STUDY OF THE ROLE OF MUTATIONS IDENTIFIED IN THE M27, M36, m139, m141, AND m143 ORFS OF THE MURINE CYTOMEGALOVIRUS (MCMV) TEMPERATURE-SENSITIVE MUTANT *TSM*5

By

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Summary

Infection with human cytomegalovirus (HCMV) is usually asymptomatic in normally healthy individuals although about 50-85% of adults are infected during their lifetime. However, it can cause severe or fatal disease in infants and immunocompromised patients. The generation of a potent protective vaccine is a necessity to protect vulnerable people. Because of host restriction, murine cytomegalovirus (MCMV) is used as a model for HCMV to study genes involved in pathogenicity. Previously, we have generated a temperature-sensitive mutant, *tsm*5, which failed to replicate to detectable levels in mice. Several mutations have been identified in this mutant. In a previous study in our laboratory, a mutation (C890Y) introduced into the M70 primase gene resulted in reduced viral replication at 40ºC *in vitro* and the mutant was severely attenuated *in vivo*. Two other previously identified mutations may also contribute to this lack of replication *in vivo*, namely an A658S mutation in a protein expressed by the M27 ORF, which is involved in interference with interferon-γ signalling, and a V54I mutation in the anti-apoptotic protein expressed by the M36 ORF.

Further mutations have been identified in this study using Comparative Genome Sequencing (GCS) (Roche NimbleGen). A total of 10 synonymous and 15 non-synonymous single nucleotide polymorphisms (SNPs) have been identified in *tsm*5 and 14 of the non-synonymous mutations were confirmed by sequencing. Among these genes are m139 (Y565X) and m141 (V195M), which have been shown to be essential for replication in macrophages but not in fibroblasts, and m143 (M232I), shown to play an important role in the inhibition of the PKR-mediated host antiviral response.

In the present study, the above mentioned mutations in M27, M36, m139, m141, and m143 were introduced individually into the MCMV K181 (Perth) variant bacterial artificial chromosome (BAC) using RecE/T homologous recombination. In addition, the M27 and M36 mutations were introduced together into the *wt* virus BAC.

An *in vitro* phenotypical analysis revealed that, apart from the double mutant (Mt[M27^{A658S}M36^{V54I}]) and the m139 (Mt[m139^{Y565X}]) mutant**,** the introduced mutations in the above mentioned genes did not show a temperature sensitive phenotype in MEF cells compared to their revertants or the *wt* virus. The M27 and M36 single mutants and the m141 and m143 mutants also showed similar growth kinetics to *wt* virus in Raw 264.7 macrophages. In contrast, replication of the M27/M36 double mutant was drastically reduced in MEFs at 40ºC and in macrophages at 37ºC. Replication of the m139 mutant was slightly reduced in MEF cells at 40ºC but not in macrophages.

Interestingly, the attenuated replication of the M70 (Mt[M70^{C890Y}]) mutant, previously noted only at 40^{o}C in MEF cells, was also observed in Raw 264.7 macrophages at 37ºC. This M70 mutation however, was unstable.

In an attempt to stabilise this mutation, many synonymous mutations have been introduced into this ORF by changing codon preferences that should reduce the efficiency of gene translation but not change protein structure. Two mutant BACs were constructed in which 403 of the 964 codons were changed to codons used less optimally in mouse cells but with either cysteine (BAC[M70^{SO403Cys}]) or tyrosine (BAC[M70^{SO403Tyr}]) at residue 890. Another two BAC constructs were produced with only 155 codons (at the distal third of M70 gene) changed to mouse cell suboptimal codons and with either cysteine (BAC[M70^{SO155Cys}]) or tyrosine (BAC[M70^{SO155Tyr}]) at residue 890. Upon transfection of

these BACs into MEF cells, none produced infectious progeny viruses except BAC[M70^{SO155Cys}] which produced mutant Mt[M70^{SO155Cys}] virus. *In vitro*, Mt[M70^{SO155Cys}] replicated similarly to its revertant and the *wt* MCMV K181 (Perth) variant.

DEDICATION

This thesis is dedicated to my father, mother and wife for their love, support and encouragement.

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Table of Contents

LIST OF FIGURES

Chapter 1: Introduction

Chapter 2: Materials and Methods

Chapter 3: Results

List of Tables

List of Abbreviations

AIDS	Acquired immunodeficiency syndrome
A-MuLV	Abselon Murine Leukaemia Virus
BAC	Bacterial artificial chromosome
BAK	BCL-2 antagonist killer 1
BAX	BCL-2 associated x protein
bp	Base pairs
CCMV	Chimpanzee cytomegalovirus
CGS	Comparative genome sequencing
CMC	Carboxymethylcellulose
CMV	Cytomegalovirus
CTL	Cytotoxic T-lymphocytes
dGTP	Deoxyguanosine triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DR	Direct repeats
E	Early
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
GAS	Gamma activation sequence
GCV	Ganciclovir
GFP	Green fluorescent protein
GM	Growth medium
HCMV	Human cytomegalovirus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
hpi	Hours post infection
HR	Homologous recombination
HSPGs	Heparan sulphate proteoglycans
HSV	Herpes simplex virus
IC	Inflammatory cytokine
ICTV	international committee on Taxonomy of Viruses
IE	Immediate early
IFN	Interferon

IL	Interleukin
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISGF	Interferon stimulated gene factor
ISRE	IFN-stimulated response elements
JAK	Janus kinase
kbp	Kilobase pairs
L	Late
LB	Luria-Bertani broth
mCBP	mCP-binding protein
MCMV	Mouse cytomegalovirus
mCP	Minor capsid protein
MCP	Major capsid protein
MEF	Mouse embryo fibroblasts
MHC	Major histocompatibility complex
MIE	Major Immediate early
MIEP	Major Immediate early promoter
MM	Maintenance medium
MOI	Multiplicity of infection
MTs	Cell microtubules
NCS	Newborn calf serum
ND10	Nuclear domain 10
NF-κB	Nuclear factor κB
NK	Natural killer
nt	Nucleotide
ORF	Open reading frame
*ori*Lyt	HCMV origin of lytic replication
PAMP	Pathogen-associated molecular patterns
pAP	Assembly protein precursor
PBM	Peripheral blood monocytes
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PFU	Plaque forming units
p.i.	Post infection

PKR	Protein kinase R
PML-NB	PML nuclear body
pPR	Proteinase precursor
RFLP	Restriction fragment length polymorphism
RIP	Receptor-interacting protein
ROI	Region of interest
RT	Room temperature
SCID	Severely compromised immuno-deficient
SD	Standard deviation
SNHL	sensorineural hearing loss
SNP	Single nucleotide polymorphism
ss	Single stranded
STAT	Signal transducer and activator of transcription
TE	Tris-HCl/EDTA
TLRs	Toll-like receptors
Tm	Melting temperature
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TR_L	Terminal repeat long
TR_S	Terminal repeat short
ts	Temperature-sensitive
tsm	Temperature-sensitive mutants
Tyk	Tyrosine kinase
UL	Unique long
US	Unique short
vIBO	Viral inhibitor of Bak oligomerization
vICA	Viral inhibitor of caspase-8-induced apoptosis
vMIA	Viral mitochondria-localized inhibitor of apoptosis
wt	Wild type

Chapter 1: **Introduction**

1.1 The *Herpesviridae*:

The taxonomy of herpesviruses has been revised recently by the international committee on Taxonomy of Viruses (ICTV), where the former *Herpesviridae* family has been divided into three families; the *Herpesviridae* retains the mammal, bird, and reptile viruses, the *Alloherpesviridae*, includes fish and frog viruses, while the *Malacoherpesviridae* holds a bivalve virus. The three families have been placed in the new *Herpesvirales* order (Davison *et al.*, 2009). This introduction will consider the mammalian Herpesviridae only.

The *Herpesviridae* have a characteristic morphology. They have a large linear double-stranded DNA genome (127-290 kb) in a T=16 icosahedral capsid surrounded by a tegument. The tegument is a proteinaceous matrix composed of more than fourteen viral proteins and some cellular proteins (Varnum *et al.*, 2004). The tegument is enclosed in a lipid envelope containing membrane-associated proteins (Varnum *et al.*, 2004). Electron microscopic studies of the envelope has a typical trilaminar appearance (Figure 1.1) (Morgan *et al.*, 1968). Furthermore, mammalian viruses of the *Herpesviridae* have common characteristic features, such as host restriction, they encodes for a large collection of enzymes involved in nucleic acid metabolism (e.g. thymidine kinase, thymidylate synthetase, dUTPase, ribonucleotide reductase), DNA synthesis, (e.g. DNA polymerase, helicase, primase) and protein processing (e.g. protein kinases), they replicate in the nucleus (viral DNA synthesis and capsid formation), and usually results in lytic infection of permissive cells and they enter a latent state (Pellett & Roizman, 2006).

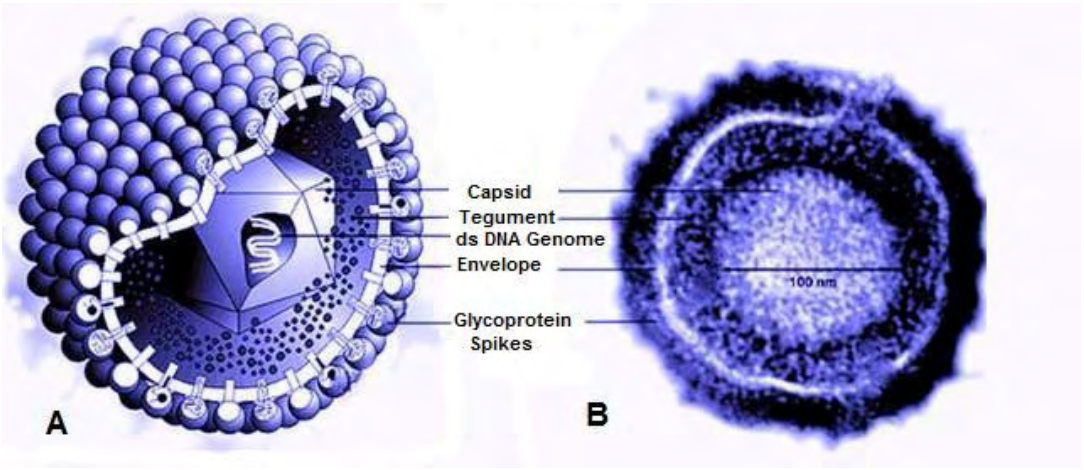


Figure 1.1: The morphology of a *Herpesviridae* virion: (A) Schematic representation of the HCMV virion. (www.biografix.de/). (B) A high resolution, computer-enhanced electron microscopy image of frozen viral capsids of Herpes simplex virus (HSV) (www.dbc.uci.edu/~faculty/wagner/hsv2f.html).

Based on their biological properties, sequence homology, arrangement of the genome and viral protein similarity, human-infecting members of the *Herpesviridae* are classified into three subfamilies; the *Alphaherpesvirinae* (α), the *Betaherpesvirinae* (β), and the *Gammaherpesvirinae* (γ) (Table 1.1).

Members of *Alphaherpesvirinae* subfamily are neurotropic and characterized by variability in host range, short reproductive cycle, ability to spread rapidly in culture, destruction of infected cells and their ability to produce latent infection in sensory ganglia. *Betaherpesvirinae* members are found in a wide range of mammalian species, HCMV as a member of *Betaherpesvirinae* is characterized by its restricted host range, long reproductive cycle and slow progress of infection in tissue culture. The characteristic common cytopathic effect (CPE) of members of this subfamily is the enlargement of infected cells (cytomegalia). The secretory glands, lymphoreticular cells and kidneys are the main sites of virus persistence as the latent form of infection. *Gammaherpesvirinae* subfamily members are characterized by their ability to infect either T or B lymphocytes (lymphotropic) and to induce latency in lymphoid tissue. The experimental host range is limited to members of the

family or order of the natural host. *In vitro* they can replicate in lymphoblastoid cells and some can cause lytic infection.

Table 1.1: Human diseases caused by *Herpesviridae* members.

Taxon and Name	Common name	Disease
Subfamily: *Alphaherpesvirinae*		
Genus: *Simplexvirus*		
Human herpesvirus 1	Herpes simplex virus type 1	Facial (skin sores), labial and ocular lesions, conjunctivitis, and encephalitis
Human herpesvirus 2	Herpes simplex virus type 2	Genital lesions
Genus: *Varicellovirus*		
Human herpesvirus 3	Varicella-zoster virus	Chicken pox shingles
Subfamily: *Betaherpesvirinae*		
Genus: *Cytomegalovirus*		
Human herpesvirus 5	*Cytomegalovirus*	**In Immunocompetent individuals:** Infectious mononucleosis, enteritis, thrombocytopenia, severe anaemia and rarely encephalitis **Congenital CMV:** birth defects including hearing and vision loss, and mental retardation, **In Immunocompromised patients:** retinitis and vision loss in AIDS patients and lymphadenopathy, hepatitis, thrombocytopenia, pneumonitis, nephritis, gastrointestinal invasion and rarely encephalitis in transplant patients
Genus: *Roseolovirus*		
Human herpesvirus 6	*Human herpesvirus 6*	Skin condition in infants called Rosella infantum or Exanthema subitum
Human herpesvirus 7	*Human herpesvirus 7*	Skin condition in infants called Rosella infantum or Exanthema subitum
Subfamily: *Gammaherpesvirinae*		
Genus: *Lymphocryptovirus*		
Human herpesvirus 4	*Epstein-Bar virus*	Infectious mononucleosis Associated with two rare forms of cancer: Burkitt's lymphoma, and nasopharyngeal carcinoma.
Genus: *Rhadinovirus*		
Human herpesvirus 8	*Kaposi's sarcoma-associated herpesvirus*	Associated with Kaposi's sarcoma

1.2 Cytomegalovirus:

1.2.1 Human Cytomegalovirus:

Cytomegaloviruses (CMVs) belong to the *Betaherpesvirinae* subfamily of the *Herpesviridae* family. HCMV is strictly species-specific infecting only humans and generally producing subclinical and persistent infections in healthy people. However, in some cases, primary infection might cause an acute febrile illness with features of mononucleosis. Studies showed that 90% primary HCMV infections in pregnant women are clinically silent, where only 3 out of 63 pregnant women with primary cytomegalovirus infection developed CMV mononucleosis and another three showed mild febrile illnesses (Stagno *et al.*, 1986). Primary CMV infection or reactivation of latent infection in immunocompromised patients, may cause a severe illness and multiorgan dysfunctions and symptoms of hepatitis, enteritis, pneumonia and cerebrospinal infection (Eddleston *et al.*, 1997).

Furthermore, HCMV has gained more importance because of the expansion in organ transplantation, immunosuppressive treatment and acquired immune deficiency syndrome (AIDS)(Moore & Chaisson, 1996; Singh, 2006). HCMV is also one of the most serious infectious diseases of infants, where 40-58%. infants with symptomatic CMV will have birth defects including hearing loss, vision impairment, and mental retardation (Dollard *et al.*, 2007).

1.2.1.1 Transmission and pathogenesis of cytomegalovirus:

Seroepidemiologic studies showed that HCMV is widely distributed in human population with an overall seroprevalence of about 60% in persons 20 years and older and

increases with age. Blood screening study showed that the base rate of CMV IgG seroprevalence in blood donors increases with age, from 34.9% at less than 20 years of age to 72.4% after the age of 50 years (Munro *et al.*, 2005).

In the United States and Western Europe, the seroprevalence of HCMV was linked to the socioeconomic status of the population where rates in young women of childbearing age range from 40% for women of middle to upper socioeconomic status to 83% for women of lower socioeconomic status (Gaytant *et al.*, 2002). In 1996, epidemiological studies showed that more than 17,000 cases of HCMV-induced sequelae or death occurred in Europe and the United States (Plotkin, 1999). For that reason, the majority of transplant donors and recipients will have had exposure to HCMV (Fishman & Rubin, 1998).

Following initial infection, HCMV infectious virus is present in body fluids such as saliva, tears semen, cervical secretions and urine for months to years (Dworsky *et al.*, 1983; Stagno *et al.*, 1982). Horizontal transmission of CMV infection usually occurs following exposure to infected body fluids which mainly occurs during sexual activity or following contact with infected young children (Chandler *et al.*, 1985; Handsfield *et al.*, 1985). Horizontal transmission of CMV also occurs via transfusion of whole blood (containing white blood cells) or transplantation of cells (Chou *et al.*, 1987; Chou, 1987). Vertical transmission of HCMV occurs either transplacentally to foetus during gestation period or at the time of birth through contact with genital organ secretions or through breast milk feeding (Dworsky *et al.*, 1983; Stagno *et al.*, 1982). Transmission of the HCMV virus can be reduced via application of good hygienic practice (such as hand washing with soap and water following exposure to contamination), which might have sufficient effect on primary infection especially in women and hence might reduce congenital infection (Cannon & Davis, 2005).

Following invasion and the lytic cycle of replication, the virus remains inactive in a latent state in infected cells, which serve as a reservoir of infection. Chronic low-grade reactivation can occur at intervals throughout the infected life span of the individual. CMV reactivation from latency to the productive virus cycle usually occurs due to a defect in the body's cell-mediated immune system, which can be due to drug-induced immunosuppression (e.g. in organ transplantation) or infection by certain pathogens (e.g. HIV) (Adler, 1983; Rubin, 1990).

Primary infection, reactivation of latent virus, and reinfection are possible and often are clinically silent. Three groups of immunocompromised hosts are susceptible to severe HCMV disease: (a) the immunologically immature foetus, (b) the transplant recipient as a result of anti-rejection treatment; and (c) AIDS patients with loss of adaptive immune response (Table 1.2) (Adler, 1983; Gaytant *et al.*, 2002; Rubin, 1990)

1.2.1.2 HCMV infection in immunocompetent individuals:

In healthy individuals, a primary CMV infection usually is clinically unapparent or subclinical and only about 10% of primary infections are symptomatic. Symptoms of HCMV disease are clearly observed in immunocompromised human (and in animal hosts with their respective CMV) after acute infection or after reactivation of latent infection (Stagno *et al.*, 1986).

The symptomatic HCMV infection result in illness similar to mononucleosis caused by Epstein-Barr virus infection. Studies showed that CMV is responsible for 20- 50% of heterophile-negative mononucleosis and it accounts for 8% of all cases of mononucleosis (Klemola *et al.*, 1970). Symptoms of CMV mononucleosis include malaise, persistent fever, myalgia and cervical lymphadenopathy and less common pneumonia and hepatitis.

Whereas laboratory findings include atypical lymphocytes, mild thrombocytopenia and elevated liver enzymes (Klemola *et al.*, 1970; Pannuti *et al.*, 1985; Stagno *et al.*, 1986).

CMV infection can rarely cause serious complications involving specific organ system and even life-threatening illness. CMV infection involving specific organ system in immunocompetent patients with problems similar to CMV in immunocompromised patients have been reported where CMV infection resulted in enteritis (Maiorana *et al.*, 2003), thrombocytopenia (Ichiche *et al.*, 2003), severe anaemia (Veldhuis *et al.*, 2004), or encephalitis (Phillips *et al.*, 1977; Studahl *et al.*, 1994) in healthy individuals.

1.2.1.3 Congenital HCMV infection:

HCMV infection in pregnant women, usually, occurs through exposure to contaminated saliva or urine via close contact with young children or through sexual transmission. After primary infection, which is usually subclinical in more than 90%, women tend to shed the virus for months to years in body fluids and excretions (Gandhi *et al.*, 2010). The high prevalence of HCMV infection in population means more pregnant women at risk of reactivation and even though reactivation is relatively infrequent for any given woman on a population level the number of affected women could be substantial as the congenital HCMV prevalence increases with maternal seroprevalence (Kenneson & Cannon, 2007). Studies showed that the rate of HCMV transmission to infants born to mothers who acquired a primary infection during pregnancy was 32% compared to only 1.4% of mothers with recurrent or past infection (Kenneson & Cannon, 2007; Lazzarotto *et al.*, 2008).

Worldwide, the prevalence of congenital HCMV is 0.7% of overall births and the percentage of infected children with CMV-specific symptoms at birth was 12.7% and symptomatic children with permanent sequelae was 40-58%. Among children with asymptomatic or subclinical CMV, 13.5% developed permanent sequelae (Dollard *et al.*, 2007).

Most of new-borns with symptomatic congenital HCMV infection will have some combination of defects, including, mental retardation, cerebral palsy, sensorineural hearing loss (SNHL), and impaired vision. The symptoms of acute congenital HCMV include premature birth (less than 37 months), petechiae, jaundice associated with hyperbilirubinemia, hepatospleenomegaly, thrombocytopenia, chorioretinitis, seizures, microcephaly, intracranial calcifications or fetal hydrops. In many cases, babies with subclinical CMV, develop permanent health problems or disabilities including hearing loss, visual disorder, brain disorders such as mental retardation, seizures, lack of coordination and epilepsy (Dollard *et al.*, 2007). Hearing loss following congenital CMV infection was reported in approximately 22%–65% of symptomatic and 6%–23% of asymptomatic children and CMV-related SNHL may be present at birth or occur later in childhood (Fowler & Boppana, 2006).

1.2.1.4 HCMV infection in sold-organ transplant recipients:

Even though considerable progress has been made in preventing HCMV infection and disease in the early stages post-transplant through the use of preventive antiviral drugs, it is still considered to be one of the main infectious agents causing complications in solid-organ transplant patients and can result in death (Fishman, 2007; Hodson *et al.*, 2005; Rowshani *et al.*, 2005; Singh, 2006).

