

6.3.2 Survival and humoral responses in mice

Recombinant MVA that was administered intramuscularly elicited detectable humoral responses only at high doses of 10^8 and 10^9 ffu (table 6.2). Doses of 10^9 ffu,

intramuscularly administered, were however necessary to achieve 70 – 80 % protection against lethal challenge (table 6.2 and figure 6.2). The recombinant MVA did not elicit any detectable humoral responses in mice that received the vaccine orally (table 6.3).

a.



b.

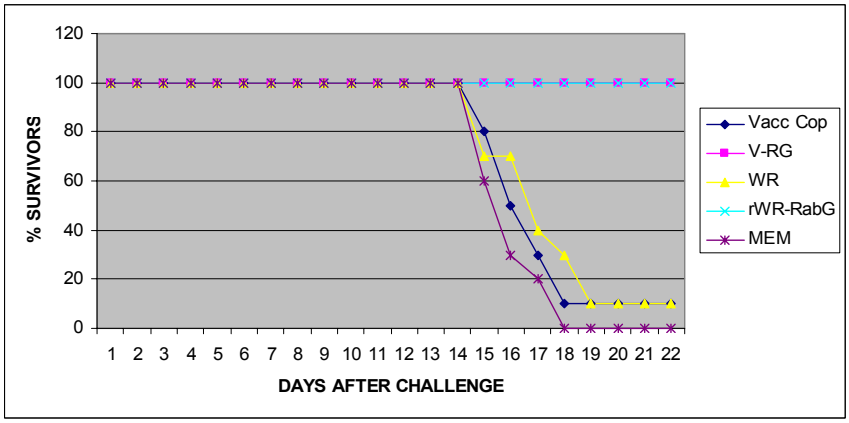


Figure 6.2: The survival of mice that received the vaccine constructs intramuscularly after lethal challenge with rabies virus.

Table 6.2 Serological responses of mice after intramuscular administration of the vaccine viruses. The geometric mean titers log base 10 (lowest value – highest value), as determined by RFFIT is provided. The percentage survivorship with the number of animals in the study is also tabulated.

GROUP		DAY 0[A]	DAY 7[B]	DAY 21[C]	% SURVIVORSHIP (NUMBER ANIMALS)
rMVA-RG (10^7 ffu)	No boost	< 1.0	< 1.0	< 1.0	0 % (0/10)
	boost	< 1.0	< 1.0	< 1.0	0 % (0/10)
rMVA-RG (10^8 ffu)	No boost	< 1.0	1.34 (1.34 -1.34)	1.34 (1.34 -1.34)	0 % (0/10)
	boost	< 1.0	1.34 (1.34 -1.34)	1.7 (1.7-1.7)	10 % (1/10)
rMVA-RG (10^9 ffu)	No boost	< 1.0	2.15 (1.76 - 2.34)	2.18 (1.92 - 2.28)	70% (7/10)
	boost	< 1.0	2.18 (1.90-2.34)	3.15 (2.85 – 3.41	80% (8/10)
V-RG	No boost	< 1.0	2.49 (1.76 - 2.80)	2.96 (2.11 – 3.50)	100 % (10/10)
	boost	< 1.0	1.7 (1.56 – 2.28)	3.38 (2.76 – 4.01)	100 % (10/10)
rWR-RabG	No boost	< 1.0	2.15 (2.10-2.30)	2.18 (2.10-2.30)	70 % (7/10)
	boost	< 1.0	2.08 (2.08-2.30)	4.18 (3.40-4.28)	100 % (10/10)
wt MVA	No boost	< 1.0	< 1.0	< 1.0	0 % (0/5)
	boost	< 1.0	< 1.0	< 1.0	0 % (0/5)
Vacc Cop	No boost	< 1.0	< 1.0	< 1.0	0 % (0/5)
	boost	< 1.0	< 1.0	< 1.0	20 % (1/5)
WR	No boost	< 1.0	< 1.0	< 1.0	0 % (0/5)
	boost	< 1.0	< 1.0	< 1.0	0 % (0/5)
MEM	No boost	< 1.0	< 1.0	< 1.0	0 % (0/5)
	boost	< 1.0	< 1.0	< 1.0	0 % (0/5)

[A] Naïve serum, before vaccination
[B] 7 days after the primary immunization
[C] 7 days after the booster immunization

The experimental V-RG vaccine administered intramuscularly fully protected mice against lethal challenge at a dosage of 10^7 pfu. The same was true for the Western

Reserve strain recombinant, which also induced protective immune responses (figure 6.1 and table 6.3). These two vaccines offered some protection against lethal challenge upon oral administration (table 6.3) and the survival of these animals was reflected in serum neutralizing antibody responses (table 6.2 and 6.3). The lower number of positive results, as well as the relatively low titers induced in the oral immunization experiments are notable and may be due to the difficulty of the oral administration procedure in mice. In all the experiments the parental vaccine viruses and the MEM control groups succumbed to challenge and these results are substantiated by the lack of responses measured by RFFIT.