HCMV primary infection, reactivation, or viral superinfection may develop in transplant recipients. Primary infection usually occurs when seronegative recipients who have not previously received immunologic therapy receive allografts from latently infected, seropositive donors (i.e., D+/R– combinations) which can result in a severe infection. Most of solid organ transplant develop CMV infection if they did not recive antiviral prophylaxis and the newly infected patients have asymptomatic viremia, although invasive disease develops in a subgroup of patients. During the first year after transplantation, seroconversion occurs in 75% of the seronegative transplant recipients who have received allografts from seropositive donors and only 25% of recipients benefit from prolonged prophylaxis where they do not undergo seroconversion (Humar *et al.*, 2005).

Cytomegalovirus infection exerts its effect in transplant patients via the direct effects of CMV replication (invasive disease) and may also results in a variety of secondary immune phenomena (Fishman & Rubin, 1998; Rubin, 1989). The direct effect of CMV or the invasive disease generally occurs within the first year following the completion of prophylaxis and usually results CMV syndrome which characterised by fever and neutropenia. In some cases, patients may also develop lymphadenopathy, hepatitis, thrombocytopenia, pneumonitis, nephritis, gastrointestinal invasion (with diffuse colitis, gastritis, ulcers, and bleeding), pancreatitis, chorioretinitis (in late stages), or rarely meningoencephalitis (Fishman, 2007).

The indirect effect of CMV infection results from the interaction of virus with the host immune response (Fishman, 2007). It is characterized by low rates of viral replication over a prolonged periods of times which are associated with an increased risk of rejection or graft dysfunction, accelerated atherosclerosis, opportunistic infections (including infections with other viruses and EBV-associated post-transplantation lymphoproliferative

disorder), malignancies, and post-transplant diabetes mellitus (Hodson *et al.*, 2005; Rowshani *et al.*, 2005; Rubin, 2000; Singh, 2006). Furthermore, CMV infection may results in vasculopathy in heart-allograft recipients and to the bronchiolitis obliterans syndrome in lung-allograft recipients (Fishman, 2007).

1.2.1.5 HCMV in patients with Acquired Immunodeficiency Syndromes:

CMV is one of the most common opportunistic diseases among the acquired Immune deficiency (AIDS) disease patients. It was linked to the impairment of immune system, resulted from low $CD4^+$ T-cells count. Before the use of highly active antiretroviral therapy (HAART), CMV infection occurs in approximately 20-40% of adult AIDS patients (Gallant *et al.*, 1992). The frequency of clinical manifestations of CMV infection largely depends on the CD4+ T-cell count. CMV end-organ disease in HIV infection is usually observed when the CD4+ T-cell count drops below 50 cells/mm^3 or when other signs of severe immunodeficiency, such as other opportunistic infections, are present (Moore & Chaisson, 1996).

Studies showed that the most common CMV end organ disease was retinitis, esophagitis, colitis and less commonly encephalitis, peripheral neuropathy, polyradiculoneuritis, pneumonitis, gastritis and hepatitis (Moore & Chaisson, 1996). CMV retinitis in HIV-1 infection was the most frequent CMV end-organ disease where it represents approximately 80–90% of all CMV end-organ manifestations (Gallant *et al.*, 1992). Cytomegalovirus infections of the gastrointestinal tract are the second most common CMV end-organ manifestation where oesophagus and the colon are most commonly affected. Intestinal involvement might results in ulcers and in rare cases complications such as bleeding, rupture or perforation of intestine (Gallant *et al.*, 1992). Neurological diseases

caused by CMV infection in AIDS patients include meningoencephalitis, polyradiculopathy and Guillian–Barré syndrome. Although involvement of the CNS in CMV-related organ disease inn HIV infection is a rare to occur (<1% of CMV-related manifestations), the mortality of untreated cases reaches almost 100% (Gallant *et al.*, 1992; McCutchan, 1995). HIV-infected patients with low CD4+ T-cell counts are at highest risk for necrotizing focal encephalitis and ventriculoencephalitis (Morgello *et al.*, 1987). In HIV patients with advanced immunodeficiency, CMV pneumonitis mostly presents as interstitial pneumonia, and pathological studies might reveal inclusion bodies in the absence of other pathogens that are more frequently the cause of pneumonia in HIV-infected patients (Rodriguez-Barradas *et al.*, 1996).

Table 1.2 Clinical manifestations of HCMV infection Britt, 2008 with modification).

Population	**Diseases associated with acute infection**	**Diseases associated with chronic infection**
Immunocompetent		
All ages	Asymptomatic infection, mononucleosis-like illness	Atherosclerotic vascular disease: inflammatory bowel disease, periodontal disease, rheumatologic disorders.
Elderly	Unknown	Immune senescence with potential decrease in immune responsiveness.
Immunocompromised		
Foetal and newborn infants (congenital infection)	90% asymptomatic infection, 10% can have hepatitis, retinitis, thrombocytopenia, neurologic disease.	Hearing loss (5-15%), neuron developmental abnormalities.
Allograft recipients	Fever, decreased bone marrow function, hepatitis, pneumonitis, bacterial super-infection.	Vascular disease (transplant vasculopathy), interstitial tubulosclerosis (renal allograft), loss of bile ducts (liver allografts); chronic graft rejection and graft loss.
Immunodeficiency syndrome (inherited and acquired)	Gastrointestinal disease (oesophagitis, colitis) retinitis, encephalitis.	Colitis (in inherited immunodeficiency)

1.3 Genetics of human cytomegalovirus:

HCMV has the largest genome (235 kb) among the eight human herpesvirus species (Dolan *et al.*, 2004). The virion's linear double stranded DNA has single unpaired bases at both ends to facilitate its circulation upon release from the capsid in infected cells (Tamashiro & Spector, 1986). The HCMV genome, like Chimpanzee cytomegalovirus (CCMV), consists of two covalently linked long and short segments designated as L and S respectively; this type of genome organization is called class E. Each segment presents a central unique region (U_L and U_S) flanked with terminal inverted repeats at both ends (TR_L, IR_L, TR_S, IR_S) giving the HCMV genome the general organization TR_L - U_L -IR_L -IR_S - U_S - TR_S (Cha *et al.*, 1996; Chee *et al.*, 1990; Davison *et al.*, 2003; Tamashiro & Spector, 1986). The TR_L region consist of a_n and *b* sequences, the IR_L -IR_S region consist of $b'a'_n\ c'$ sequences and the TR_S region consist of *c* and a_n sequences where prime designations indicate sequences in reverse alignment to sequences without primes. The resultant repeat organization of the genome is thus $a_n\ b$ -U_L-$b'a'_n\ c'$ -U_S– ca_n. The two domains of HCMV and CCMV can generate genomic isomers as a result of the ability of the terminal a_n sequences to recombine with the internal a'_n sequence (Figure 1.2) (Cha *et al.*, 1996; Chee *et al.*, 1990; Davison *et al.*, 2003; Tamashiro & Spector, 1986).

The *a* segments of the HCMV genome consist of multiple head-to-tail repeats in the *a* sequences that can vary in number among strains and different passages of the same strain (Dollard *et al.*, 2007; Tamashiro & Spector, 1986). These include two *cis*-acting packaging elements, *pac1* and *pac2* (Kemble & Mocarski, 1989), conserved among all herpes virus genomes that direct cleavage and packaging of viral genomes. Since MCMV lacks α sequences, *pac-1* and *pac*-2 are located at opposite ends of the genome outside the terminal repeats (McVoy *et al.*, 2000).

The complete genome sequence of HCMV (strain AD169varUK) showed that it is 230,283 bp in length and was predicted to contain 189 open reading frames (ORFs), of which fourteen are duplicated in an inverted repeat (Chee *et al.*, 1990). A later study revised the annotation of the AD169 HCMV ORFs and revealed that it contains only 145 genes (Davison *et al.*, 2003). Furthermore, another laboratory HCMV strain (Towne) was sequenced and found to encode at least 162 unique ORFs and that most of them have homologues in the AD169 strain (Dunn *et al.*, 2003).

HCMV AD169, clinically isolated from the adenoids of a child, was passaged under certain conditions in fibroblasts to attenuate the virulence of the virus so that it could be used as a live attenuated vaccine (Elek & Stern, 1974; Rowe *et al.*, 1956). After 25 years of serial passage of the virus in different laboratories, its genome was plasmid cloned for sequencing (Chee *et al.*, 1990). Studies comparing the prototype laboratory strain AD169 with other laboratory and wild type isolates of human cytomegalovirus showed that AD169, as a result of these many passages, contains frame-shift mutations in three genes (Akter *et al.*, 2003), and 19 genes at the right end of the UL region (UL133–UL150) have been lost by a reversed duplication of the a sequence close to the left genome end, leading to a substantial expansion of IR_L (Cha *et al.*, 1996; Prichard *et al.*, 2001). In other stocks of the virus it was found that a replacement of a nucleotide results in inactivation of the UL36 gene (Skaletskaya *et al.*, 2001) while deletions of UL42 and UL43 genes have been observed in other virus stocks (Dargan *et al.*, 1997).

Comparative studies with other low passaged human cytomegalovirus strains showed that three of five isolates passaged in cell culture have defects in certain genes, UL128, UL130 and UL131 (Akter *et al.*, 2003), leading to the conclusion that there is no laboratory strain with an intact genome. In another comparative study, four clinical isolates

(Toledo, FIX, PH and TR) have been passaged to a limited extent in the laboratory were cloned into Bacterial Artificial Chromosomes (BACs). Sequences comparison of these four isolates to the AD169 and Towne strains, showed that as many as 252 ORFs with the potential to encode proteins were preserved in the four clinical isolates (Murphy *et al.*, 2003b). A fifth clinical isolate, Merlin, has been sequenced after only three serial passages in fibroblasts and is thought to represent the wild-type complement of 165 ORFs, comprising no noticeable mutations apart from a single base substitution that results in truncation of the UL128 gene (Dolan *et al.*, 2004).

Using the whole-genome shotgun technique, the complete DNA sequence of the laboratory passaged Smith strain of MCMV was determined from virion DNA. The sequence showed that the genome consists of 230,278 bp, arranged as a single unique sequence (Figure 1.2) with short (31-bp) terminal direct repeats and several short internal repeats; its G/C content was 58.7%. MCMV was suggested to encode more than 170 ORFs. It was also demonstrated that there is considerable similarity between MCMV and the HCMV strain AD169 genome sequence, especially in the central part of the genome that encodes 78 ORFs. In addition, both murine and human cytomegalovirus genomes showed similarity in distribution of G+C content (Rawlinson *et al.*, 1996). In contrast to the observed variations in the HCMV sequence (among different strains and passages), the genomic sequence of the K181 (Perth) laboratory strain of MCMV, as well as three low passaged wild derived strains, showed that there was no major genetic rearrangements in MCMV. Furthermore, the study showed that the genome size was extremely conserved between these MCMV strains with no major deletions or insertions (Smith *et al.*, 2008). In addition, there are only a few changes in 2 isolates of the K181 strain, K181 (Perth) and

K181 (Birmingham), passaged in 2 different laboratories over many years (Smith *et al.*, 2008; Timoshenko *et al.*, 2009a).

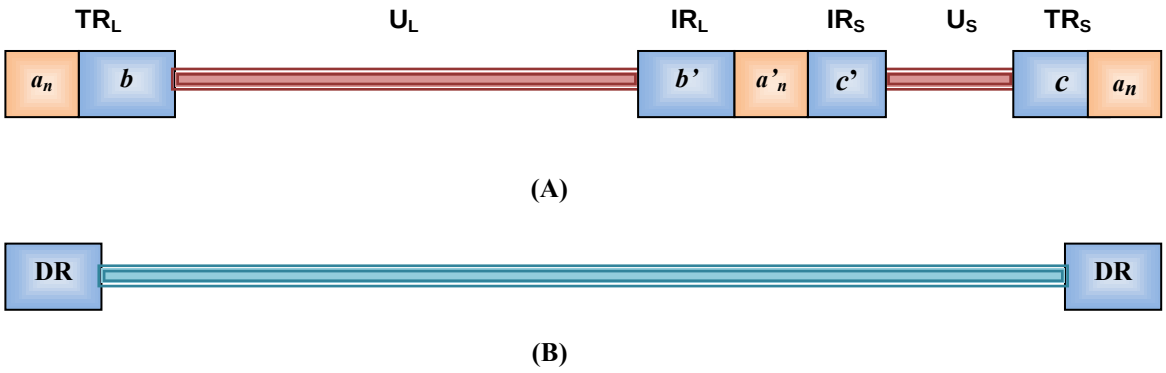


Figure 1.2: A diagram representation showing (A) The HCMV genome (class E) composed of the unique long sequence (U_L), flanked by terminal repeat long (TR_L) and inverted repeat long (IR_L), and unique short sequence (U_S), flanked by terminal repeat short (TR_S) and inverted repeat short (IR_S). (B) The MCMV genome (class F) contains a single unique sequence with two terminal direct repeats (DR) (Mocarski *et al.*, 2006; Rawlinson *et al.*, 1996) Not to scale.

1.4 The Virus Replication Cycle, Entry:

HCMV has a wide cellular tropism where many cell types such as endothelial cells, epithelial cells, smooth muscle cells, fibroblasts, monocytes/macrophages, stromal cells, neutrophils, hepatocytes, and neuronal cells, as well as tissue culture cell lines of these cells, are susceptible to HCMV infection. Like other herpesviruses, HCMV entry into cells occurs at neutral pH through direct fusion of the viral envelope with the plasma membrane (Compton *et al.*, 1992). In contrast, in certain cell lines, including retinal pigmented epithelial and endothelial cells, HCMV enters the cell through receptor-mediated endocytosis that requires low-pH (Bodaghi *et al.*, 1999; Ryckman *et al.*, 2006).

Analysis of the proteins from purified HCMV AD169 virion preparations revealed that the envelope contain at least 19 virally encoded glycoproteins (Varnum *et al.*, 2004). The laboratory HCMV AD169 strain suffer from deletions and rearrangements as a result of the long-term passage in tissue culture (Akter *et al.*, 2003; Cha *et al.*, 1996; Davison *et al.*, 2003). Analysis of other minimally passaged HCMV strains predicted that HCMV encodes more than fifty proteins that are potentially glycosylated or contain predicted transmembrane domains (Davison *et al.*, 2003; Dolan *et al.*, 2004; Murphy *et al.*, 2003a). Among them, only 5 glycoproteins, glycoprotein B (gB UL55), gM/gN (UL100/UL73) and gH/gL (UL75/UL115), participate in virus replication *in vitro* (Compton *et al.*, 1993; Kari & Gehrz, 1992; Kari & Gehrz, 1993; Navarro *et al.*, 1993). The major envelope glycoprotein, glycoprotein M, together with gN interacts to form the gM/gN complex that serves as an attachment protein. Glycoprotein B is considered to be an attachment and fusion protein (Compton *et al.*, 1993; Kari & Gehrz, 1992). Another complex formed from gH, gL and gO (gH/gL/gO), where gH acts as a fusion protein and gL is an essential chaperone for gH localization. The gH/gL/gO complex enhances HCMV entry (Huber & Compton, 1997; 1998; Keay & Baldwin, 1991). A recent study has showed that interaction of UL131A-UL128 gene products with gH and gL resulting in a gH/gL/UL131A-UL128 complex, is involved in HCMV entry into endothelial/epithelial cells (Dolan *et al.*, 2004; Revello & Gerna, 2010; Wang & Shenk, 2005).

The capability of HCMV to infect a broad range of cells indicated the existence of a great number of cellular receptors and/or ubiquitous molecules on the cell surface that allow the recognition and entrance of HCMV into the host. The current attachment model for HCMV starts with the initial tethering step to heparan sulphate proteoglycans (HSPGs) on the cell surface with viral glycoprotein complexes, the heterodimer gM/gN and the gB

homodimer of the virion (Compton *et al.*, 1993; Kari *et al.*, 1992; Kari & Gehrz, 1992). A recent study showed that gB is essential for virus entry and cell-to-cell spread of the virus but it is not essential in attachment to the cell surface where gB-null virions (Δ UL55) were able to attach to the cell surface like wild-type virions (Isaacson & Compton, 2009).This suggests that the initial binding is mediated by the gM/gN complex to the HSPGs followed by a high affinity binding to one or more cellular receptors. Epidermal growth factor receptors (EGFR) are thought to play a role in attachment and entry of HCMV as it has been shown that attachment and entry of HCMV was repressed following EGFR-neutralizing antibody treatment of fibroblasts (Wang *et al.*, 2005). On the other hand, many cell types permissive to HCMV, such as haematopoietic cells, do not express EGFR suggesting that it is not a universal receptor for HCMV. Furthermore, EGFR-neutralizing antibody treatment of epithelial and endothelial cells followed by challenge with a clinical isolate did not affect viral gene expression (Isaacson & Compton, 2009). Interaction of gB and gH with integrins may result in several downstream signalling processes essential for entry of virus and/or gene expression (Feire *et al.*, 2004; Wang *et al.*, 2005). Finally, interaction of gB and gH in the gH/gL/gO complex with cellular integrins facilitates the fusion of the viral envelope and the cellular membrane enabling the deposition of viral components (virion and tegument proteins) into the cytoplasm (Figure 1.3) (Feire *et al.*, 2004; Wang *et al.*, 2005).

Following fusion of the viral envelope to the cell membrane and entry of the virus into the cytoplasm of a permissive cell, capsids containing DNA are transported to the nucleus utilizing the intact cell microtubules (MTs) (Sinzger *et al.*, 2000). Indirect immunofluorescence showed that the tightly associated tegument protein pp150 is detected associated with MTs. Nocodazole, a drug causing microtubule de-polymerization, results in

detection of pp150 diffusely distributed in the cytoplasm inhibiting virions from delivering HCMV DNA to the nucleus and consequently block the immediate early gene expression (Ogawa-Goto *et al.*, 2003).

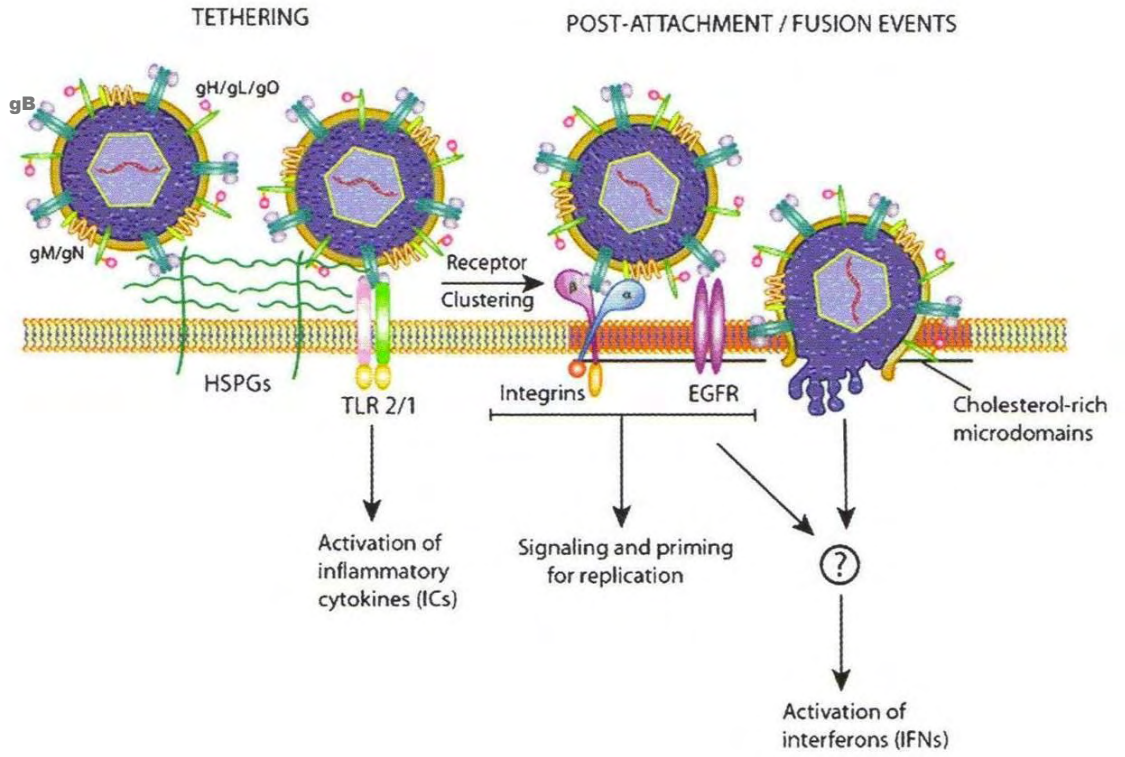


Figure 1.3: Model for entry of HCMV: Initial attachment of HCMV in tethering interactions to heparan sulphate proteoglycans (HSPGs) through gM/gN and/or gB glycoproteins. A stable docking step allows gB to cooperate with the epidermal growth factor receptor (EGFR) in HCMV permissive cell types and other receptors in haematopoietic cells. HCMV envelope glycoproteins and cellular integrins allow receptor clustering that leads to fusion facilitating internalization of virion components (Isaacson *et al.*, 2008).

Tegument proteins pUL47 and pUL48 interact to form the UL47/UL48 complex, which can play a role in the transport of capsids to the cell nucleus and possibly injecting the viral DNA into the nucleus through the nuclear pore complex. Mutants lacking the UL47 protein were found to be less infectious than the wild-type virus and have a delayed onset of IE gene expression (Bechtel & Shenk, 2002).

1.4.1 Regulation of gene transcription:

Transcription of CMV genes takes place in the nucleus of infected cells by host cell RNA-polymerase II and the associated basal transcription machinery (Fortunato & Spector, 1999). Like other herpesviruses genes expressions of cytomegaloviruses is conventionally divided into three kinetic phases which are defined on the time of expression and sensitivity to inhibition by protein or viral DNA synthesis inhibitors. The phases are: immediate-early (IE or α), early (E or β), and late (L or γ) (Fortunato & Spector, 1999).

1.4.1.1 IE gene regulation:

In the nucleus, the viral genome is associated with cellular histones and packaged into chromatin. Some viral genomes are deposited adjacent to a subnuclear structures named PML (promyelocytic leukaemia) nuclear bodies (PML-NBs) [or the nuclear domain 10 (ND10)] (Ishov *et al.*, 1999). The PML-NBs are dynamic intranuclear domains bound to the nucleare matrix and contain cellular proteins that involved in many cellular functions, including regulation of gene transcription and expression, chromatin structure, DNA repair, mitosis and apoptosis (Bernardi & Pandolfi, 2003; Dellaire & Bazett-Jones, 2004). Studies showed that PML-NBs are preferred sites for HCMV genome expression where only the HCMV genome found next to PML-NBs can be transcribed (Ishov *et al.*, 1999). Many cellular proteins are associated with the PML-NBs including Sp100, hDaxx, SUMO-1, p53, STAT1, STAT2, ATRX (Choi *et al.*, 2006; Ishov *et al.*, 2004; Lukashchuk *et al.*, 2008; Negorev & Maul, 2001; Paulus *et al.*, 2006; Tang *et al.*, 2004).

PML-bodies are now recognized to act as an intrinsic barrier to virus infection where RNAi knockdown of either PML or hDaxx significantly enhanced HCMV or HSV-1 replication (Everett, 2006; Lukashchuk *et al.*, 2008; Tavalai *et al.*, 2006; 2008). The HCMV

tegument protein pp71 counteracts the repression effect hDaxx where it promotes proteosome-mediated degradation of hDaxx, and thereby relieves hDaxx-mediated repression of IE gene expression (Saffert & Kalejta, 2006).

The human major IE protein (IE1-72) found to be associates with metaphase chromatin, recruiting both PML and STAT2. hDaxx, STAT1 and IE2-86K did not re-locate to metaphase chromatin; the fate of hDaxx is particularly important as this protein contributes to an intrinsic barrier to HCMV infection. While IE1-72K participates in a complex with chromatin, PML, STAT2 and Sp100, IE1-72K releases hDaxx from ND10

1.4.1.2 Immediate-early gene expression

IE gene expression occurs immediately after entry and does not rely on the expression of any viral genes or viral protein synthesis. Proteins produced during the IE phase are important for initial viral gene expression by acting as autostimulators of their own gene expression or as transactivators and stimulators for other viral genes, especially those of the next phase.

Following virus entry and delivery of viral DNA to the cell nucleus, the tegument phosphoprotein pp71 encoded by UL82 is released from the virion and transported to the nucleus, where it is detected at ND10 (Hofmann *et al.*, 2002). pp71 can activate expression from the major immediate-early promoter (MIEP) and other promoters and may stimulate the expression from the entire HCMV genome. Studies showed that UL82 null HCMV is severely impaired for IE expression especially at low multiplicity of infection (MOI) which proves the importance of pp71 in initiation of IE gene expression (Bresnahan & Shenk, 2000). It has been shown that pp71 binds to Daxx at ND-10 sites resulting in the reversal of

Daxx-mediated repression of viral IE gene expression (Cantrell & Bresnahan, 2005; Lukashchuk *et al.*, 2008).

IE genes include the major IE genes (MIE) UL123 (IE1) and UL122 (IE2) and auxiliary genes (UL 36-UL38, UL115-UL119, IRS1/TRS1 and US3) (Colberg-Poley, 1996; Stenberg, 1996). Expression of IE genes is controlled by a composite DNA component, the MIEP, which is composed of multiple sequence motifs that function as binding sites for cellular proteins that regulate transcription (such as NF-κB/Rel, Sp1, NF-1, AP-1, CREB/ATF, YY1 and others), both positively and negatively. The MIEP is located down-stream from a strong enhancer located between ~-50 and -550 bp from the transcription start site. The MIE region produces a primary transcript that undergoes differential splicing to produce numerous mRNA species. The predominant gene products are IE72 (IE1) and IE86 (IE2). They share exons 2 and 3 but vary in having either exon 4 (IE1) or exon 5 (IE2) (Akrigg *et al.*, 1985; Kerry *et al.*, 1995; Stenberg *et al.*, 1985).

Both IE72 and IE86 act as transactivators of viral and cellular proteins. IE72 also associates with PML-NB and disrupts their integrity (Dimitropoulou *et al.*, 2010; Wilkinson *et al.*, 1998). IE86 also acts as an autorepressor, by binding to the *cis* repression sequence located between the TATA box and the transcription initiation sites of these genes that results in repressing transcription of the IE1 and IE2 genes (Liu *et al.*, 1991). IE86 plays a role in down-regulation of cellular cytokine and chemokine promoters, controlling cell cycle progression and is necessary for viral DNA synthesis (Taylor & Bresnahan, 2005; 2006b). In addition to that, IE86 plays a main role in regulation of the transition from the IE to the E and L phases of HCMV expression (Kalejta & Shenk, 2002; Lu & Shenk, 1996). In addition to the importance of the MIE products in viral replication, auxiliary IE genes play roles in regulation (IRS1/TRS1), inhibition of apoptosis (UL36 and UL37) and

modulation of host cell MHC class I expression (US3(Child *et al.*, 2004; Colberg-Poley *et al.*, 1992; Colberg-Poley, 1996; Jones & Sun, 1997).

In MCMV, genes ie1 (m123) and ie3 (M122) are the functional homologues of the HCMV IE1 and IE2 genes, respectively (Cardin *et al.*, 1995). IE2, the third MCMV IE gene product, has no functional and or sequence homology with the HCMV genome. MCMV ie1 protein is involved in overcoming the cellular repression of viral DNA replication. Temporal analysis of MCMV ie1 protein shown its initial segregation into ND10 by binding to PML and/or Daxx and IE1-dependent recruitment of the transcriptional repressor histone deacetylase-2 (HDAC-2) to this site (Tang & Maul, 2003). IE1 binding to HDAC-2 resulted in decrease in deacetylation activity. Studies shown that HDAC inhibition by trichostatin-A resulted in an increased viral protein synthesis, formation of prereplication compartments and increase in the progeny virus yields (Tang & Maul, 2003). The IE3 protein encoded by MCMV gene ie3 is the important transactivator of viral E-phase genes (Angulo *et al.*, 2000).

1.4.1.3 Early gene expression

Early gene expression is dependent on the availability of IE gene products. Genes of this phase are not a target of viral DNA replication inhibitors, i.e. they do not require viral DNA synthesis to be expressed. Depending on the time of expression, the early phase may be divided into two subclasses, β1 (or true early) and β2 (early-late), where expression of β1 genes for HCMV occurs within 4-8 hrs post infection, while β2 gene expression occurs 8-24 hr post infection. E genes encode mostly non-structural proteins, including viral DNA replication factors [UL112-UL113, DNA polymerase (UL54), polymerase accessory protein (UL44), single-stranded DNA binding protein (UL57), helicase-primase complex

(UL70, UL105, UL102)] (McMahon & Anders, 2002; Pari, 2008b; Pari & Anders, 1993; Park *et al.*, 2006), repair enzymes, proteins involved in immunomodulation (US2 and US11) and an early glycoprotein (UL4/gp48) involved in transcriptional and posttranscriptional controls (Jones & Sun, 1997; Wiertz *et al.*, 1996). A family of phosphoproteins (encoded by the UL112-UL113 genes) regulates expression of core DNA replication genes and organizes viral DNA replication compartments within the nucleus (Park *et al.*, 2006).

1.4.1.4 Late gene expression

In a similar way late or γ phase genes are subdivided into two subsets designated as γ_1 (partial-late) genes, where their expression is amplified by the onset of viral DNA synthesis, and γ_2 (true-late) genes, whose expression is inhibited by DNA synthesis inhibitors and thus are totally dependent on viral DNA synthesis. The L genes encode structural proteins and are involved in the assembly and morphogenesis of virions (Chambers *et al.*, 1999; Fortunato & Spector, 1999).

1.4.2 DNA replication

Replication of the HCMV genome, virus particle assembly and packaging occurs in the nucleus of the infected cell. Within 4 hours post infection (hpi) the linear HCMV genome circularizes in the nucleus of permissive cells. DNA replication starts about 16 hpi and reaches its optimal level at 60-80 hpi yielding thousands of viral genome copies per infected cell. DNA synthesis requires six herpesvirus-conserved replication-fork proteins; the DNA polymerase (UL54), polymerase accessory protein that prevents dissociation of the polymerase from the template (UL44), a single-stranded DNA-binding protein (UL57) that prevents the re-annealing of DNA strands following unwinding by the three subunit

proteins of the helicase-primase complex (UL70, UL102, UL105) (McMahon & Anders, 2002). For optimal replication, the products of additional HCMV genes (UL36-38, UL84, UL112/UL113, UL114, IRS1/TRS1, and IE1/2) are also required. UL84 is a multifunctional protein that plays an important role in the regulation of transcriptional activation mediated by IE2, and is required for the initiation of *ori*Lyt-dependent DNA synthesis (Pari, 2008a). UL114 protein has a functional uracil DNA glycosylase activity required for efficient viral DNA replication in post-mitotic cells (McMahon & Anders, 2002; Pari *et al.*, 1993; 2008b; Pari & Anders, 1993; Park *et al.*, 2006)

Replication occurs via a bidirectional theta (θ) mechanism from a single origin (*ori*Lyt) of replication, that initiates binding of the replication complex and the rolling cycle mechanism is followed by viral DNA synthesis in the form of large head-to-tail genome concatemers (Masse *et al.*, 1992; Zhu *et al.*, 1998b). In HSV-1 the alkaline nuclease, the product of the UL12 gene (HCMV homologue UL98), is required for resolving these recombination intermediates prior to DNA packaging (Goldstein & Weller, 1998).

1.4.3 Virion assembly, maturation, egress:

HCMV capsid formation and packaging of viral DNA takes place in the nucleus of infected cells. Nucleocapsid particles accumulate in the nucleus as nuclear inclusion bodies giving infected cells the typical cytopathic effect (owl's eye). Synthesis of nucleocapsid proteins occurs in the cytoplasm where the major capsid protein (MCP pUL86) interacts with the scaffolding assembling protein precursor (pAP, pUL80.5) and proteinase precursor (pPR, pUL80a) to form putative complexes. The putative complexes are then translocated into the nucleus. Similar to MCP, a homodimer of the minor capsid protein (mCP, pUL85) interacts with mCP-binding protein (mCBP, pUL46) to produce triplexes that then

translocate into the nucleus. The HCMV portal protein (pUL104) may also interact with pAP in a way similar to that in HSV or it may be translocated to the nucleus via its own nuclear localization sequence (Figure 1.4) (Dittmer *et al.*, 2005).

In the nucleus, procapsids form from the interaction of pAP, pPR and MCP complexes with the triplexes (mCP and mCBP). The procapsids are less angular than mature capsids and they contain the scaffolding protein but not viral DNA (Newcomb *et al.*, 1999; Newcomb *et al.*, 2000). The immature capsids undergo proteolytic maturation to form some B-capsids that have an angular appearance and contain cleaved scaffolding proteins but not viral DNA. These B-capsids are ready for packaging viral DNA and further mature into infectious C capsids. Correct timing of scaffolding protein cleavage and release is very critical where delayed scaffold protein processing might result in sealing of capsid pores prior to release of cleaved protein and B capsid formation. While quick or premature scaffolding processing and release of scaffolding protein before recruiting DNA to the procapsid results in empty A capsids (Gibson, 2008; Yu *et al.*, 2005).

Newly synthesized genomes mature through an inversion process and cleavage to unit lengths at specific cleavage/packaging signals *pac*1 and *pac*2 located within the *a* sequence of the genomic DNA (Landolfo *et al.*, 2003). This cleavage utilizes ATP and is believed to proceed concurrently with insertion of the DNA into the preformed B capsids leading to the formation of mature C capsids (Adamo *et al.*, 2004). This may not be true in all cases as the MCMV temperature-sensitive mutant (*tsm*), *tsm*5, makes few capsids at 40^{o}C and these lacked DNA. DNA synthesis was significantly reduced in *tsm*5-infected cells at 40^{o}C but DNA cleavage occurred with close to wild type (*wt*) efficiency (Sweet *et al.*, 2007).Two gene products (pUL56 and pUL89) form the herpesvirus DNA cleavage/packaging enzyme (terminase). pUL56 (96 kDa) is the larger subunit of the

terminase and has similar properties to the bacteriophage terminase large subunit (Scheffczik *et al.*, 2002). It mediates specific binding to the *pac* sequences on the concatemers, has an ATPase activity essential for providing energy for translocation of unit length DNA into the procapsids, and binds to capsid (Hwang & Bogner, 2002). pUL89 (77 kDa), the smaller subunit, has nuclease activity essential for cleavage of viral DNA (Scheffczik *et al.*, 2002). In HCMV, retention of viral DNA in the capsid is stabilized by the HSV pUL25 homologue, the portal capping protein pUL77 (71 kDa), as mutant viruses with defective pUL77 were not able to stably package DNA (Ogasawara *et al.*, 2001; Sheaffer *et al.*, 2001). It is unlikely that this is the reason for the lack of DNA packaging in *tsm*5-infected cells as no mutations were identified in M77, the MCMV homologue of UL77 (Timoshenko *et al.*, 2009a).

After packaging of a unit length linear DNA genome, mature nucleocapsids accumulate in the nucleus and gather at the inner nuclear membrane prior to budding into the perinuclear space cisternae thus acquiring a primary envelope (Buser *et al.*, 2007). Two conserved viral proteins (in HCMV, pUL50 and pUL53) are required for this process where they form a complex at the nuclear envelope, referred to as the nuclear egress complex (Sanchez & Spector, 2002).

HCMV-encoded protein kinase UL97 and cellular protein kinase C (PKC) are involved in phosphorylation of many kinds of nuclear lamins. The activity of pUL97 in lamin-phosphorylation is associated with a recognition of nuclear lamin A/C and targeted by Cdc2/cyclin-dependent kinase 1 (Hamirally *et al.*, 2009). Studies showed that in transiently transfected or HCMV-infected cells, either pUL97 or PKC has the ability to induce distinct punctate lamin-depleted areas at the periphery of the nuclear envelope (Milbradt *et al.*, 2010). In MCMV, it was shown that the homologue of pUL34 (MCMV

M50) interacts with cellular protein kinase C for phosphorylation and dissolution of the nuclear lamina that facilitates viral budding (Muranyi *et al.*, 2002). In addition, M53 viruses with mutations in their M53 ORF, the homologue of UL53, were affected in their nuclear distribution and steady-state levels of the nuclear egress complex and export of viral capsids was completely blocked (Popa *et al.*, 2010).

The enveloped nucleocapsids then fuse with the outer nuclear membrane or the endoplasmic reticulum (ER) membrane resulting in release of de-enveloped nucleocapsids into the cytoplasm. The nucleocapsids acquire tegument proteins in the nuclear and the cytoplasmic phases (Eickmann *et al.*, 2006). In the cytoplasm, HCMV nucleocapsids acquire their tegument, and tegumented capsids obtain their final envelope by budding into vesicles of the *trans*-Golgi network. Enveloped capsids leave the cell to the extracellular environment via an exocytotic-like pathway (Eickmann *et al.*, 2006; Mettenleiter, 2002).

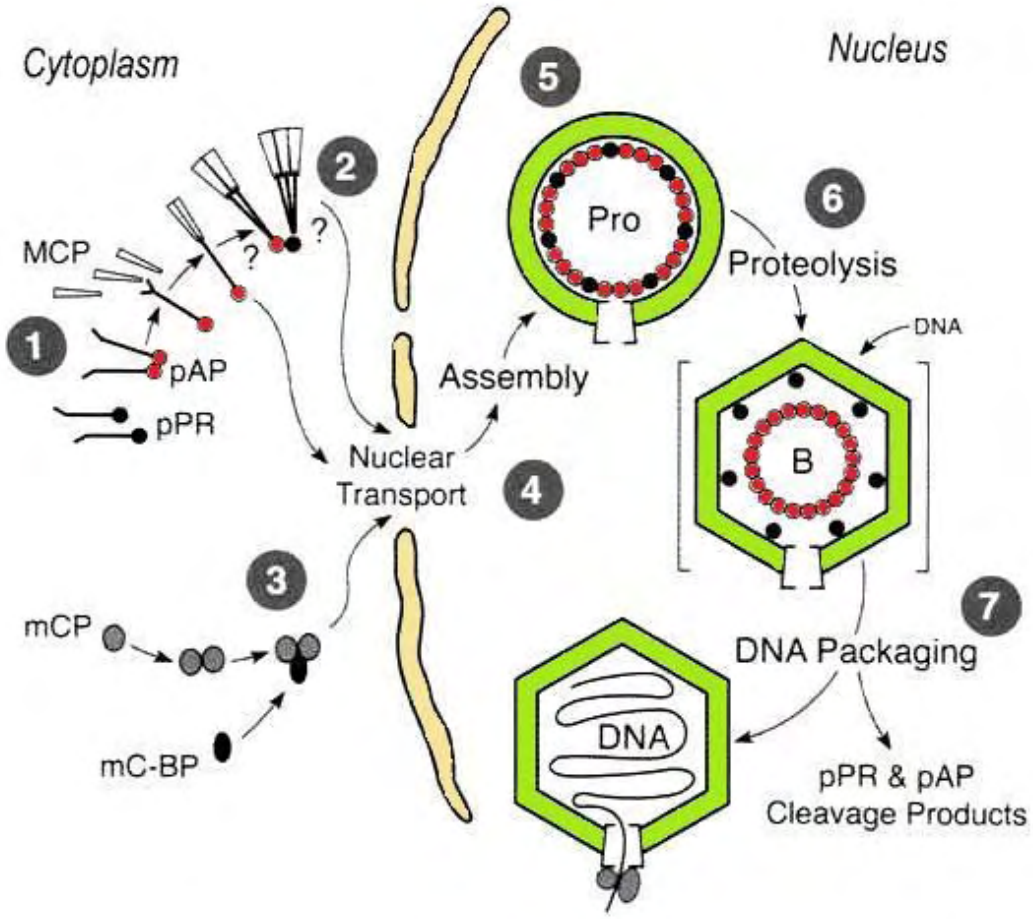


Figure 1.4: Capsid assembly model showing: (1) Major capsid protein (MCP, pUL85, *narrow trapezoids*) interaction with assembly protein precursor (pAP, pUL80.5, *lines with red filled circles*) and protease precursor (pPR, pUL80a, *lines with black filled circles*) results in the formation of putative complexes. (2). Minor capsid protein (mCP, pUL85, 35 kDa, *light ovals*) interaction with mCP-binding protein (mCBP, pUL46, 33 kDa, *dark ovals*) results in formation of heterotrimers, called triplexes (3). Translocation of the two formed oligomers into the nucleus (4). Interaction of the oligomers in the nucleus to form the procapsid (Pro), incorporating the portal-protein complex (pUL104) (5). Maturation process of the procapsid into the B capsid involves proteolytic cleavage and elimination of the scaffolding proteins (pPR and pAP) from the capsid (6), before or during the process of DNA packaging (7). Viral DNA packaging and formation of C capsids completes virion assembly. (From Gibson, 2008, with modification).

1.5 Activation of Immune Responses:

1.5.1 Activation of innate immunity:

Upon HCMV binding to permissive cell membrane receptors, HCMV glycoproteins result in triggering and activation of signal transduction pathways leading to the activation of cellular transcription factors including nuclear factor κB (NF-κB), Sp1 and interferon regulatory factor 3 (IRF3) (Boehme *et al.*, 2004; Boehme *et al.*, 2006; Navarro *et al.*, 1993; Yurochko *et al.*, 1997). Studies have revealed that HCMV entry can affect hundreds of cellular genes, the most strongly induced are genes involved in antiviral responses which belong to the inflammatory cytokine (IC) family and the interferon-stimulated gene (ISG) family including RANTES, interleukin-6 (IL-6), IL-8, ISG-45-kDa protein, and IRF-7 (Browne *et al.*, 2001; Simmen *et al.*, 2001; Zhu *et al.*, 1997; Zhu *et al.*, 1998a). Stimulation of these two classes of molecules, which play a role in innate immunity, is triggered by structural viral components and does not require virus replication or cellular protein synthesis (Browne *et al.*, 2001; Zhu *et al.*, 1997). The innate immune response play a role in limiting viral replication and spread of virus to adjacent cells and organs. It also recruits immune cells to the infection site, and initiates and modulates adaptive immune responses by T and B lymphocytes to clear the infection.

In HCMV infected fibroblasts and monocytes, NF-κB is activated as shown by its nuclear translocation and increased DNA-binding activity. Activation of NF-κB results in stimulation of inflammatory cytokines such as IL-1, IL-6, IL-8, IL-12 , IL-18 and TNF α, essential for recruitments of phagocytic leukocytes to the site of infection (Laroux, 2004). Studies show that induction of transcription of NF-κB can be inhibited by pre-treatment of HCMV with neutralizing antibodies to gH and gB. Furthermore, the use of purified soluble

gH and anti-idiotypic antibody to imitate gH resulted in stimulation of NF-κB and Sp1 (Yurochko *et al.*, 1997).

NF-κB can also be activated by many other stimuli including ligand stimulation of the Toll-like receptors (TLRs) that can identify microbial pathogens on the bases of structural motifs designated as pathogen-associated molecular patterns (PAMPs) that vary from those found in the host cell (Takeda & Akira, 2003). PAMPs can be detected in a wide range of viruses, bacteria, and fungi and initiate immune responses HCMV's gB and gH can activate TLR2, which in turn can induce IC synthesis via TLR2-dependent activation of NF-κB (Boehme *et al.*, 2006; Compton *et al.*, 2003).