Table 6.3 Serological responses of mice after oral administration of the vaccine viruses. The geometric mean titers log base 10 (lowest value – highest value), as determined by RFFIT is provided. The percentage survivorship with the number of animals in the study is also tabulated.

GROUP		DAY 0[A]	DAY 7[B]	DAY 21[C]	% SURVIVORSHIP (NUMBER ANIMALS)
rMVA-RG	No boost	< 1.0	< 1.0	< 1.0	0 % (0/10)
	boost	< 1.0	< 1.0	< 1.0	0 % (0/10)
V-RG	No boost	< 1.0	<1.0	<1.0	0 % (0/10)
	boost	< 1.0	1.14 (<1.0 – 2.18)	1.86 (<1.0 – 2.23)	40 % (4/10)
rWR-RabG	No boost	< 1.0	2.15 (<0.1 -2.83)	2.20 (<0.1 – 2.28)	40 % (4/10)
	boost	< 1.0	2.16 (<0.1 -2.83)	2.78 (<0.1 -3.38)	60 % (6/10)
wt MVA	No boost	< 1.0	< 1.0	< 1.0	0 % (0/10)
	boost	< 1.0	< 1.0	< 1.0	0 % (0/10)
Vacc Cop	No boost	< 1.0	< 1.0	< 1.0	0 % (0/10)
	boost	< 1.0	< 1.0	< 1.0	0 % (0/10)
WR	No boost	< 1.0	< 1.0	< 1.0	0 % (0/10)
	boost	< 1.0	< 1.0	< 1.0	0 % (0/10)
MEM	No boost	< 1.0	< 1.0	< 1.0	0 % (0/10)
	boost	< 1.0	< 1.0	< 1.0	0 % (0/10)

[A] Naïve serum, before vaccination
[B] 7 days after the primary immunization
[C] 7 days after the booster immunization

6.3.3 Anamnestic responses in previously vaccinated dogs and raccoons

Anamnestic responses to the recombinant MVA constructs in previously vaccinated and challenged animals were tested (table 6.4 – 6.5). The dogs and raccoons that received recombinant MVA intramuscularly, exhibited booster responses. The level of neutralizing antibodies boosted up to 100 fold and 14 fold respectively. No secondary responses could be measured in animals that received parental vaccine virus and MEM preparations administered intramuscularly or orally. Animals did not exhibit booster responses to the recombinant MVA when administered per mouth.

Table 6.4: Anamnestic responses in beagles after administration of the vaccine viruses. The geometric mean titers log base 10 (lowest value – highest value), as determined by RFFIT is provided.

GROUP	DAY 0	DAY 7	NUMBER OF ANIMALS IN GROUP
rMVA-RG, intramuscular	**2.16 (1.78 – 2.53)**	**3.88 (3.20 – 4.56)**	2
rMVA-RG, per os	2.50 (1.78 -3.88)	2.52 (1.78 – 3.88)	4
MVA, intramuscular	2.20 (1.81 -2.60)	2.60 (2.50 – 2.67)	2
MVA, per os	2.02 (1.88 – 2.16)	2.01 (1.85 – 2.16)	2
MEM, intramuscular	1.78	1.78	1
MEM, per os	1.93	1.93	1

Table 6.5: Anamnestic responses in raccoons after administration of the vaccine viruses. The geometric mean titers log base 10 (lowest value – highest value), as determined by RFFIT is provided.

GROUP	DAY 0	DAY 7	NUMBER OF ANIMALS IN GROUP
rMVA-RG, intramuscular	**2.91 (2.64 - 3.18)**	**3.83 (3.78 - 3.88)**	2
rMVA-RG, per os	1.82 (< 0.1 – 2.40)	1.85 (<0.1 – 2.43)	5
MVA, intramuscular	3.60	3.62	1
MVA, per os	3.15 (2.68 – 3.62)	3.12 (2.62 – 3.60)	2
MEM, intramuscular	<1.0	<1.0	1
MEM, per os	<1.0	<1.0	1

6.4 Discussion

MVA has been identified as a safe and immunogenic carrier of foreign antigens and have in many applications become a vector of choice for recombinant vaccine development (Sutter and Staib, 2003; Drexler *et al*., 2004). The use of MVA as vaccine carrier has been extensively investigated in a number of disease models and its potential as vaccine carrier shown in several clinical trials employing different regimens of administration (including human immunodeficiency virus vaccines) (Hanke *et al*., 1998; Hanke *et al*., 2002; Gomez *et al*., 2002; Schneider *et al*., 1998).