Interactions of gBs with members of the TLR family can also result in activation of interferon (IFN)-α/β responses. Activation of TLRs results in the activation of the key regulatory transcriptional factor, IRF3, which is the primary factor in controlling HCMV-induced interferon signalling (DeFilippis *et al.*, 2006; Navarro *et al.*, 1998). Signal transduction results in virus-mediated phosphorylation of IRF3 followed by homodimerization and translocation of IRF3 to the nucleus. IRF3 then interacts with the co-activators, CREB binding protein (CBP), and p300, to form a complex that results in transcription of IFN-β and a subset of ISGs (Simmen *et al.*, 2001). In an autocrine and paracrine manner, the secreted IFN-β acts through the IFN-α/β receptor resulting in the activation of the janus kinases (JAK) and the signal transducers and activators of transcription (STAT) signal transduction cascade to induce expression of the full complement of ISGs. This event leads to the expression of the IFN-α genes. Like IFN-β, IFN-α, can stimulate ISG expression, which results in a rapid amplification of the interferon response that can lead to an antiviral state (Stark *et al.*, 1998).

Apart from TLR4, which resides on the surface of cells, the majority of TLRs are intracellular (Akira *et al.*, 2006). They are involved in IFN activation where they are able to recognize viral ligands early through uncoating or degradation processes and require low pH for their activation (Ahmad-Nejad *et al.*, 2002). TLR9 can recognize HSV-1, HSV2 and MCMV via their CpG-rich genome (2004b; Krug *et al.*, 2004a; Lund *et al.*, 2003). In MCMV, TRL3, which is activated by dsRNA formed during bidirectional transcription of the viral genome during the late stage of infection, stimulates IFN production (Tabeta *et al.*, 2004).

One of the innate immunity first lines of defence is natural killer (NK) cells that play an essential role in primary CMV infection before the onset of the adaptive immune response. A case study reported that humans having a defect in NK cells are more susceptible to HCMV infection (Biron *et al.*, 1989). The same was demonstrated experimentally in mice, where newborn mice and mice with genetic deficiencies in NK cells are more susceptible to MCMV and transfer of NK cells protects against MCMV infection (Bukowski *et al.*, 1985). Some mouse strains are comparatively resistant to MCMV such as C57BL/6, while other mouse strains including BALB/c and DBA/2 are more susceptible to MCMV infection (Scalzo *et al.*, 1990). It was attributed to the Cmv1 genetic locus on chromosome 6 encoding Ly49 NK cell inhibitory and activation receptors that recognize MHC class I-like molecules on target cell surface (Scalzo *et al.*, 1995). m157 gene product of the MCMV is a ligand for both the activating receptor Ly49H and inhibitory receptor Ly49I (Smith *et al.*, 2002). Therefore, the effects of m157 gene expression rely on Ly49 receptor expressed on NK cell surface, which varies in mouse strains. In C57BL-6 mice, Ly49H is expressed on about 50% of NK cell. The MCMV-

encoded protein m157 on the surface of infected cells can be recognized by the Ly49H receptor resulting in a MCMV-specific NK response (Arase *et al.*, 2002).

1.5.1.1 Humoral immune response:

In general, antiviral antibodies exhibit their protective activity either by direct neutralization or indirectly by complement activation and activation of antibody-dependent cellular cytotoxicity (Hangartner *et al.*, 2006). Therefore, antibodies against CMV may suppress the spread of virus either by direct virus neutralization or by cytotoxicity of cells carrying the virus. Experimental and clinical studies showed that humoral immunity to CMV plays an essential role in prevention of virus dissemination following reactivation from latency or reinfection (Jonjic *et al.*, 1994; Polic *et al.*, 1998). While viral envelope glycoproteins and tegument components are able to induce antibody responses, the envelope glycoprotein gB is considered to be the major target for neutralising antibodies (Landolfo *et al.*, 2003). A recent study showed that the antibody response to infected endothelial cells is potent, happens very early and is directed mainly to combinations of two or three gene products of the UL131A, UL130, UL128 genes. Whereas the antibody response elicited by infected fibroblasts appears late and relatively weak in potency and the neutralizing antibodies are directed against gH and gB (Revello & Gerna, 2010).

Despite the fact that maternal seropositivity against HCMV prior to pregnancy does not necessarily provide full protection against prenatal infection and disease (Boppana & Britt, 1995), congenital transmission of HCMV infection to the foetus is at a lower rate than in women with primary infections (Fowler *et al.*, 1992). Studies also showed that CMV-specific hyperimmune globulin treatment of pregnant women was effective in prevention of congenital CMV infection (Nigro *et al.*, 2005). In adult AIDS and transplant patients, the

presence of antiviral antibodies has been associated with slower progression of HCMV disease (Boppana & Britt, 1995; Schoppel *et al.*, 1998).

Mice immunized against MCMV gB were protected from a lethal dose challenge of MCMV (Rapp *et al*., 1992) and pregnant guinea pigs immunised with GPCMV gB showed reduced infection and disease of their foetuses (Schleiss *et al*., 2004). In the mouse, memory B-cell transfer offers long-term protection from lethal viral challenge in immune deficient animals (T-cell- and B-cell-deficient RAG-1(-/-) mice). Furthermore, transfer of memory B-cells was also effective in protection from an already on-going viral infection, which demonstrated the therapeutic importance of virus-specific memory B-cells while T-cells were not involved in this process (Klenovsek *et al.*, 2007).

1.5.1.2 Cellular immune response:

The evidence for the importance of cell-mediated immunity was provided by the observations that HCMV infection is most severe in patients with dramatically impaired cell-mediated immunity, such as bone marrow transplant recipients and patients with AIDS. The severity of CMV disease parallels the degree of T cell impairment in AIDS patients (Tamarit *et al.*, 2004). Early studies in the murine model showed that cytotoxic T lymphocytes (CTL) immunity played a central role in control of active viral disease and in reactivation from latency. Mice were protected from lethal challenge after adoptive transfer of virus specific $CD8^+$ cytotoxic T cell clones (specific for both structural and non-structural proteins) and this protection was independent of $CD4^+$ T helper cells (Reddehase *et al.*, 2004). In contrast, in humans adoptive transfer of protective $CD8^+$ cytotoxic T cells (with specificity for HCMV tegument proteins) was dependent on the presence of $CD4^+$ T helper cells (Hakki *et al.*, 2003). HCMV gene products of UL123 (IE-1), UL122 (IE-2), and

UL83 (pp65) are the most immunodominant antigens to which HCMV-specific $CD8^+$ T cells respond (Crough & Khanna, 2009).

In mice, depletion of $CD4^+$ T-lymphocytes resulted in an increase in the incidence of recurrent infection (Polic *et al.*, 1998). In humans, children with a persistent and selective deficiency of HCMV-specific $CD4^+$ T-cell immunity, when infected with HCMV usually have prolonged viral urinary and salivary virus shedding (Tu *et al.*, 2004). Furthermore, in lung transplant recipients, decreased levels of HCMV-specific $CD4^+$ T-cells resulted in higher susceptibility to infectious complications (Sester *et al.*, 2005).

Studies showed that a large proportion of $CD8^+$ and $CD4^+$ T-cells is dedicated to the anti-HCMV response. Therefore, up to 10% of $CD8^+$ and 9.1 % of $CD4^+$ circulating T-lymphocytes are HCMV-specific in healthy virus-carriers. In donors of different ages, the magnitude of the cellular immune response to virus pathogens such as influenza virus and varicella-zoster virus decreases with progressing age (Asanuma *et al.*, 2000; Deng *et al.*, 2004). In contrast, the cellular immune response (HCMV-specific $CD8^+$ T-lymphocytes) to HCMV may expand up to 40% in elderly individuals, causing what is called immune inflation (Khan *et al.*, 2002; Khan *et al.*, 2004a; Ouyang *et al.*, 2003). The HCMV-specific $CD4^+$ cell response is also increased in the elderly (Pourgheysari *et al.*, 2007). Consequently, this immune inflation and expansion of HCMV-specific $CD8^+$ T cells, in which a large fraction of the entire $CD8^+$ T cell subset may be directed to one single viral epitope, results in shrinking of the T cell stock available for responses to other antigens. Therefore, it may contribute to immune senescence, which characterize by a reduction in levels of naive cells, the accumulation of clonally expanded CD28 memory T cells, a decline in immune responsiveness and increases in the incidence of infectious disease in elderly people (Ouyang *et al.*, 2003; Pawelec *et al.*, 2005).

1.6 Cytomegalovirus Immune Evasion:

CMV is considered to be a classic example of viral immune evasion because of its distinctive and varied immune-modulatory strategies that enable the virus to avoid elimination by the immune system. Many CMV gene products are capable of modulating both innate and adaptive immune responses at different stages of the virus replication cycle. Antiviral interferon-induced genes were downregulated during cytomegalovirus infection. The HCMV IE86 protein inhibits IFN-β, TNF-α and other NF-κB- dependent cytokine and chemokine gene expression by blocking the binding of the transcription factor NF-κB to their promoters (Taylor & Bresnahan, 2005; 2006a; 2006b). Binding of the IE1 (IE72) protein to STAT1 (signal transducer and activator of transcription 2) and STAT2 resulting in formation of a physical complex in the nuclei if infected cells and *in vitro*, prevents association of STAT1, STAT2 and IFN regulatory factor 9 (IRF9) with the promoters of IFN-responsive genes *in vivo* (Paulus *et al.*, 2006).

In HCMV infection, an effective immune response is critically dependent upon the recognition of infected cells by CTL via presented antigenic peptides in complex with major histocompatibility complex (MHC) class I molecules (Groothuis *et al.*, 2005). One of the most important HCMV evasion mechanisms relies on the inhibition of MHC class I-restricted antigen presentation. Many HCMV genes are involved in inhibition of antigen presentation to cytolytic T cells by retention of MHC class I heavy chain/β-2 microglobulin complexes in the endoplasmic reticulum (US3) (Ahn *et al.*, 1996) or even destruction of MHC class I heavy chain molecules by interference with antigen presentation and by phosphorylation of a potentially important CTL target, the 72-kd immediate-early protein, preventing antigen processing (Gilbert *et al.*, 1996; Jones & Sun, 1997; Lehner *et al.*, 1997; Wiertz *et al.*, 1996). In addition, US2 genes are involved in degradation of MHC class II

proteins, which consequently prevent the presentation of antigen and the host $CD4^+$ T lymphocyte response to CMV (Tomazin *et al.*, 1999). The most surprising is the ability of CMV to activate or inhibit activatory and inhibitory receptors found on NK cells, NKT cells and T cells as well as auxiliary cells of the immune system. Passage of MCMV in mice lacking adaptive immunity but carrying the Cmv1r allele, which encodes the Ly49H activation receptor on NK cells, resulted in emergence of viruses carrying mutations in m157 ORF (encoding the only known ligand for Ly49H). These mutants were characterized by an increased virulence in naive Cmv1r mice which was attributed to the presence of single-amino-acid substitutions or premature stop codons (French *et al.*, 2004). Whereas the adjacent ORFs (m156 and m158) show no mutations which suggests that mutation emergence (and may be reversion) of mutation in both HCMV and MCMV occurs under selective pressure (Cheng *et al.*, 2010).

CMV's other immune evasion mechanisms are binding and sequestration of β-chemokines (US27, UL28, UL33) (Bodaghi *et al.*, 1998), competition with the TNF receptor superfamily through its viral homologue (UL144) (Benedict *et al.*, 1999), interference with apoptosis (Zhu *et al.*, 1995), down-regulation of MHC class II expression on the surface of macrophages (Fish *et al.*, 1996) and protection of infected cells from complement-mediated lysis (Spiller *et al.*, 1996).

1.6.1 Interference with the induction of interferons:

Interferons (IFNs) are a large family of multifunctional secreted proteins that play a role in the antiviral response, cell growth and regulation, and immune activation. IFNs induce the expression of many IFN-stimulated genes (ISGs) whose products limit the growth and replication of virus at different stages (Samuel, 2001). The IFNs are classified

into two types. Members of type I IFNs, IFN-α and IFN-β, are synthesized in response to viral infection. IFN-α is mainly produced by leukocytes, while IFN-β is produced by many cell types but especially by fibroblasts. IFN-γ represents the type II IFNs that are produced in response to virus infection, and synthesized by activated T lymphocytes and NK cells upon recognition of infected cells (Goodbourn *et al.*, 2000). The biological activity of IFNs is a result of binding of IFN-α/β and IFN-γ to their receptors on the cell surface. The result of this signalling is the activation or upregulation of transcription of IFN-dependent ISGs.

Binding of IFN-α/β to type I IFN receptors (IFNAR1 and IFNAR2) results in phosphorylation of tyrosine kinase 2 (Tyk2) and Janus kinase 1(JAK1) that then phosphorylate STAT2 and STAT1 respectively. Phosphorylation of STAT1 and STAT2 results in formation of STAT1/STAT2 heterodimers. The STAT1/STAT2 heterodimers then disassociate from the receptor and are translocated to the nucleus where they bind IRF9 to form the ISGF3 complex (IFN-stimulated gene factor 3) that accumulates in the nucleus. Binding of ISGF3 to IFN-stimulated response elements (ISREs) contained in the promoters of many ISGs leads to upregulation of their transcription (Shuai & Liu, 2003) (Figure 1.5). Most IFN-α/β inducible genes have a conserved upstream regulatory sequence AGTTTCNNTTCNC/T, while IFN-γ inducible genes have a unique constituent named as the gamma activation sequence (GAS) that contains the consensus sequence TTC/ANNNG/TAA.

The biological outcome of the activation of the IFN signalling pathways is the activation of the transcription of the target genes. Products of these genes have antiviral activity. Protein kinase R (PKR) and the 2'-5' oligoadenylate synthetases are two well-known IFN-inducible components that exert the antiviral response. Other factors, such as

molecules involved in the cell cycle or in cell death may also be induced. These factors limit viral replication. Usually these IFN-inducible enzymes are in an inactive form that can be activated when the cells are virally infected. It is thought that viral co-factors such as dsRNA can play a role in the IFN-inducible enzyme activation (Jacobs & Langland, 1996).

For viruses to replicate efficiently *in vivo*, they need to develop means to limit the production, or block the actions, of interferons (Goodbourn *et al.*, 2000). Cytomegalovirus is an example of viruses that have developed means to interfere with IFN-signalling pathways at several points. In HCMV infected cells, JAK1 levels are decreased compared to non-infected cells resulting in inhibition of IFN-α-stimulated signal transduction. In HCMV-Towne infected fibroblasts, levels of phosphorylated IFNAR-1, STAT2, STAT1α, and Tyk2 are all reduced, with JAK1 overall levels very impaired. Studies showed that JAK1 mRNA levels were constant during HCMV infection and JAK1 degradation is mediated by the proteasome. Consequently, loss of JAK1-mediated signal transduction, results in inhibition of both ISGF3-dependent and –independent gene expression. Furthermore, in HCMV infection the level of p48 is also reduced which will further disrupt the formation of the ISGF3 complex (Miller *et al.*, 1998; 1999). STAT protein function is another target of HCMV at different levels. During HCMV infection of fibroblasts, the STAT2 level is reduced due to proteasomal degradation (Le *et al.*, 2008).

The HCMV immediate-early 86 kDa protein (IE2-86) inhibits transcription of IFNβ by blocking NF-κB DNA-binding and NF-κB-induced gene expression (Taylor & Bresnahan, 2006a). In addition, HCMV has been shown to interfere with JAK/STAT signal transduction. HCMV IE 72 kDa protein interacts with STAT2 and hinders association of the activated ISGF3 complex with ISRE elements in the nucleus, thus preventing

upregulation of IFN-α/β gene stimulation (Paulus *et al.*, 2006). Rather than interfering with STAT2 protein levels, phosphorylation, or formation of the ISGF3 complex, IE72 acts after nuclear translocation of ISGF3. The acidic domain close to the carboxy-terminal end of IE72 is responsible for the interaction with STAT2, and studies have showed that this domain is essential for efficient viral growth especially in IFN-treated cells (Huh *et al.*, 2008).

Beside inhibition of IFN-γ-stimulated genes, which is linked to JAK1 degradation, HCMV interferes with IFN-γ signalling by impairing STAT1 phosphorylation. Upon IFN-γ signalling, levels of phosphorylated STAT1 in HCMV-infected cells are reduced as is activation of GAS-mediated transcription (Baron & Davignon, 2008).

The M27 protein product of MCMV binds to and degrades STAT2, and subsequently blocks both IFN type I and II signalling (Zimmermann *et al.*, 2005). In contrast, UL27, the HCMV homologue of M27, is not involved in the induction of STAT2 downregulation and is not required for viral growth in cell culture, suggesting that the HCMV mechanisms for STAT2 interference are UL27-independent (Le *et al.*, 2008).

Furthermore, HCMV was shown to interfere with ISG function, where HCMV proteins pTRS1 and pIRS1 block the 2'-5'oligoadenylate synthetase mediated eIF2α phosphorylation and decrease RNA degradation by RNase L (Child *et al.*, 2004; Zimmermann *et al.*, 2005).

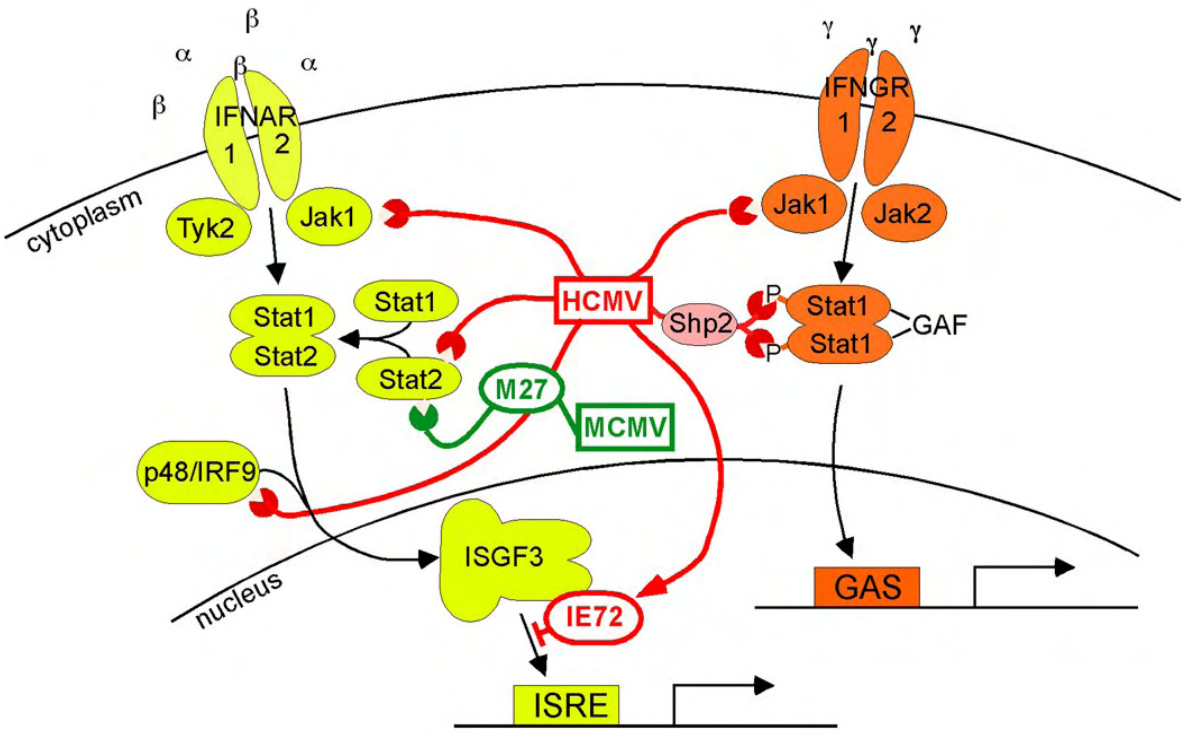


Figure 1.5: IFN-α/β and IFN-γ signalling pathways. IFNs bind to their respective receptors on cell surfaces and this results in activation of receptor-associated tyrosine kinase and consequent phosphorylation of STAT1 and STAT2. STAT1/STAT2 heterodimers are then translocated into the nucleus where they bind to IRF9 to form the ISGF3 complex that then binds to the ISREs contained in the promoters of many ISGs leading to their activation. HMCV (red) and MCMV (green) counteract these pathways at multiples steps as detailed in the text (Marshall & Geballe, 2009).

1.7 Control of apoptosis:

Another means of controlling viral infection of cells is programmed cell death (PCD) or apoptosis, which is a genetically regulated cell suicide process. A result of apoptosis is the elimination of damaged and infected cells that subsequently limits viral replication (Meier *et al.*, 2000). Cell morphology is changed during apoptosis, which involves cell volume reduction, chromatin condensation, membrane blebbing, and lastly the fragmentation of the cell into vesicles called apoptotic bodies (Kroemer *et al.*, 2009). In contrast, cell morphology in necrosis is characterized by enlargement of cytoplasmic organelles, moderate chromatin condensation, loss of membrane integrity, and escape of cellular components into the intercellular space (Festjens *et al.*, 2006; Kroemer *et al.*, 2009). In contrast to necrosis, apoptosis does not lead to the release of intracellular enzymes (Festjens *et al.*, 2006).

Apoptosis occurs in response to either extrinsic or intrinsic signals. Extrinsic signals, which may be induced by immune cells, result from activation of the tumour necrosis factor (TNF) superfamily receptor leading to the recruitment of Fas-associated death domain (FADD) which in turn results in recruitment and subsequently autocatalytic activation of initiator caspase-8. Intrinsic signals, which result from DNA damage, ER stress, or other stresses that alter mitochondria membrane permeability, lead to the release of cytochrome C and other death factors with subsequent activation of initiator caspase-9. The intrinsic and extrinsic pathways ultimately merge with activation of the downstream executioner caspase-3 that targets specific proteins (Barnhart *et al.*, 2003).

As a result of extrinsic or intrinsic pro-death signalling, apoptosis occurs as signalling cascades in which caspases (a group of aspartate-specific cysteine proteases) are

essential signal transducers and their activation is regulated by the Bcl-2 family. Based on the difference in Bcl-2 homology (BH) domains, Bcl-2 family proteins are divided into anti- and pro-apoptotic proteins. The anti-apoptotic Bcl-2 family proteins [which include BCL-2, BCL-w, BCL-xL (BCL-2 related gene, long isoform), A1 and MCL-1 (Myeloid cell leukaemia 1)] contain BH domains 1–4 and are usually integrated within the outer mitochondrial membrane. In the apoptotic pathway, these proteins directly bind and inhibit the pro-apoptotic BCL-2 proteins and prevent caspase activation resulting in preservation of mitochondrion integrity (Chipuk & Green, 2008; Youle & Strasser, 2008). Members of the pro-apoptotic family proteins are functionally divided into two classes; the effector molecules and the BH3-only proteins. The effector molecules [BAK (BCL-2 antagonist killer 1) and BAX (BCL-2 associated x protein)], contain BH1-3 domains and create the proteolipid pore in the outer mitochondrial membrane that increases its permeability and results in the release of cytochrome C (Leber *et al.*, 2007). The BH3-only proteins [which include BAD (BCL-2 antagonist of cell death), BID (BCL-2 interacting domain death agonist), BIK (BCL-2 interacting killer), BIM (BCL-2 interacting mediator of cell death), BMF (BCL-2 modifying factor), bNIP3 (BCL-2/adenovirus E1B 19-KD protein-interacting protein 3), HRK (Harakiri), Noxa and PUMA (p53-upregulated modulator of apoptosis)] act in different cellular stress pathways and, by protein–protein interactions with other BCL-2 family members (i.e. anti-apoptotic BCL-2 proteins and/or the effector molecules), signal that a cellular stress has occurred. The collective signalling within the BCL-2 family determine whether or not to permeabilize the mitochondrial outer membrane and consequently the fate of the affected cell (Chipuk & Green, 2008). Many cellular organelles such as the nucleus, Golgi apparatus, endoplasmic reticulum, and the lysosome have sensors that can promote death through apoptosis (Ferri & Kroemer, 2001).

Cytomegalovirus has many genes that encode pro-survival or anti-apoptotic factors that function by different mechanisms at different levels to prevent apoptosis and ensure that the cell remains alive and metabolically active (Figure 1.6). The HCMV UL36 protein blocks the extrinsic apoptotic pathway by binding to procaspase-8 [called viral inhibitor of caspase-8-induced apoptosis (vICA)] and a recent study has shown that UL36 can also block a caspase-independent cell death pathway. Although, viral replication of vICA-deficient viruses (HCMV and MCMV) was not affected, the ability of the virus to replicate in macrophages was massively impaired and apoptosis was induced (McCormick *et al.*, 2010). In MCMV, deletion of M36, the homologue of UL36, resulted in attenuation of virus growth in macrophages *in-vitro* and replication of the mutant was severely impaired in mice and produced more apoptotic cells (Cicin-Sain *et al.*, 2008; Menard *et al.*, 2003). The UL37×1 gene encodes a viral mitochondria-localized inhibitor of apoptosis (vMIA) that is analogous to cellular Bcl2 proteins and is effective against many extrinsic and intrinsic stimuli (Goldmacher, 2005). Furthermore, the HCMV UL38 protein suppresses apoptosis, and prevents premature death of host cells to promote efficient virus replication (Terhune *et al.*, 2007).

In MCMV beside M36, there are products of at least three genes, M45, m38.5, and m41.1, that are essential in prevention of infection-induced cell death in macrophages (Brune *et al.*, 2001; Cam *et al.*, 2010; Manzur *et al.*, 2009). The MCMV M45 protein plays a role in preventing premature death of endothelial cells during murine cytomegalovirus infection by inhibiting receptor-interacting protein (RIP) activation and RIP1/RIP3-dependent programmed necrosis (Upton *et al.*, 2008). The M38.5 ORF encodes a Bax-specific vMIA and protects virally infected leukocytes from apoptosis, whereas the m41.1 ORF encodes a small mitochondrion-localized protein that functions as a viral inhibitor of

Bak oligomerization (vIBO). It prevents Bak-mediated cytochrome C release and Bak-dependent induction of apoptosis (Cam *et al.*, 2010; Manzur *et al.*, 2009).

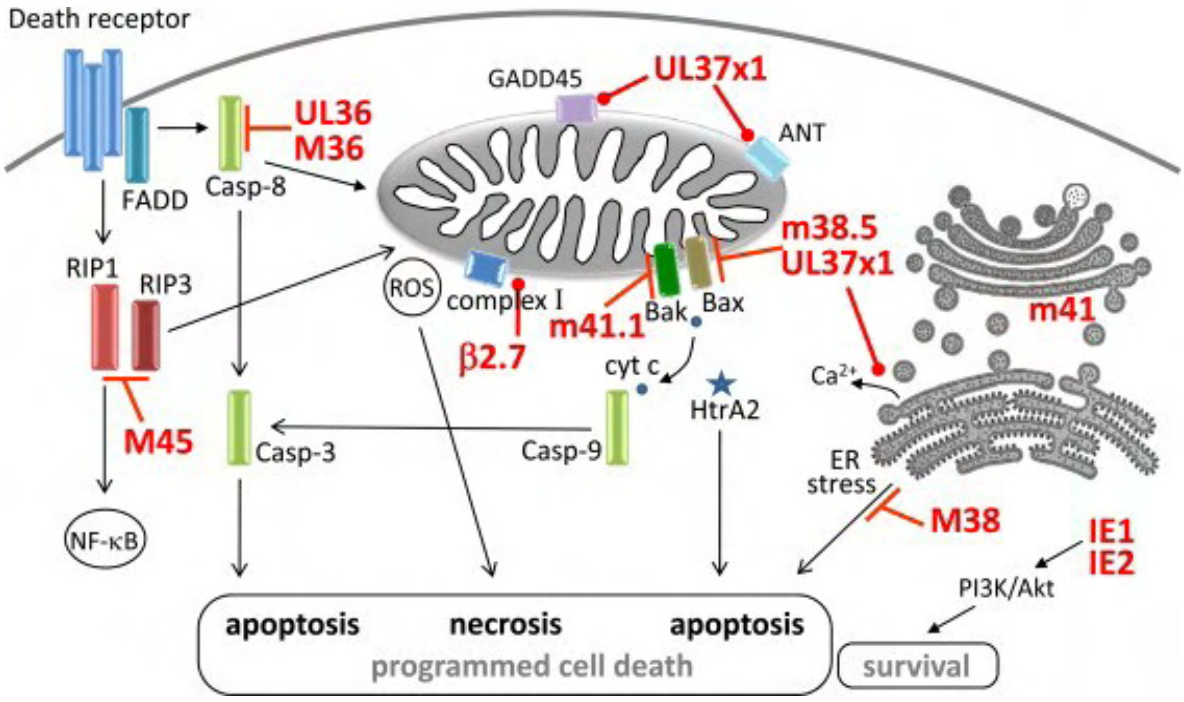


Figure 1.6: HCMV and MCMV cell death inhibitors, and their cellular target proteins (Brune, 2011)

1.8 Latency and reactivation:

CMVs, like other herpesviruses are able to establish a lifelong persistent infection where the viral genome is maintained in the host cells with limited gene expression and no infectious virions are produced. The viral genome is able to reactivate from latency, which occurs routinely in healthy virus carriers and is usually contained by the healthy immune system. In immunosuppressed patients, HCMV reactivation from latency is very serious and can result in morbidity and mortality (Rubin, 1990). Clinical studies revealed that using leukocyte depleted blood drastically reduced HCMV transmission from seropositive blood donor to healthy recipients, and leads to the suggestion that latent HCMV can be carried in peripheral blood leukocytes of healthy carriers (de Graan-Hentzen *et al.*, 1989). Using highly sensitive techniques like PCR has unveiled that peripheral blood monocytes are sites for virus latency (Taylor-Wiedeman *et al.*, 1991). Later studies showed that progenitor $CD34^+$ cells in bone marrow carry the virus (Mendelson *et al.*, 1996). $CD34^+$ cells are progenitors of many cell lineages such as T and B lymphocytes, polymorphonuclear phagocytes and monocytes. Use of reverse transcription PCR (RT-PCR) showed that among these cell types, only $CD34^+$ progenitor cells and their derivatives, monocytes, are the naturally latent cells which are not associated with lytic infection (Mendelson *et al.*, 1996; Taylor-Wiedeman *et al.*, 1994). *In vitro* studies showed that induction of differentiation of naturally latent peripheral blood monocytes (PBMs) using granulocyte colony stimulating factor and hydrocortisone resulted in reactivation of viral IE and E gene expression (Taylor-Wiedeman *et al.*, 1994). Later studies showed that monocytes are true latent cells and non-permissive for lytic infection; differentiation of $CD34^+$ cells and monocytes to mature dendritic cells reactivated viral lytic gene expression and resulted in infectious virus production (Reeves *et al.*, 2005).

Recent studies suggest that HCMV latency results from an intrinsic cellular anti-viral response in which viral gene expression of the incoming genome is silenced via chromatinisation. Histone proteins are recruited to the MIEP of the HCMV genome and are then subjected to post-translational modifications to form a repressive chromatin structure preventing transcriptional activity (Reeves & Sinclair, 2010; Sinclair, 2010). Inflammatory and/or differentiation signals encourage chromatin remodelling of the MIEP to a transcriptionally active state that results in reactivation of viral lytic IE gene expression and production of infectious virus (Reeves & Sinclair, 2010; Sinclair, 2010).

1.9 Antiviral treatment of CMV

For the treatment of HCMV disease, five drugs, ganciclovir/valganciclovir, cidofovir, foscarnet and fomivirsen have been approved so far (Schreiber *et al.*, 2009). Apart from fomivirsen, all of the above mentioned drugs share the same target molecule, the viral DNA polymerase. For prevention and control of CMV disease in immunocompromised patients, the use of these drugs has been shown to reduce or eliminate viraemia.

In solid organ transplant recipients, ganciclovir (GCV) is the first-line treatment for HCMV infection and disease (Razonable & Emery, 2004). GCV is a purine analogue which is phosphorylated first by a viral protein (protein kinase encoded by the HCMV UL97 gene) and then cellular enzymes to ganciclovir triphosphate, the chemically active form (Sullivan *et al.*, 1992). During DNA synthesis, which is catalysed by the viral DNA polymerase (encoded by the UL54 gene), the ganciclovir triphosphate competes with deoxyguanosine triphosphate (dGTP) and consequently terminates viral DNA elongation (Gilbert & Boivin, 2005). Prolonged HCMV antiviral therapy of AIDS patients using GCV

lead to the emergence of resistance to ganciclovir due to UL97 and UL54 gene mutations (Foulongne *et al.*, 2004). The side effects of GCV treatment include haematological disorders and it can result in reproductive toxicity (Markham & Faulds, 1994). Valganciclovir is the L-valyl ester of ganciclovir. Following oral administration, it is rapidly metabolised to the active form (GCV) in the intestinal wall and liver. Initially valganciclovir was approved for treatment of HCMV retinitis in AIDS patients then later for HCMV prophylactic treatment of solid organ transplant recipients (Cvetkovic & Wellington, 2005).

Cidofovir is an acyclic nucleoside phosphonate that has a broad antiviral spectrum with high potency against herpesviruses as well as other DNA viruses. After administration, host kinases convert cidofovir to the active diphosphoryl form which is a competitive inhibitor of the viral DNA polymerase and results in termination of elongation of viral DNA during DNA synthesis (De Clercq E., 2001). In 1996, cidofovir was approved for treatment of HCMV retinitis in AIDS patients. The side effect of the use of cidofovir is renal toxicity and neutropenia (Ho *et al.*, 2000).

Foscarnet sodium (trisodium salt of phosphormophonic acid) is an inhibitor of DNA polymerase (UL54) activity via binding to the pyrophosphate binding site and blocking cleavage of pyrophosphate from the terminal nucleoside triphosphate added to the extending DNA chain (De Clercq E., 2001). Resistance to foscarnet emerged due to UL54 mutations. Foscarnet's major side effects are the renal impairment that can lead to cardiac and neurological disorders due to electrolyte imbalance.

Fomivirsen is a 21 nucleotide anti-sense RNA targeted against the HCMV IE2 mRNA. It was approved for clinical use in 1998 as a second-line therapy for local treatment of HCMV retinitis administered by intraocular injection (De Clercq E., 2001).

1.10 Vaccination against HCMV

All HCMV antiviral drugs available for clinical use suffer from complications with prolonged use, including drug toxicity, carcinogenicity and teratogenicity as well as drug resistant strain emergence. Because of limitations of use and side effects of HCMV antiviral drugs, many HCMV vaccines have been evaluated in clinical studies. A variety of approaches has been used to produce HCMV vaccines, which can be divided into two major groups: live attenuated vaccines and subunit vaccines that target specific proteins (Adler *et al.*, 1995; Pass, 2009; Pass *et al.*, 2009).

1.10.1 Live attenuated HCMV vaccine

For more than 40 years, scientists and researchers have attempted to develop a live attenuated HCMV vaccine. The laboratory adapted HCMV AD169 strain was the first HCMV vaccine tested in humans followed by another laboratory-adapted clinical isolate, the HCMV Towne strain. Live attenuated vaccines were able to stimulate both humoral and cellular immune responses. HCMV Towne vaccination of renal transplant recipients failed to prevent HCMV infection after transplantation, but it protected against the development of HCMV disease (Plotkin *et al.*, 1994). Also, the Towne vaccine failed to protect seronegative mothers from acquiring HCMV infection from their HCMV-infected children, but the vaccine protected seropositive women against acquiring a new strain of HCMV from their children (Adler *et al.*, 1995). The relative failure of the Towne vaccine was attributed to inadequate antigen-specific IFN-γ responses by $CD4^+$ and $CD8^+$ cells post

vaccination. Co-administration of recombinant IL-12 with the Towne vaccine improved its immunogenicity, revealed by the elevated levels of both HCMV-specific humoral and cellular responses after vaccination (Jacobson *et al.*, 2006).

Another strategy to improve the immunogenicity of the Towne vaccine was to generate a series of genetic recombinant viruses from the genomes of the Towne strain and the un-attenuated Toledo strain. Four chimaeric vaccine viruses were produced and tested in a double-blinded, placebo-controlled study. Post vaccination assessment revealed that the vaccines were well tolerated and no virus shedding was detected by viral and PCR analyses of blood and body fluids (Heineman *et al.*, 2006).

1.10.2 HCMV subunit vaccines

In this approach, one or combinations of immunogenic viral proteins are used as a subunit vaccine to protect against HCMV infection. The envelope glycoprotein gB, pp65 and IE1 vaccines are in clinical or preclinical trials. A recombinant gB molecule expressed in Chinese hamster ovary cell culture was used as a subunit vaccine combined with MF59 adjuvant was shown to be safe, immunogenic, and produced high levels of gB-specific antibodies and total virus-neutralising activity in several clinical trials. This vaccine trial showed that the gB vaccine had the potential to reduce the incidence of cases of maternal and congenital HCMV infection (Pass, 2009; Pass *et al.*, 2009).

In another approach the extracellular domain of gB was covalently linked to multiple Human leukocyte antigen (HLA) class I and class II-restricted T- cell epitopes from multiple HCMV antigens as a contiguous polypeptide in a replication deficient adenoviral vector Ad5/F35. Immunisation using this vaccine produced strong gB- specific

neutralizing antibody and a wide range of HCMV- specific pluripotent $CD8^+$ and $CD4^+$ cells (Zhong & Khanna, 2009).

Similar strategies were used for generation of pp65 and IE1-based vaccines. A combination of three immunodominant viral proteins [Soluble (s) UL55 (surface glycoprotein), UL83 (tegument protein) and UL123/e4 (nuclear protein)] were also used as a subunit vaccine (Wang *et al.*, 2006).

Other vaccine strategies include DNA immunisation with viral glycoproteins (gN, gM, gH, gO, gL), non-structural proteins [DNA polymerase (UL54) or helicase (UL105)] (Morello *et al.*, 2007) or structural, early/late proteins (pp28, pp50, US2, US3, US6 and UL18) and using dense bodies and peptide vaccines. Bacterial artificial chromosome (BAC) cloned CMV viral genomes allowed the generation of genetically modified viruses which have been shown to induce protective immunity in mouse and guinea pig models (Redwood *et al.*, 2005).

1.10.3 Murine Cytomegalovirus:

Studies of the pathogenesis of human cytomegalovirus are limited because of its restricted host specificity and the fact that there are no suitable animal models for studying HCMV infection *in vivo* for growth and disease characteristics. Studies of HCMV have therefore been limited to tissue culture cells of human origin. Thus, murine cytomegalovirus in the mouse, as well other rodent cytomegaloviruses in their respective hosts, have been used as models for studying pathogenesis, tissue tropism, persistent infection, and latency (Abenes *et al.*, 2001). The high similarity of HCMV and MCMV in genome organization, temporal regulation of gene expression, and amino acid sequence of the predicted protein products of many genes makes the latter an ideal model (Rawlinson *et*

al., 1996). Therefore, understanding of the biology of MCMV and the function of its genes is a key to understanding the pathogenesis of HCMV.

Infection of mice with MCMV is similar to HCMV infection where there are three stages in MCMV infection. Acute infection of central visceral organs is rapidly controlled but is followed by persistent infection of the salivary gland for several weeks. Lastly, it enters the latent phase for the life of the host (Lu *et al.*, 2006). In the immunocompromised or immunologically immature mouse, MCMV can result in severe infections whereas in immunocompetent mice it can be re-activated from latency by immunosuppression, which results in similar clinical syndromes (Sweet, 1999). Furthermore, MCMV has been widely used for experimental vaccine development (Morley *et al.*, 2002) and used as a model for CMV antiviral resistance studies (Scott *et al.*, 2005).

1.11 Mutagenesis:

To understand the role of virus genes in pathogenesis and replication many researchers have used the mutagenesis approach in which the effect of gene mutation on virus phenotype is studied (Abenes *et al.*, 2001). Reliable and rapid mutagenesis methods are required for analysing CMV gene functions during the disease course *in vivo*. CMV replicates slowly and since mutagenesis approaches depend on rare recombination events in eukaryotic cells, many mutagenesis approaches are considered to be difficult, laborious, and time-consuming (Kemble *et al.*, 1996). Chemical mutagenesis was used in herpesvirus studies more than thirty years ago for the generation of temperature-sensitive mutants resulting from random point mutations, insertions or deletions. Despite the large number of mutants generated by this technique (from a single treatment), identification of the mutation(s) responsible for an observed phenotype was difficult (Sweet *et al.*, 2007).

Creation of mutated virus with mutations in specific (targeted) genes, followed by studying their phenotype in tissue culture and in animal models has greatly improved our understanding of CMV pathogenesis. This approach has helped in mapping the genome of herpesvirus in relation to specific functions, such as the genes concerned in replication, tropism, virulence, and other biological phenomena such as viral immune evasion.

Generation of herpesvirus mutants was first reported using site-directed homologous recombination and transposon-mediated insertional mutagenesis. Transposon mutant libraries helped to classify all known genes of HCMV with regards to their impact on the *in-vitro* growth of virus (Yu *et al.*, 2003). Other approaches have used overlapping cosmid DNA fragments to generate mutants of HCMV and other herpesviruses (Kemble *et al.*, 1996). The main advantage of this approach is that only recombinant virus is produced

obviating selection against non-recombinant wild type virus. Nevertheless, it remains that the produced mutant is the product of many recombination events in eukaryotic cells that are not easy to control. Confirmation of correct reconstitution of the viral genome can be achieved after growth and isolation of the generated mutant virus (Ruzsics & Koszinowski, 2008).

1.11.1 BAC Technology:

A recent advance in molecular microbiology allowed the cloning of foreign DNA larger than 300 kbp in size, in a fertility factor (F-factor) replicon based bacterial artificial chromosome (BAC) that can maintain the cloned DNA with an incredible sequence stability if propagated in suitable strains of *E.coli* such as DH10B and its derivatives (Shizuya *et al.*, 1992). Since then most herpesvirus (including HCMV and MCMV) genomes have been cloned as BACs (Messerle *et al.*, 1997; Redwood *et al.*, 2005).

The CMV BAC is generated using a recombinant plasmid containing the BAC cassette flanked by viral DNA sequences homologous to the target insertion site in the viral genome that is transfected (or electroporated) into eukaryotic cells previously infected (~2 hrs) with virus. Homologous recombination (HR) between the BAC cassette and virus DNA results in insertion of the BAC vector (~8 kbp) into the CMV genome (Redwood *et al.*, 2005). Since herpesviruses can tolerate only up to ~5 kbp of extra DNA in their genomes, insertion of the BAC vector requires deletion of part of the herpesvirus genome but this can be repaired later (Messerle *et al.*, 1997; Redwood *et al.*, 2005). The BAC vector contains *gpt* (guanosine phosphoribosyl transferase) and *gfp* (green fluorescent protein) gene, which serve as selection and screening markers respectively. In the presence of mycophenolic acid and xanthine in the medium, the *gpt* gene allows the selection of

recombinant viruses containing the BAC. While mycophenolic acid blocks the synthesis of purine by cells, replication of recombinant BAC-containing viruses is supported by the *E. coli gpt* enzyme that is able to utilize xanthine to synthesize and provide purine. In the infected cell, the linear, double-stranded DNA genome of herpesviruses circularises during replication, this circular viral DNA can be isolated and transferred to a suitable *E. coli* strain by DNA transformation. Once the BAC is transferred to bacteria, it can be repaired and the segment lost during insertion of the BAC can be reintroduced into the genome; this results in flanking of the BAC cassette by repeat sequences. Transfection of the virus BAC into permissive eukaryotic cells allows homologous recombination between the repeat sequences, which leads to reconstitution of viral progeny and excision of the BAC cassette from the herpesvirus genome (Adler *et al.*, 2003; Wagner *et al.*, 1999).