Here the efficacy of MVA recombinants as a rabies vaccine was investigated, a cause that has been called for by other investigators in the field (Lodmell *et al*., 2004). In our hands a recombinant MVA vaccines expressing a rabies virus glycoprotein gene, were effective in mice when administered peripherally, but significant protection was only attained at higher doses of recombinant MVA namely 10^9 ffu. Doses of 10^8 ffu have however been reported sufficient to elicit humoral responses in mice in other disease models. A dosage effect was apparent as the level of humoral immune responses clearly correlated with the dosage of recombinant MVA vaccine administered. At a dosage of 10^9 ffu, recombinant MVA primed the immune response efficiently so that after a booster administration up to 10-fold elevated levels of virus neutralizing antibodies were observed. In contrast with experiences with the experimental V-RG and Western Reserve recombinants that protected fully at doses of 10^7 pfu. It is well known from previous studies even lower doses of the vaccinia based-vaccines can provide protective efficacy. At these effective doses the titers of virus neutralizing antibodies induced by these recombinant vaccines tested here were comparable. Similar observations have been reported before by Ramírez *et al*., 2000. In the latter study a dosage effect was also apparent for serological data, and higher titers of recombinant MVA was required to attain the same humoral responses compared to an equivalent Western Reserve recombinant.

In accordance with numerous previous works, recombinant MVA boosted virus neutralizing antibody levels up to a 100-fold in pre-vaccinated and challenged raccoons and dogs when administered peripherally. In a study involving recombinant MVA

expressing measles virus antigens, the MVA recombinants boosted low-levels of vaccine-induced immunity much more efficiently than live attenuated measles virus vaccine (Stittelaar *et al*., 2000). Booster responses induced by MVA recombinants upon mucosal delivery, specifically after intranasal delivery, have also been indicated in macaques (Goonetilleke *et al*., 2003; Bertley *et al*., 2004). Probably the most prominent value of recombinant MVA vaccines lie in this so-called "booster effect". As such, recombinant MVA have potential application in the field if used in conjunction with current live attenuated vaccines to attain long lasting protective responses. On the other hand, used as a primary vaccine, multiple doses may be required to attain efficacy upon peripheral administrations (de Waal *et al*., 2004).

It was in the scope of this study to determine specifically the oral innocuity of the rabies glycoprotein recombinant MVA. Although successful vaccination through intragastric intubation with MVA recombinants have previously been shown, successful vaccination directly *via* the oral route has not yet been investigated to our knowledge (Bender *et al*., 1996). The efficacy of MVA recombinants *via* other mucosal routes have been shown in a number of studies (Gherardi and Esteban, 2005). As mentioned, the recombinant rabies glycoprotein MVA proved immunogenic in mice under certain conditions when administered peripherally, but failed to elicit any detectable humoral responses upon oral administration. Judging from the data of the experimental V-RG and Western Reserve strain recombinants, it appears that the mouse model is not an optimal model for testing oral immunization. These vaccines have known efficacy in other models by the oral route, but in the mouse model only 40% and 60 % protection could be achieved. The ability of the MVA recombinants to elicit memory responses in previously vaccinated dogs and raccoons, however, also failed through the oral route. Both these species of animals have been shown to be susceptible to oral inoculation with V-RG (Rupprecht *et al*., 1986, Rupprecht *et al*., 2005).

The failure of oral immunization with MVA recombinants used in this study is most likely the result of the severe attenuation of the vaccine carrier leading to its lack of replication in the vaccine recipient. Considering the mechanics of per mouth administration of the vaccine virus, a lot of the virus is likely to be "lost" due to sloughing of cells in the mouth and throat. In the case of the V-RG and Western Reserve

recombinant, the viruses are thought to be able to readily infect and replicate in these tissues which may explain its potency *via* this route.

A recombinant MVA vaccine may still be of use if included in immunization schemes in the presence of maternally derived antibodies (for example against rabies) or in the presence of preexisting immunity to vaccinia virus. These constructs might therefore have a potential for the vaccination of younger animals with maternally derived immunity or previously vaccinated animals that require boosting (Belyakov *et al*., 1999).

In summary, the immunogenicity of recombinant MVA expressing rabies virus glycoprotein has been shown in this study. The recombinant vaccine proved efficacious upon peripheral administration in mice, dogs and raccoons. The booster potential of MVA recombinants has also been confirmed in the application described here. However, a lack of oral innocuity of the recombinant MVA rabies glycoprotein, likely due to its severe attenuation, would rule this candidate unsuitable for replacement of V-RG or other effective oral rabies vaccines.