Use of the CMV-BAC containing the whole sequence of the wild-type virus in *E. coli* facilitates the manipulation and introduction of any kind of mutation (including deletions, insertions, and point mutations) into the genome in a manner independent of the biological properties of the mutant virus and the recombinant genome can be characterized and controlled prior to reconstitution of viral progeny. Mutated virus progeny are then generated after transfection of permissive cells that support virus production with the MCMV BAC plasmid. The BAC-based mutagenesis approach allows detailed studies of viral gene functions both *in vitro* and *in vivo* because it provides a powerful and handy approach to generate viral mutants (Wagner *et al.*, 1999).

1.11.2 BAC targeted mutagenesis by Red-mediated recombination:

Homologous recombination is important for many cellular processes, such as maintenance of genomic integrity, appropriate segregation of chromosomes in meiosis, and the rescue of stalled replication forks. It provides a means for repair of DNA double-

stranded breaks, which occur during DNA replication, or DNA damage caused by external factors such as irradiation (Muyrers *et al.*, 2000).

Researchers have utilized homologous recombination as an insertional mutagenesis technique for disruption and deletion of CMV genes (Spaete & Mocarski, 1987). In eukaryotic cells, the frequency of homologous recombination is low, and to some extent, this reduces the effectiveness of this approach in eukaryotic cells. Also, unwanted deletions or the creation of undesired recombinant viruses has been detected (Spaete & Mocarski, 1987; Vieira *et al.*, 1994). Even though selection procedures have been used to develop the original technique, generation of CMV mutants remains a laborious, time-consuming, and often unsuccessful task (Messerle *et al.*, 1997).

Homologous recombination in prokaryotic cells presents another way to manipulate DNA, and many approaches have been used in *E. coli* including recombination between independent replicons, for example, between a plasmid with a conditional replication origin and the *E. coli* genome, or a BAC (Yang *et al.*, 1997).

Recombination-mediated genetic engineering (recombineering) using the three γ Red-encoded genes, allowed the introduction of mutations into the cloned BAC genome. The use of this powerful recombination reaction has encouraged the development of the BAC-based mutagenesis approach for studying viral gene functions in tissue culture and in laboratory animal models (Brune *et al.*, 2001). The γ Red-encoded genes; *exo*, *bet*, and *gam* can mediate recombination of 30-50 nucleotide homologous regions therefore allowing insertion of a linear DNA fragment carrying the desired mutation at any site of the BAC plasmid (Ruzsics & Koszinowski, 2008). *Exo* (*redα*) encodes a 5'-3'exonuclease that produces 3'overhangs from introduced double stranded DNA targeting cassettes (dsDNA).

Bet (*redβ*), is a pairing protein that binds to the 3'overhangs and facilitates its annealing and homologous recombination with complementary DNA present on the BAC (Figure 1.7). At the same time, *gam* (the inhibitor of *E. coli* RecBCD exonuclease) protects the linear DNA-targeting cassette from degradation by RecBCD. γ Red (or the corresponding RecE and RecT genes of the prophage Rac) can be expressed from a multicopy plasmid via an inducible promoter (such as pKD46 where RecA/T are expressed under the control of L-arabinose) (Muyrers *et al.*, 2000).

An alternative method has been used recently (as in *E. coli* SW102) in which *exo*, *bet* and *gam* are expressed from a stably integrated defective λ prophage, and controlled by the strong phage promoter *p*L, under strict control of the temperature-sensitive repressor, *cI857* (Lee *et al.*, 2001; Yu *et al.*, 2000a). In this system, when the bacteria are grown at 32ºC, *exo*, *bet* and *gam* are not expressed, but they are rapidly induced to very high levels when the incubation temperature is shifted to 42ºC for as little as 15 min with a very efficient homologous recombination (Warming *et al.*, 2005).

In the first step of this technique, a linear DNA fragment comprising a selectable marker (such as an antibiotic resistance marker or using *galK* positive/negative selection) flanked by an homologous sequence of the target site in the BAC, is introduced into the BAC. In the second step, a linear DNA fragment containing the desired mutation replaces the selection marker (Figure 2.4). Suitable permissive cells are then transfected with the viral BAC to yield the recombinant virus containing the desired mutation (Muyrers *et al.*, 2000).

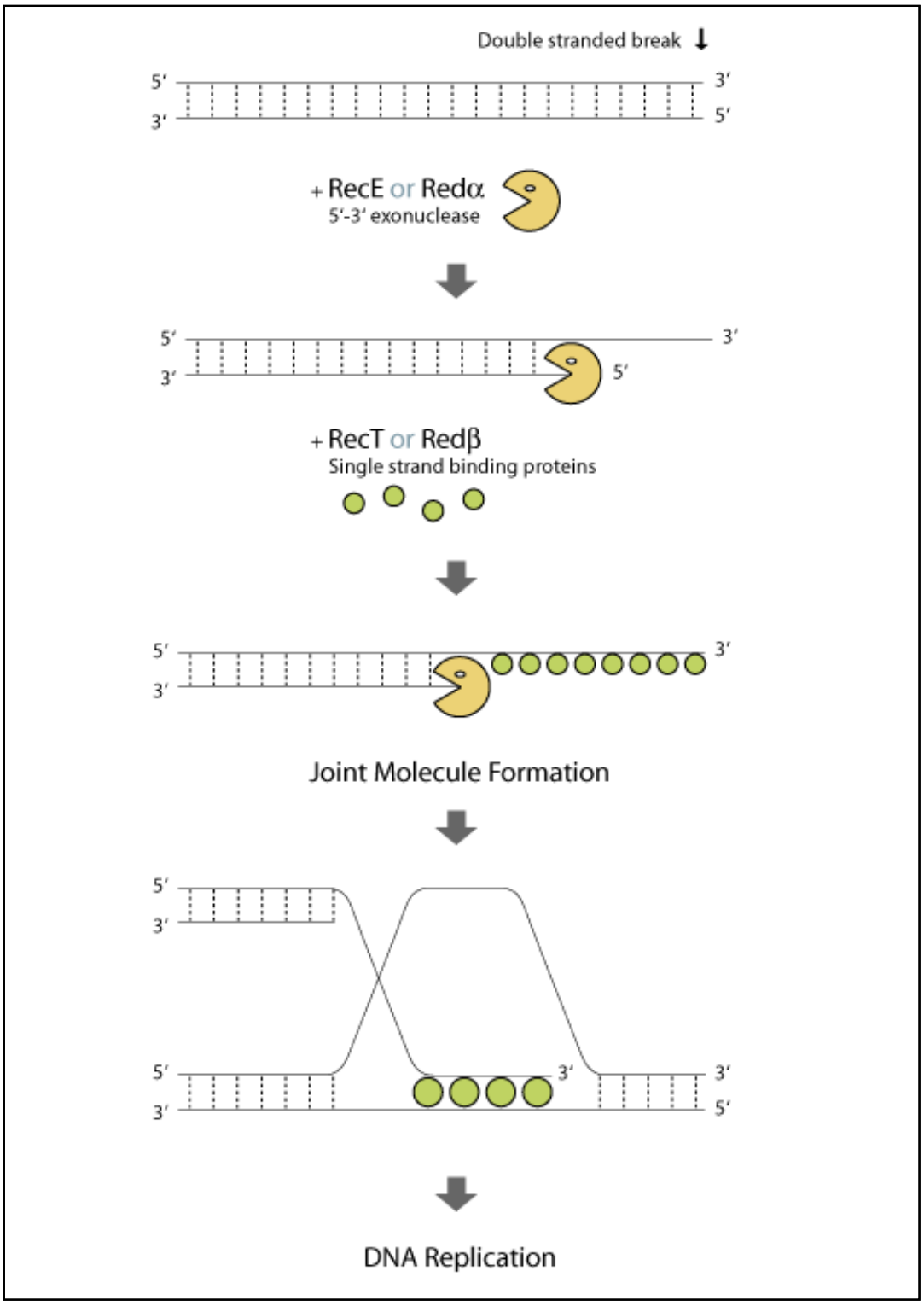


Figure 1.7: Mechanism of Red/ET recombination. During the process of BAC replication, recombinase protein pairs (RecE/RecT or Redα/Redβ) starts double-stranded break repair. In this process RecE (Redα) digests one strand of the DNA from the double-stranded break, leaving the other strand as a 3` hanging strand. RecT (Redβ) recognises and coats the DNA overhang to form the protein-nucleic acid filament aligning with homologous DNA. Then the 3` end acts as primer for DNA replication.

1.12 Background of the study:

MCMV is used as a model for HCMV in studying genes involved in pathogenicity. Generation of MCMV with mutations in genes allows the analysis of the phenotype of generated mutant viruses *in vitro* and *in vivo* to help in identification of virulence determinants (Akel *et al.*, 1993).

In our laboratory, 31 temperature-sensitive mutants of the MCMV-K181 (Birmingham) strain were generated using chemical mutagenesis (N-methyl-N-nitro-N-nitrosoguanidine) and selection for mutants restricted in their growth at the non-permissive temperature for replication at 39°C and above (Akel *et al.*, 1993; Akel & Sweet, 1993; Sammons & Sweet, 1989). One of these temperature sensitive mutants, *tsm5*, demonstrated an interesting phenotype, where it was able to express immediate-early, early and late phase genes in the tissues of infected BALB/c mice, but failed to produce infectious progeny virus at any time point from 1–21 days post infection and was not reactivated from latency following immunosuppression with cortisone acetate and anti-lymphocyte serum (Bevan *et al.*, 1996). *Tsm5* was able to protect mice against subsequent sublethal wild-type challenge when inoculated with as little as 4 pfu of *tsm5* where no virus could be isolated from any tissue at 3-42 days after challenge (Morley *et al.*, 2002). This suggested that *tsm5* was replicating at undetectable levels, which can be reflected by induction of high titre neutralizing antibody and a $CD8^+$ CTL response. In addition, an electron microscope investigation of *tsm5* infected MEF cells grown at the non-permissive temperature revealed an accumulation of *tsm5* virus capsids devoid of DNA, which suggests an inability of *tsm5* to package viral DNA into pre-formed capsids (Morley *et al.*, 2002). To investigate the regions of *tsm5* contributing to this phenotype, recombinant chimaeric viruses were generated using a *tsm5* cosmid library and a Smith MCMV cosmid library. One of the

recombinant viruses (Smith/*tsm5*DGIK) was designed to contain the central part of *tsm5* and the 3' and 5' ends of Smith virus genome. This virus was temperature sensitive although not to the same extent as *tsm5* suggesting that a temperature sensitive gene (s) is located in the central region of the chimaeric virus encoded by *tsm5* genes. This region contains herpes virus conserved genes involved in DNA synthesis, packaging and cleavage. These genes were sequenced in *tsm5* and mutations found in M56 (G439R), M70 (C890Y) and M98 (P324S). The recombinant virus now replicated in the salivary glands of immunocompetent adult mice at levels similar to those of the *wt* Smith virus. From this observation it was concluded that mutations in the *tsm5* genomic region that had been replaced by the Smith genome (i.e. ORFs m01 to M54 and/or m145 to m170), were important in the inability of *tsm5* to replicate in immunocompetent mice. Some of these genes (m04, m06, m144, m152, m157) are known to interfere with MHC peptide presentation, NK cell killing or recognition and thus are called immune-evasion proteins (Reddehase *et al.*, 2004).

Other genes such as M27 and M33 interfere with cell signalling, while genes M36, M37 and m38.5 inhibit apoptosis. These genes were also sequenced in *tsm5* and revealed that there were no mutations present in ORFs m04, m06, M33, M37, m38.5, m144, m152, or m157 but genes M27 and M36 each contained a single point mutation resulting in amino acid changes (Sweet *et al.*, 2007).

1.12.1 Aim of the Study:

In this study, the aim is to investigate the role of the mutations identified in ORFs M27 and M36 of *tsm5* in the observed *in vitro* and *in vivo* phenotype of *tsm5*. The mutation in the *tsm5* M27 gene was characterized at nucleotide position 32,324, where an adenosine

substitution for cytosine results in replacement of an alanine with a serine at residue 658 (A658S). The mutation in the M36 ORF resulted in an isoleucine substitution for valine at residue 54 (V54I) resulting from a cytosine mutation to a thymine, at nucleotide position 49,108 (Sweet *et al.*, 2007). These mutations will be introduced into the wild type K181 virus BAC-DNA, both individually and together, using RecE/RecT homologous recombination. Such mutations and their revertants, in which the mutations have been repaired, will subsequently be used to study of the role of each mutation *in vitro*.

Furthermore, in this study, a comparative genome sequencing (CGS) approach will be used to analyze both the MCMV-K181 (Birmingham) strain and *tsm5* genome to identify all possible differences of MCMV-K181 (Birmingham) from the published MCMV K181 (Perth) sequence and any further mutations in *tsm5* that may be involved in generation of the *tsm5* phenotype.

Chapter 2: **Materials and methods**

2.1 Maintenance of cell lines

All *in vitro* cell culture work was carried out in sterile conditions in a Class II biological safety cabinet. The cells were incubated at either the permissive (37°C) or non-permissive (40°C) temperature using a Sanyo CO_2 Incubator in a humid atmosphere supplemented with 6% CO_2.

2.1.1 Mouse embryo fibroblasts

2.1.1.1 NIH 3T3 cells

The immortalized mouse embryo fibroblast cell line, NIH 3T3 (ATCC CRL-1658), was cultured in growth medium (GM1) comprising Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Dorset, UK) supplemented with 10% (v/v) newborn calf serum (NCS) (Sigma-Aldrich), 4mM L-glutamine (Invitrogen, Paisley, UK) and 200 U/ml penicillin and 200 μg/ml streptomycin (Invitrogen).

2.1.1.2 Primary mouse embryo fibroblasts

Primary mouse embryo fibroblasts (MEF) were isolated from the foetuses of 14-day-old BALB/c mice. Between 10 and 24 embryonic sacs were removed from the uterine horns of the mouse and placed in a Petri dish in 10 ml of phosphate buffered saline (PBS) (Oxoid, Hampshire, England). Each embryonic sac was opened and the embryos were released into the Petri dish. The placenta, membranes and foetal visceral tissues were discarded and the embryos placed in a clean Petri dish and washed with 10 ml of PBS. The embryos were subsequently forced through a 20 ml syringe and 10 ml of trypsin: EDTA (0.5% v/v

trypsin; 5.3 mM EDTA) (Invitrogen) was added to the cells which were then well shaken. The mixture was then incubated for 10 minutes at 37°C. The cell suspension was vigorously shaken and filtered through a fine metal sieve to extract cellular debris. The sieving should take only a couple of minutes as prolonged sieving time reduces viability and attachment of the cells due to over-exposure to trypsin. Two ml NCS and 10 ml GM1 were added and all the homogenate transferred to a centrifuge tube. After centrifugation at 720 x g for 10 minutes at room temperature (RT), the pellet was re-suspended in 10 ml GM1. The cells from the equivalent of three embryos were seeded into a 162 cm^2 tissue culture flask containing 15 ml GM1 and incubated at 37°C in 94% air/6% CO_2 until confluent. Cells were then passaged as described in section 2.1.1.3 or seeded as required for the experiments. MEF cells were used within 3 passages, after which time the attachment and growth properties of the cells were poor.

2.1.1.3 Subculturing of cells

Cells were subcultured when 80-95% confluent. Growth medium was removed from the 162 cm^2 tissue culture flask, the cells washed with 10 ml of phosphate buffered saline (PBS) followed by a rinse with 2ml of 10x trypsin:EDTA (TE). Incubation of the flask proceeded at 37°C for 1 minute. Detachment of the cells from the flask surface was monitored using an inverted microscope, after that the cells were resuspended in 10 ml of GM1. The cells were seeded into a 162 cm^2 tissue culture flask containing 20-40 ml GM1 at a split ratio of 1:10, cultured at 37°C until 80-90% confluent and passaged further as required.

2.1.1.4 Long term storage of NIH 3T3 cells (Cryopreservation)

Healthy cell cultures, free from contamination, were used for preparation for stock cells. Cells were maintained in log phase growth, in 162 cm^2 tissue culture flasks, for several days. Cell were then trypsinised as described above (section 2.1.1.3) and harvested by centrifugation at 850 x g for 10 minutes at room temperature. The cell pellet was resuspended in GM1 supplemented with 10% (v/v) dimethyl sulphoxide (DMSO) (Sigma-Aldrich). The cell concentration was adjusted to be 2×10^5-5×10^6 cells/ ml/cryovial. Cryovials were placed in a freezing vessel containing isopropanol and subsequently cooled in a -80°C freezer. Twenty four hours later, the cryovials were moved to a liquid nitrogen-filled storage vessel for long-term storage.

2.1.1.5 Resuscitation of NIH 3T3 cells

A cryovial containing the NIH3T3 cells stored in liquid nitrogen was thawed in a 37°C water bath with constant moderate agitation until ice in the cryovial was no longer visible. The cryovial was then warmed in the water bath for 30 seconds with gentle agitation and disinfected with 70% ethanol. Working in a class II safety cabinet, thawed cells were transferred to 1.5 ml pre-warmed GM1 (37°C) and allowed to stand at room temperature for five minutes. Three ml of pre-warmed GM1 were added and allowed to stand for another five minutes at room temperature. Six ml of pre-warmed GM1 were then added to the cells and allowed to stand for a further five minutes. The cells were centrifuged at 200 x g for 10 minutes at room temperature. The supernatant was discarded and the cell pellet resuspended in 25 ml fresh GM1 and seeded into a 75 cm^2 tissue culture flask. The cells were then incubated in a 37°C incubator (94% air /6% CO_2) for several days and monitored daily until 80-90% confluent. Cells were then passaged into a 162 cm^2 tissue culture flask.

2.1.2 Mouse (Raw 264.7) Macrophage cell line

Raw 264.7 macrophages (ECACC 91062702) are mouse monocyte macrophages established from ascites of a tumour induced in a male mouse by intraperitoneal injection of Abelson Murine Leukaemia Virus (A-MuLV) (Ralph & Nakoinz, 1977; Raschke *et al.*, 1978). Raw cells were cultured in growth medium (GM2), comprising RPMI-1640 (Sigma-Aldrich) supplemented with 10% (v/v) NCS, 4mM L-glutamine, 200 U/ml penicillin and 200 μg/ml streptomycin. For virus growth curves, Raw 264.7 cells were seeded at 3.5×10^5 per well in 24 well plates and infected at an MOI = 0.1.

2.1.2.1 Subculturing of Raw 264.7 cells

Cells were subcultured when 80-90% confluent (i.e. every 3 days). GM2 was removed from the 75 cm^2 tissue culture flask, the cells were rinsed with 10 ml of PBS, followed by addition of 15 ml of ice-cooled PBS and incubation at room temperature for 15 minutes. Cells then were scraped gently from the flask surface using a rubber scraper. After that the cells were resuspended in PBS by gentle pipetting and collected in a 50 ml centrifuge tube. The cells were harvested by centrifugation at 200 x g for 10 minutes at 4°C and resuspended in 10 ml GM2. The cells were then seeded into a 75 cm^2 tissue culture flask containing 20 ml GM2 at a split ratio of 1:3 -1:5, cultured at 37°C and 94% air/6% CO2 in a humidified atmosphere until they reached 80-90% confluency (usually 3 days) and passaged further as required. Harvested Raw 264.7 macrophages were prepared for long-term storage as described for NIH 3T3 cells in sections 2.1.1.4 whereas, resuscitation of cryopreserved macrophages was performed as described in section 2.1.1.5

2.1.3 Cell counting

Cell number was determined by counting cells in a Neubauer haemocytometer. One hundred µl of cell suspension was mixed with 100 µl of Trypan Blue (Sigma-Aldrich) and the mixture was introduced into the counting chamber. The haemocytometer consists of nine 1 mm^2 squares divided into smaller squares. One of the 1 mm^2 squares represents a volume of 0.1 mm^3 or 10^{-4} ml. Cells were counted using an inverted microscope in the four corner 1 mm^2 squares and the total number of cells recorded was divided by 4 giving the average number of cells per 1 mm^2 square. The obtained number was multiplied by 2 taking into consideration the dilution 1:2 with Trypan Blue. To calculate the concentration of the cells the following formula was applied: $c = n/v$

Where

c = cell concentration in cells/ml;

n = average number of cells per mm^2;

v = volume counted = 10^{-4} ml

Thus $c = n \times 10^4$ cells/ml.

2.2 Viruses

2.2.1 The MCMV K181 (Birmingham) variant:

The K181 strain of MCMV was obtained from Professor C. A. Mims (Department of Microbiology, United Medical and Dental Schools of Guy's and St. Thomas' Hospitals, Guy's Campus, London Bridge, UK). The parent K181 strain of MCMV was originally received from Dr J. Osborn (Department of Medical Microbiology, University of Wisconsin, Madison, Wis., USA) who had isolated it and described it as a virulent variant of the Smith strain after many passages in mouse salivary glands (Mims & Gould, 1979; Smith *et al.*, 2008). The parental Osborn strain of virus has been designated K181 (Birmingham) in this laboratory. The K181 (Birmingham) virus was further passaged in mice and isolated from salivary glands to produce a seed stock. Working stocks were produced from propagation of K181 (Birmingham) virus (from seed stock) *in vitro* either on NIH 3T3 cells or MEF cells as described in sections 2.3.3 and 2.3.4.

2.2.2 *Tsm5* virus

Temperature-sensitive mutant viruses were previously produced in our laboratory from K181 (Birmingham) MCMV by chemical mutagenesis using N-methyl-N'-nitro-N-nitrosoguanidine. These viruses were defined on the basis of their restricted growth at non-permissive high temperature (>39°C) (Sammons and Sweet, 1989). Among these viruses, one temperature sensitive virus, *tsm5*, was not able to produce detectable infectious virus in the salivary glands of mice 2-3 weeks after inoculation with 10^2 plaque forming units (PFU), and so *tsm5* was grown on MEF cells to produce working stocks (Akel *et al.*, 1993; Akel & Sweet, 1993; Sammons & Sweet, 1989). In this study *tsm5* virus was propagated either on NIH 3T3 cells or MEF cells to produce seed and working stocks (Sections 2.3.3

and 2.3.4) from virus working stocks produced previously on 21/11/1990, 4/02/1991, 16/12/1995, 22/09/1998, 22/08/2002 and 27/07/2004.

2.2.3 The MCMV K181 (Perth) Variant:

The K181 (Perth) variant of MCMV was kindly provided by Dr Alec Redwood (University of Western Australia, Australia). It was cloned and propagated as a BAC plasmid (pARK25) in the DH10B strain of *E. coli* (Redwood *et al.*, 2005). It was originally obtained from Dr D. Lang (Duke University, Durham, N.C., U.S.A.) and designated as the Smith strain. Later, restriction enzyme digestion analysis of the viral DNA revealed that it had a pattern identical to that of the K181 strain (Hudson *et al.*, 1988; Shellam *et al.*, 2007; Xu *et al.*, 1992).

Because mutant *tsm5* was originally derived from the K181 (Birmingham) strain of MCMV, the K181 (Perth) strain was used in this study to construct mutant as well as revertant BACs (Table 2.1) using homologous recombination as the Birmingham variant is not yet available as a BAC. The K181 (Perth) variant, mutant and revertant MCMV were reconstituted by transfection of the corresponding BAC DNA into permissive NIH 3T3 cells (Section 2.3.1).

Table 2.1 MCMV BAC plasmids and reconstituted viruses used in this study.

	Name	Description
1-	K181 (Perth)	*Wt* K181 (Perth) variant of MCMV.
2-	Mt[M27^{A658S}]	Mutant virus with C:T point mutation creating A658S amino acid change in M27 protein.
3-	Rv[M27^{S658A}]	Revertant virus with *wt* M27 gene.
4-	Mt[M36 ^{V54I}]	Mutant virus with C:T point mutation creating V54I amino acid change in M36 protein.
5-	Rv[M36 ^{I54V}]	Revertant virus with *wt* M36 gene.
6-	Mt[M27^{A658S} M36 ^{V54I}]	Mutant virus with point mutations creating amino acid changes A658S in M27 protein and V54I in M36 protein.
7-	Rv[M27^{S658A} M36 ^{I54V}]	Revertant virus with *wt* M27 and *wt* M36 genes.
8-	Mt[m139^{Y565X}]	Mutant virus with C:T point mutation resulting in a stop codon insertion Y565X, creating a truncated m139 protein.
9-	Rv[m139^{X565Y}]	Revertant virus with *wt* m139 gene.
10-	Mt[m141^{V195M}]	Mutant virus with C:T point mutation creating V195M amino acid change in m141 protein.
11-	Rv[m141^{M195V}]	Revertant virus with *wt* m141 genes.
12-	Mt[m143^{M232I}]	Mutant virus with C:T point mutation creating V195M amino acid change in m143 protein.
13-	Rv[m143^{I890M}]	Revertant virus with *wt* m143 genes.
14-	Mt[M70^{C890Y}] *	Mutant virus with C:T point mutation creating C890Y amino acid change in M70 protein.
15-	Mt[M70 ^{403Cys}]	The M70 mutant virus with whole M70 gene codons converted to sub-optimal codons but containing *wt* cytosine at residue 890.
16-	Rv[M70 ^{403Cys}]	Revertant virus with *wt* M70 gene sequence.
17-	Mt[M70 ^{403Tyr}]	The M70 mutant virus with whole M70 gene codons converted to sub-optimal codons but containing mt tyrosine at residue 890.
18-	Mt[[M70 ^{155Cys}]	The M70 mutant virus with the carboxyl terminal of M70 gene codons converted to sub-optimal codons but containing *wt* cytosine at residue 890.
19-	Rev[M70 ^{155Cys}]	Revertant virus of Mt[[M70 ^{155Cys}] with *wt* M70 gene codons
20-	Mt[M70 ^{155Tyr}]	The M70 mutant virus with the carboxyl terminal of M70 gene codons converted to sub-optimal codons but containing mt tyrosine at residue 890.

* Mt[M70^{C890Y}] construct was designed by Dr. Olga Timoshenko (Timoshenko *et al.*, 2009b).

2.2.4 HCMV Merlin BAC

The HCMV Merlin BAC was kindly provided by Professor Gavin Wilkinson (Department of Infection, Immunity and Biochemistry, School of Medicine, Cardiff University) and designated as PAL1111 in *E. coli* SW102 strain (Stanton *et al.*, 2010). Unlike its parent strain *E. coli* DH10B, SW102 does not need transformation with a plasmid containing λ Red-encoded genes: *exo*, *bet* and *gam*. It contains a stably integrated defective λ prophage and *exo*, *bet* and *gam* are expressed under the influence of the strong phage promoter *p*L under the control of the temperature sensitive repressor, *c1857*. *Exo*, *bet* and *gam* genes are not expressed at 32ºC but they are expressed efficiently by shifting the incubation temperature to 42ºC for 15 minutes (Warming *et al.*, 2005).

2.3 Virology methods

All virus work and cell infection was carried out under sterile conditions in a Class II biological safety cabinet. The infected cells were incubated in a Sanyo CO_2 Incubator in a humid atmosphere supplemented with 94% air/6% CO_2.

2.3.1 Transfection of MCMV BAC plasmids into NIH 3T3 cells

MCMV BAC plasmid DNA was isolated using mini preps (alkaline lysis method) as described in section (Section 2.5.3.1) or maxi preps [BACMAX™ DNA purification kit (EPICENTRE® Biotechnologies)] (Section 2.5.3.2). NIH 3T3 cells were seeded at a concentration of $4x10^5$ cells per well of a 6-well plate (Corning Incorporated, USA) in 3 ml of GM1 24 hours before transfection. At the time of transfection the cell monolayers were 80-90% confluent. ExGen500 *in vitro* transfection reagent (Fermentas, York, UK) was used according to the manufacturer's instructions with modifications. ExGen500 is a sterile

apyrogenic solution of linear 22 kDa polyethylenimine in water, which belongs to an efficient new class of non-viral, non-liposomal gene delivery reagents. BAC DNA (20-80 µl), isolated by the alkaline lysis method, or 5 µg of BAC DNA, isolated with the BACMAX^{TM} DNA purification kit, was added to a tube containing 150 mM NaCl to make a final volume of 300 µl. The mixture was gently mixed by tapping for 10 seconds and spun down briefly. Fifteen µl of ExGen500 reagent was added to the tube, the solution gently mixed by tapping for 10 seconds and then left to stand for 10 minutes. Three hundred µl of the ExGen500/DNA mixture was added slowly to the monolayer in each well. The plate was gently rocked back and forth and from side to side to achieve even distribution of the transfection mixture. The cells were incubated at 37°C in a humid 94% air/6% CO_2 atmosphere for 24-48 hours and then transferred to a 75 cm^2 tissue culture flask (Section 2.1.1.3). Plaque formation was monitored for up to 10 days using an inverted microscope. As the BAC contains the *gfp* gene it was possible to monitor plaque formation using a fluorescence microscope. When 100% CPE was observed the supernatant containing virus was harvested as described in section 2.3.3.

2.3.2 Plaque purification of BAC-derived virus

BAC-derived virus was plaque purified in order to isolate the viral clone and to excise the BAC cassette from the viral genome. Either NIH 3T3 or MEF cells were seeded as $4x10^5$ cells in 3 ml of GM1 into each well of a 6-well flat bottomed plate and cultured overnight at 37°C. A 10 fold dilution series of each harvested supernatant containing virions was prepared in 1 ml of maintenance medium (MM) comprising DMEM, 2% (v/v) NCS, 4 mM L-glutamine, 200 U/ml penicillin and 200 µg/ml streptomycin. When cell confluence was about 80-90% the GM1 was removed from each well and 500 µl of viral dilution was added. The cells were incubated at 37°C for 1 hour and then 2 ml of MM was

added. Plaque formation was monitored using an inverted fluorescence microscope. Three-to-four days p.i., plaques that did not show green fluorescence, and thus may have excised their BAC cassette, were picked by scraping the area of cells containing the plaque using a sterile tip. An inverted microscope was used to aid the accuracy of scraping. Each isolated plaque was transferred to 0.5 ml of MM. The virus-infected cells were harvested (Section 2.3.3) and viral DNA extracted (Section 2.5.2). Virus-containing supernatants were prepared for long term storage (Section 2.3.3) and labelled as 1st passage. A 6-well plate with 4x10^5 cells in 3 ml of GM1 in each well was prepared the day before and 1 ml of MM containing the isolated plaque was added to the cells in a well. Cells were incubated at 37°C for 1 hour and a further 2 ml of MM was added to the cells. The cells were incubated at 37°C until formation of new plaques was observed. The plaques were picked and used to inoculate new cell monolayers in 6-well plates as described above.

2.3.3 Virus propagation and harvesting

NIH 3T3 or MEF cells were seeded in a 162 cm^2 tissue culture flask in 25 ml of GM1 and cultured at 37°C until 80-90% confluent. The GM1 was removed from the flask and 0.5-1.0 ml of virus stock, or virus-containing supernatant harvested after virus passaging (Section 2.3.2), in 5 ml of MM was added. The flasks were incubated at 37°C for 1 hour to allow virus attachment to the cells. A further 15 ml MM was then added and the flasks incubated at 37°C until the cell monolayer showed 100% CPE. At this point the medium from the flask was transferred to a centrifuge tube. The cells left in the flask were detached using a cell scraper and transferred to the same centrifuge tube. The contents of the tube were then centrifuged at 850 x g for 10 minutes at room temperature. After centrifugation, the supernatant, containing virus, was supplemented with 10% DMSO and stored at -80°C. The cell pellet was kept at -20°C for DNA extraction (Section 2.5.2).

2.3.4 Production of seed and working stocks of virus

Viral seed stocks were produced on NIH 3T3 or MEF cells as described in section 2.3.7. Infection of cells with virus was carried out as described in section 2.3.3 with the exception that only 15 ml of MM was used per flask to yield higher titres of virus. When all cells showed 100% CPE, the virus-containing supernatant was harvested and centrifuged at 850 x g for 10 minutes at RT to remove cellular debris. The virus-containing supernatant was supplemented with 10% DMSO, aliquoted in 2 ml cryogenic vials and stored at -80°C. In order to produce stocks with a higher virus concentration, the supernatant was filtered through a Whatman filter paper directly into Oakridge-style centrifuge tubes and centrifuged at 29,000 x g for 40 minutes at 4°C. The viral pellet was re-suspended in MM supplemented with 10% DMSO. The viral stock was aliquoted and stored at -80°C. The cell pellet was kept at -20°C for DNA extraction (Section 2.5.2).

2.3.5 Virus titration using a plaque assay

Based on the ability of MCMV to form plaques in a close-to confluent monolayer of permissive cells, viral stocks were assayed on MEF cells to determine viral titres. The amount of virus present in viral stocks (Section 2.3.4), and tissue culture supernatants (Sections 2.3.6 and 2.3.7) are expressed in PFU per ml.

MEF cells were seeded at $1x10^5$ cells in 0.5ml of GM1 in each well of a 24 well tissue culture plate. Cells were allowed to adhere during overnight incubation at 37ºC, this density of cells being established to provide a confluent monolayer after attachment. The GM1 was replaced by 200µl of serially diluted virus samples diluted in MM. The control well only contained MM. After 1 hour incubation at 37°C, 1ml of overlay medium [2/3 carboxymethylcellulose (CMC) and 1/3 GM1] was added to each well and the plates

returned to the incubator. Medium containing CMC was used to limit the spread of virus from distinct virus replicating loci to distant uninfected cells. After 5 days for viral plaques to form the cells were fixed and stained. At room temperature, 0.5ml of formal saline fixative (4% formaldehyde in PBS) was added directly to each well. After 10 minutes, the overlay was replaced by another 0.5 ml of fixative. The plates were incubated at room temperature for 30 minutes and then the fixative was replaced by 0.5-1.0 ml of 0.3% crystal violet solution [0.3% (w/v) crystal violet dissolved in 10% (v/v) methanol in water] in each well. The plates were left for 1 hour at room temperature, when the wells were washed gently with running tap water and the plates left on the bench to air dry. The plaques were counted using a light microscope.

2.3.6 *In vitro* growth curves of viruses on MEF cells

To study the growth properties of viruses used in this study, MEF cells from the second culture passage were seeded at a density of $1x10^5$ cells in 0.5ml of GM1 per well of 24 well tissue culture plates; three wells were infected for each virus. Cells were allowed to adhere during overnight incubation at 37ºC, this density of cells being established to provide a confluent monolayer after attachment. GM1 was removed and the cells were infected at an MOI of either 0.05 plaque forming units (PFU) per cell in 200 µl. After one hour incubation at 37 ºC, the culture medium was removed and the cells washed with PBS. To each well, 1000µl of GM1 was added and the cells incubated at 37ºC. At different time points, two 500µl aliquots were collected from each well and stored at -80°C; the medium being replaced by a further 1 ml of GM1. The samples were thawed and viral titres were determined by plaque assay (Section 2.3.5).

2.3.7 *In vitro* growth curves of viruses on Macrophage cell-lines

Raw 264.7 macrophage cells were seeded at $3.5x10^5$ cells in 0.5ml of GM2 into each well of a 24 well tissue culture plate. Cells were allowed to adhere during overnight incubation at 37ºC, this density of cells being established to provide a confluent monolayer after attachment. Raw 264.7 cells were infected at an MOI of 0.1 PFU per cell in 200 µl. After one hour incubation at 37 ºC, the culture medium was removed and the cells washed with PBS. To each well, 1000 µl of GM1 was added and the cells incubated at 37ºC. At different time points, three 500 µl aliquots were collected from each well and stored at -80°C; the medium being replaced by a further 1 ml of GM1. The samples were thawed and viral titres were determined by plaque assay (section 2.3.5).

2.3.8 Statistical analysis

Virus titres and genome copy numbers were log_{10}-transformed and processed using the computer program GraphPad Prism (GraphPad Prism Software, Inc.). Results were considered to be significantly different when $P<0.05$ was achieved. Values reported represent mean ± standard deviation (SD).

2.4 Bacteriology methods

2.4.1 Bacterial hosts and plasmids

E. coli strain DH10B (*rec*A$^-$), containing the MCMV K181 (Perth) BAC and designated as pARK25, was kindly provided by Professor G. R. Shellam (University of Western Australia, Australia) (Redwood *et al.*, 2005). The pRpsL-neo plasmid carrying a kanamycin resistance and a streptomycin susceptibility gene and the RecE/RecT protein expression plasmid pKD46 (Datsenko & Wanner, 2000) were originally obtained from Gene Bridges (Dresden, Germany).

A stab culture of SW102 (developed from the *E. coli* DH10 B strain) containing the HCMV Merlin BAC was kindly provided by Professor Gavin Wilkinson (Department of Infection, Immunity and Biochemistry, School of Medicine, Cardiff University) and designated as PAL1111. Hosts and plasmids used in this study are shown in Table 2.2.

Table 2.2: Bacterial hosts and plasmids used in this study.

Plasmid name	Host	Antibiotic selection	Growth Temp.	Source or reference
pARK-25 [*wt* MCMV K181 (Perth) BAC]	DH10B	12 µg/ml Cm[1], 80 µg/ml Sm[2]	37°C	(Redwood *et al.*, 2005)
pKD46	TOP10	50 µg/ml Carb[3]	30°C	(Datsenko & Wanner, 2000)
pRpsL-neo	DH10B	20 µg/ml Kan[4]	30°C	Gene Bridges, Germany
PL1111 (HCMV Merlin BAC)	SW102	12 µg/ml Cm	30°C	Prof. Gavin Wilkinson, Cardiff University
pGEM®-T Easy vector	XL1-Blue	100 µg/ml Amp[5], 0.1 mM IPTG[6], 20 µg/ml X-Gal[7],	37°C	Promega, USA
pPCR-Script Amp SK(+) plasmid	XL1-Blue	100 µg/ml Amp	37°C	ShineGene Molecular Biotech, Inc., Shangai, China

1- Cm, Chloramphenicol.
2- Sm, Streptomycin
3- Carb, Carbenicillin
4- Kan, Kanamycin
5- Amp, Ampicillin
6- IPTG, isopropyl-beta-D-thiogalactopyranoside
7- X-Gal 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside

2.4.2 Media for bacterial cultures

Bacterial cultures were grown in Luria-Bertani (LB) medium prepared by dissolving 10 g tryptone, 5 g yeast extract and 10 g NaCl in 800 ml distilled water. All culture media were autoclaved for 15 minutes at 121°C and, where required, mixed with appropriate antibiotics. For making agar plates, 100 to 200 ml aliquots of LB medium supplemented with 15g/l bacto-agar (L-Agar) were autoclaved, cooled and stored at 4°C. The L-Agar was dissolved by boiling, cooled down to 50°C and supplemented with appropriate antibiotics (Table 2.2). In a laminar flow hood, 20-25 ml of L-Agar was poured into each Petri dish (agar plate) and left to solidify for 20 to 30 minutes.

2.4.3 Cultivation and long term storage of bacteria

Single colonies isolated from agar plates were transferred aseptically into 5 or 10 ml of LB medium supplemented with appropriate antibiotics. Bacteria were then grown overnight at a suitable temperature (Table 2.2) on a shaker at 200 rpm. For long term preservation of bacteria, 500 µl of overnight culture were transferred to 2 ml cryovials followed by addition of 500 µl 80% (w/v) glycerol, mixed by pipetting, and stored at -80°C.

2.4.4 Preparation and transformation of electrocompetent cells

A single colony was picked from an LB agar (LBA) plate and grown in 5 ml LB medium (supplemented with appropriate antibiotics) for overnight incubation with shaking at 37°C. A 500 µl aliquot of the overnight culture was added to 25 ml of pre-warmed LB medium supplemented with appropriate antibiotics. Cells were grown at 37°C with shaking until growth reached an absorbance OD_{600} of 0.6. The culture was then transferred to a 50

ml centrifuge tube and placed on ice for 10 minutes. The cells were harvested by spinning down for 10 minutes at 4,000 x g in a pre-cooled centrifuge (4°C). The cells were washed by resuspension in 10 ml of 10% (w/v) glycerol followed by centrifugation for 10 minutes at 4,000 x g. The supernatant was discarded and the washing step was repeated twice. The supernatant from the final wash was discarded and the cells resuspended in the remaining liquid (about 250 μl). For long term storage, the cells were then aliquoted into 0.5 ml centrifuge tubes and snap-frozen in liquid nitrogen prior to storage at -80°C.

2.4.4.1 Transformation of pKD46 into Electrocompetent cells:

pARK-25 (*E.coli* DH10B) containing the K181 (Perth) BAC was used for manipulation of the virus and construction of the mutated and reverted constructs by homologous recombination. In order for these cells to be ready for homologous recombination they have to contain the red recombinase plasmid, pKD46 (GenBank Acc. No: AY048746.1, size = 6329 bp) (Figure 2.1) (Yu *et al.*, 2000b). pKD46 is a temperature sensitive plasmid that can be maintained in bacteria at 30° C but is lost if the cells are grown for 24 hours at 37-40° C. It encodes the lambda Red genes (*exo*, *bet*, *gam*) that are required for recombination with the native terminator (tL3) after the *exo* gene. The expression of these genes is under the control of an arabinose-inducible promoter, P_{araB}. It also encodes *araC* for repression of the P_{araB} promoter. Bacteria containing pKD46 can be selected on ampicillin or its derivatives.

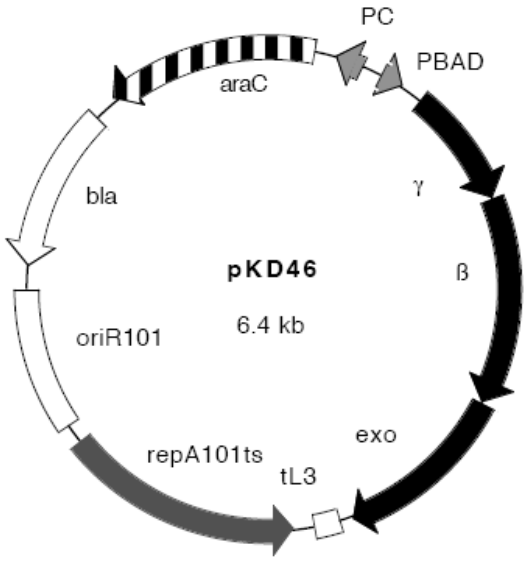


Figure 2.1: Plasmid pKD46 expresses lambda *red* under Para (the arabinose-inducible promoter) control. Expression is regulated by L-arabinose. The plasmid carries *bla* [the beta-lactamase gene (ampicillin resistance gene)]and can be maintained under ampicillin selection. The plasmid origin of replication is temperature sensitive, and the plasmid will be lost on temperature shift from 30°C to 37-40°C on overnight incubation.

For transformation of pARK-25, electrocompetent cells, prepared as described in section 2.4.4, were thawed on ice. Up to 5 µl (100-300 ng) of pKD46 plasmid solution were added to the thawed cells, mixed by gentle pipeting and placed on ice for 5 minutes. The cells and plasmid mixture was then transferred to an ice-cold 1mm gap cuvette and electroporation was performed using a Bio-Rad Gene Pulser set to 25µF, 1.85 KV with pulse controller set at 200 ohm. One ml of SOC medium (2.0g tryptane, 0.5g yeast extract, 1ml 1M $MgCl_2$, 1ml 1M $MgSO_4$, 1ml 1M NaCl, 250µl 1M KCl and 1.8µl 20% glucose in 100ml distilled water) was added to the cells in the cuvette immediately after electroporation. The cells were transferred to a 1.5 ml centrifuge tube and incubated at 30°C for 70 minutes with shaking spread on a plate containing 12 µg/ml chloramphenicol and 50 µg/ml carbenicillin and then incubated for 24-48 hours at 30°C.

2.4.4.2 Electrocompetent cell preparation and transformation with linear DNA:

A single colony of pARK-25 containing the pKD46 plasmid was picked and grown in 5 ml LB medium containing 12 μg/ml choramphenicol and 50 μg/ml carbanacillin overnight with shaking at 30°C. An aliquot of the 440 μl overnight culture was added to 20 ml of pre-warmed LB broth supplemented with 12 μg/ml choramphenicol and 50 μg/ml carbanacillin (Table 2.2). Cells were grown at 30°C with shaking until the absorbance at an OD 600 reached 0.3. 10 ml of the culture were then transferred to a 25 ml Universal tube. 300-500 μl of 10% L-arabinose was added to the culture to induce the recombinase proteins expression. Cells were further grown at 37°C for one hour (to induce production of proteins necessary for recombination). The cells were then placed on ice for 10 minutes and electrocompetent cells prepared as described in section 2.4.4. Electrocompetent cells were then transformed with 100-300 ng linear DNA as described in section 2.4.4.1. 100 μl of the cells were spread onto a plate (supplemented with appropriate antibiotics) and incubated for 24-48 hours at 30°C in order not to lose the temperature sensitive pKD46 plasmid needed for all steps of BAC mutagenesis.

2.4.5 Transformation of XL-1 Blue competant cells

XL-1 Blue competent cells (Stratagene, UK) was used as a host for cloning of pGEM®-T Easy plasmid containing UL70 gene DNA frament insert (Section 2.6.1). XL1-Blue cells were thawed on ice, mixed gently and then 100 μl of the cells were transferred into a pre-chilled 14 ml Falcon polypropylene round-bottom tubes. A 1.7μl sample of β mercaptoethanol was then added to cells and incubated one ice for 10 minutes, whilst being swirled gently. A 2μl sample of the mutated pGEM T Easy plasmid was then added to the cell and incubated on ice for a further 30 minutes. The cells were then heat shocked for 45

seconds in a 42^{o}C water bath, after which they were incubated on ice for a further 2 minutes followed by addition of 900 µl of preheated (42^{o}C) SOC medium. The cells were then incubated at 37^{o}C for 1 hour, on a shaking plate at 225 rpm and then streaked directly onto a previously prepared LB agar plate containing 100µg/ml ampicillin, 80µg/ml X-Gal and 0.5mM of IPGT. Plates were incubated at 37^{o}C overnight. Successfully transformed cells (white colonies) were grown in LB medium containing 100µl/ml ampicillin at 32^{o}C for 24 hours.

2.5 Molecular biology methods:

2.5.1 Isolation of DNA from viral particles:

Five 162 cm^2 flasks of NIH 3T3 cells were infected with MCMV and, when the CPE had reached 100%, the cells were harvested by scraping. Cell debris was pelleted at 850 x g for 10 minutes at 4°C, and the supernatant filtered through a Whatman filter paper directly into a sterile Oakridge-style centrifuge tube and the cell pellet stored at -20 for viral DNA extraction as described in section 2.5.2. Virus in the supernatant was centrifuged at 29,000 x g for 30 minutes at 4°C and the pellet resuspended in 500 µl of DNase I buffer [50 mM Tris HCl (pH 8.0), 5 mM $MgCl_2$, 0.1 M sodium acetate, 100 µg of bovine serum albumin per ml]. Extra-virion DNA was digested for 1 hour in 0.2 U of DNase I (Sigma-Aldrich, DN25) at room temperature, and the reaction was stopped with 20 µl of 500 mM EDTA (pH 8.0). Virus was treated with 500 µl of 1% sodium dodecyl sulphate (SDS) and 40 µl of 20 mg/ml proteinase K (Sigma-Aldrich) for 16 hours at 56°C. The liberated viral DNA was purified twice by phenol-chloroform extraction and 0.1 volume 3M sodium acetate was added to the collected aqueous phase. Ethanol (2.5 x volume) was added and the viral DNA precipitated at 15,000 x g for 15 minutes at 4°C. The pellet was washed twice with 5 ml 70% ethanol at RT and centrifuged at 15,000 x g for 10 minutes at 4°C. The supernatant was carefully poured out and the tube was centrifuged for a short time and the remaining ethanol removed using a 1 ml pipette. The pellet was air-dried at RT for 5 minutes and then resuspended in 250-500 µl TE [1 mM Tris HCl (pH 7.6) and 10 mM Tris EDTA (pH 8.0)] elution buffer.

2.5.2 Isolation of viral DNA from infected tissue culture cells

Isolation of total DNA from virus-infected tissue culture cells was performed using a DNeasy Tissue Kit (Qiagen, Crawley, UK), according to the manufacturer's instructions. Virus infected and virus lysed cells were harvested from 162 cm^2 tissue culture flasks using a cell scraper and pelleted by centrifugation at 850 x g for 10 minutes at RT. Alternatively, cell debris stored at -20ºC was thawed at RT. The pellet was resuspended in 200 µl PBS, 20 µl proteinase K and 200 µl buffer Al was added to the sample, which was vortex-mixed thoroughly and incubated at 56°C for 10 min. To this homogeneous solution, 200 µl of 100% ethanol was added and mixed thoroughly by vortexing. The mixture was then pipetted into the DNeasy mini spin column placed in a 2 ml collection tube followed by centrifugation at 6,000 x g for 1 minute in a bench top centrifuge at RT. The flow-through and collection tube was discarded and the column was placed in a new 2 ml collection tube. 500 µl buffer AW1 was added to the column and the column was centrifuged for 1min at 6,000 x g at RT. The flow-through and collection tube were again discarded and the column placed in a new 2 ml collection tube. 500 µl buffer AW2 was added to the column, followed by centrifugation for 3 min at 20,000 x g at RT to dry the DNeasy membrane. After discarding the flow-through and collection tube, the column was placed in a clean 1.5 ml microcentrifuge tube and 200 µl buffer AE was pipetted onto the DNeasy membrane. The column was incubated at RT for 1 minute and then centrifuged for 1 minute at 6,000 xg RT to collect the eluate. The eluted DNA was stored at 4°C.

2.5.3 Plasmid DNA isolation

2.5.3.1 Small scale preparation of plasmid DNA

BAC DNA used for PCR screening (Section 2.5.7) and transfection of eukaryotic cells (Section 2.3.1) was isolated from 1.5-5 ml of overnight bacterial cultures using an alkaline lysis method. The culture was centrifuged at 5,000 x g for 8 minutes at 4ºC. The supernatant was discarded and the pellet re-suspended in 300 µl of re-suspension buffer (15 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase). After resuspension, 300µl of freshly prepared solution-II (0.2M NaOH; 1% SDS) was added to lyse the cells and the tube was inverted gently several times. The lysis reaction was allowed to proceed for a maximum of 5 minutes at RT. To stop the reaction, 300µl of solution-III (117.78 g potassium acetate; 46 ml glacial acetic acid in 400ml distilled water) was added and incubated on ice for 15 minutes before centrifugation at 15,000 x g for 15 minutes at RT. The supernatant was then transferred to a fresh Eppendorf tube containing 700 µl isopropanol which was then centrifuged at 15,000 x g for 10 minutes at RT. The supernatant was discarded and the pellet, containing DNA, was washed by adding 500 µl of 70% ethanol to the tube, and then the tube was centrifuged 15,000 x g for 5 minutes at RT. The supernatant was then fully removed and the pellet air dried for 5-10 minutes. DNA was re-suspended in 50 µl TE buffer and stored at 4°C for use or at -20 for long-term storage.

2.5.3.2 Isolation of high quality BAC plasmid DNA

The BACMAX^{TM} DNA purification kit (EPICENTRE® Biotechnologies, Cat. No. BMAX04) was used for isolation of MCMV BAC DNA for transfection and RFLP study purposes. 50 ml of LB medium, containing 15 µg/ml of chloramphenicol and 50 µg/ml streptomycin, in a 250 ml flask was inoculated with a single bacterial colony from a freshly

streaked plate and incubated at 37°C with vigorous shaking for 14-16 hours (~ 250 rpm). When the culture reached an OD_{600} of 3-4, the cells were transferred into centrifuge tubes. The cells were pelletted by centrifugation at 5,000 x g for 8 minutes at 4ºC and the supernatant was discarded. Three ml of chilled BACMAX solution 1 were added to the pellet and vortexed vigorously until completely resuspended. Six ml of BACMAX solution 2 were added and mixed very gently by inverting the tube 2-3 times; the suspension was incubated at RT for not more than 5 minutes. Chilled BACMAX solution 3 (4.5 ml) was added and the tube mixed gently by inversion 2-3 times and then incubated on ice for 15 minutes prior to centrifugation at 15,000 x g for 15 minutes at 4ºC to pellet cellular debris. The supernatant was transferred carefully to a 40 ml Oakridge-style centrifuge tube using a 10 ml pipette, avoiding aspiration of the white precipitate. Room temperature isopropanol (0.6 volumes) was added and mixed thoroughly by inverting the tube 4-6 times. Nucleic acid was precipitated by centrifugation at 15,000 x g for 15 minutes at 4ºC. Isopropanol was carefully decanted and the tube was briefly centrifuged then excess isopropanol was pipetted off without disrupting the pellet. The pellet was air-dried at RT for 3-5 minutes and then resuspended in 500 µl of TE buffer by tapping and swirling. Eighteen µl of RiboShreder RNase Blend were added to the tube and incubated at 37ºC for 30 minutes. An additional 500 µl of TE buffer were added and mixed by tapping the tube. One ml of chilled BACMAX solution 4 was added, the tube mixed gently by tapping and then incubated on ice for 15 minutes followed by centrifugation at 15,000 x g for 15 minutes at 4ºC. The supernatant was carefully transferred to a 40 ml Oakridge-style centrifuge tube without disrupting the pellet. Four ml of absolute ethanol were added to the recovered supernatant and mixed gently by inverting the tube 4-6 times. The DNA was precipitated by centrifugation at 15,000 x g for 15 minutes at 4ºC and the supernatant discarded. The pellet was air-dried at RT for 5 minutes and resuspended in 200 µl of TE buffer.

2.5.3.3 Quick method for BAC DNA preparation for PCR screening

For fast and efficient BAC preparation for PCR screening, 200 µl of overnight bacterial culture was centrifuged at 6,000 x g for 5 minutes at RT, the supernatant removed and the cell pellet re-suspended in 200 µl of sterile distilled water. The cell suspension was boiled for 10 minutes and then centrifuged at 15,000 x g for 5 minutes at RT to remove cell debris. 1-2 µl of supernatant containing DNA was used in 25 µl of PCR reaction.

2.5.3.4 Isolation of plasmid DNA (mini preps)

Small plasmid DNA (up to 10 kb) was isolated from 1-5 ml of overnight bacterial culture using a QIAprep Spin Miniprep Kit (Qiagen). The bacterial cells were harvested by centrifugation at 6,800 x g for 3 minutes at RT and then re-suspended in 250 µl of buffer P1 containing RNase A. The cells were lysed by adding 250 µl of buffer P2 (contains NaOH, SDS). The mixture was incubated for a maximum of 5 minutes and 350 µl of buffer N3 was added to neutralise the lysate and precipitate cellular debris, denatured proteins and chromosomal DNA. The mixture was then centrifuged at 17,900 x g for 10 minutes at RT and the supernatant applied to a QIAprep spin column. The column was centrifuged at 17,900 x g for 30 seconds at RT and the flow-through was discarded. The column was washed by adding 750 µl of buffer PE and centrifuged at 17,900 x g for 30 seconds at RT. The flow-through was discarded and the column was centrifuged for an additional 1 minute. To elute plasmid DNA, 50 µl of buffer EB (10 mM Tris-HCl, pH 8.5) was added to the centre of the QIAprep spin column, left to stand for 1 minute, and centrifuged at 17,900 x g for 30 seconds at RT.

2.5.4 DNA quantification

DNA was quantified using the NanoDrop™ ND-1000 Spectrophotometer (Labtech International Ltd., UK). Two µl of sample was used and the concentration of the sample was presented as ng/µl based on absorbance at 260 nm. Ratios 260/280 and 260/230 were also obtained to indicate the purity of DNA. A 260/280 ratio of 1.8 and 260/230 ratio of 1.8-2.2 is accepted as pure for DNA. If the 260/280 ratio is lower than 1.8 it may indicate the presence of contaminants such as proteins, phenol, salts or other contaminants that absorb at or near 280 nm. A lower ratio for 260/230 also indicates the presence of contaminants.

2.5.5 Primer design and preparation

Most of the primers used in this study were designed using the Primer-BLAST computer program, available at http://www.ncbi.nlm.nih.gov/tools/primer-blast/. The program identified all possible primer pair combinations within the proximities of the screened sequence of interest. The best primer combinations were selected and the sequences underwent any modification, before being sent to Eurogentec S.A, for primer synthesis.

Primers used for amplification of the RpsL-neo cassette containing DNA fragments and for insertion of the RpsL-neo cassette into the gene of interest, were designed to contain 50 bases homologous (homology arm) to the 5'end of the wild-type gene sequence and 20-22 bases from the pRpsL-sequence at the 3' end. Primers used for 'QuikChange™, site directed mutagenesis (Stratagene, UK) of the Merlin strain of HCMV were designed to contain a single base alteration in the middle of the 30 base primer (Table 2.10).

2.5.6 Preparation of Primers

All primers received from Eurogentec S.A were in solution at a concentration of 100µM. Primers used in PCR reactions were diluted to a 10µM concentration by combining 20µl of the primer solution to 180µl dH_20. All primers used within the sequencing reactions were diluted to a 3µM concentration by combining 3µl of the primer solution into 97µl dH_20.

Table 2.3: Primers used for the M27 gene mutagenesis and sequencing.

Primer name	Forward sequence	Tm (°C)
M27FA	CGGAAGTTATCGCCAAAACC	65.2
M27FB	CAAGAGGTCCATGAACTTGG	62.1
M27FC	CGCGTAGCCCAGATCGATGT	61.3
M27FD	ATCGTAGATGACCTCCATGG	56.5
M27F5	CGACCTCTATTCCCCTGAGT	62.0
M27F6	AGGTGAAGACCTGGCCCAC	62.0
M27RA	TGGCTGCTTGCTGTGTAGG	69.6
M27RB	ATCGAGTTCGTCCCCTTCGC	69.6
M27RC	ACATCGATCTGGGCTACGCG	69.5
M27RD	ACACCTTCTTCATCAAGAG	58.3
M27R5	GCATGCGGCTGCGCTACTAC	70.1
ET M27 F*	TCCGCAACTCATCCCCTTGGAACCGCCGGCGACCGCGAC CGCCTCCGAGGC***CCTGGTGATGATGGCGGGATC***	82.2
ET M27 R*	ACAGGCTCATCCCCTACTACATGTTTGTCGGCGGATACCG CCGGCGCGACGG***TCAGAAGAACTCGTCAAGAAGGA***	96.7

Table 2.4: Primers used for the M236 gene mutagenesis and sequencing.

Primer name	Forward sequence	Tm (°C)
M36F1	CCCGGCGTCGCCCGTGAGGC	83.3
M36F2	ATCGATGATGAGGATGAGCC	64.0
M36F3	TCCTTTTAGGGAGGACGTCG	65.2
M36F4	CCTTGATCACGGGGTCAGAG	64.0
M36F5	CCTGATTCTCCGCGACGATG	64.0
M36R1	AACGCGAGTCGCTGTTCAAA	68.0
M36R2	GAGATCGTCCCTTACCAGGA	63.0
M36R3	CAGCACCTTTGACATCTTCG	63.4
M36R4	TCTGCATGTCGCGGATGACC	64.0
M36R5	GGAAACTGGTCCACCGTCTG	64.0
ET M36 F*	GAAGAGCATCACGAACCTCGTGGTGTCCAGGTCCCGTCC ACGCAGCCGGCCG***CCTGGTGATGATGGCGGGATC***	80.6
ET M36 R*	ATAAAGTTGCTGCTTGCCTCAAAATGCTCGAATGCGGTCT TGTTTTTCGCGT***TCAGAAGAACTCGTCAAGAAGGA***	93.2

Table 2.5: Primers used for the m139 gene mutagenesis and sequencing.

Primer name	Forward sequence	Tm (°C)
m139 F1	TAGCGATCGGAGCCGGTTTC	64
m139 F2	CCCTCGGTAGCATTGCCTTC	64
m139 F3	AGGAGGCAGAGTGCGATCAG	64
m139 F4	TCCTCGGCGATCCTGAAGAC	64
m139 F5	CGGGATCAGGTACCAGTTCTC	66
m139 R1	GGCTTCGTCTTCGAGGTGTC	64
m139 R2	GAAGAGGCAGCTGACCCATC	64
m139 R3	TGCGCGACTTCAAGAGCTTCC	66
m139 R4	CGAGAGCGTGCTAACGACAC	64
ET m139 F*	GATGCGGCACGGCTTTTATAGACTGATGGTTGCTGCGCG CCCCCCCGCGAGCGG***CCTGGTGATGATGGCGGGATC***	
ET m139 R*	CCGCCGCTCTGTCCCCACAGGGACGAAGACCGGCCGCC GATCGCCACGTCGC***TCAGAAGAACTCGTCAAGAAGG***	

Table 2.6: Primers used for the m141 gene mutagenesis and sequencing.

Primer name	Forward sequence	Tm (°C)
m141 F1	GAAGAATTCGGCCACGCTGTC	66
m141 F2	GTGGCGATGTTGACAGGCAG	64
m141 F3	AACGACCACGAGCAGTGCAAC	66
m141 R1	ATCGGGCAAGAGTTGCTGGTC	66
m141 R2	ATGTGCGACCCCGAGTTCAAG	66
m141 R3	CGTTTCGGGATCTCCCGAC	62
m141 R4	CACTTCTGGCTCTGGTCTCTC	66
ET m141 F*	GAACTTGTCGGCCACGATCACGTGGTCGGCCCTGAACTC CGCGTCCGGCAGGGG***CCTGGTGATGATGGCGGGATC***	
ET m141 R*	GATACGGGGTGTCCCTCGTGCTGTCGGGCGAGTACGGAC GGGTGTACGTGTAT***CAGAAGAACTCGTCAAGAAGG***	

Table 2.7: Primers used for the m143 gene mutagenesis and sequencing.

Primer name	Forward sequence	Tm (°C)
m143 F1	ACGTGAGATCCACGCGAACTG	66
m143 F2	TCGCCCAAGTGTGAGAGAGG	64
m143 F3	TCTTCAGCAGTCCCATGCGAC	66
m143 F4	AGTTCGAGCCGGTCCAGTAG	64
m143 R1	ATGGGCAAGGAGTCGAGGATG	66
m143 R2	TATCGCTGATCAGCGGCGAG	64
m143 R3	TGACCGGCTACGACCTGAAC	64
m143 R4	TCCCGGATCCCAGCATTTGAG	64
ET m143 F*	TCGGGAGTGGAACCACAGCTCCTGGTCGGGTGGGGGCT CGAGCCGGACGCTCGG***CCTGGTGATGATGGCGGGATC***	
ET m143 R*	CTACAGGGTGCCGCTGTCGATACCGTTGAAGAAAGACCC GGTCTGGGACGCC***TCAGAAGAACTCGTCAAGAAGG***	

Table 2.8: Primers used for the M70 gene mutagenesis and sequencing.

Primer name	Forward sequence	Tm (°C)
M70ExtLargeF	TTTCCAGTTGGGATGAGAGG	60
M70ExtSmallF	CGGAAATGTGGCTAGGTGTT	60
M70F	ACGTTAGTTAGGGCCGGGTG	57.5
M70F3	TCAAGCATTCCAGGTCGTCGG	67.3
M70F4	CGGTAGTGGTTACGCGTGTG	61.5
M70F6	GTCCGCAGCATGGTTTCGCC	60.5
M70F7	CACCGTCTCCTGGCGAGTGT	63.0
M70R1	ACTCCCACCCCGTCTTCTTC	66.5
M70R3	CGTCGAGCGATTACGAGGAG	70.3
M70R4	CTGCTGCCCAAGGACCTGAT	70.8
M70R7	GCCGGCATCCTCGATCACAC	74.2
M70ExtSmall Rev	CTTCCGGTCTATAAGCGCTGC	62
M70ExtLarge Rev	AAATCCAAACGTGTCATGCA	56
ETM70F*	GTGCGCTGCCGGAACGGGGGTCGTCGTCGCCCACGCGC TCGTCGACGCGT***CCTGGTGATGATGGCGGGATC***	
ETM70R*	GATGATGACCGACTTCGGCGGTGGCGGGACTGGCACTA GTAATGCT***TCAGAAGAACTCGTCAAGAAGG***	
M70HA-RpsL-Rev1*	AGAACGCCGCGGGGGCGGCGGACGAGGCGATGCTGTAC GGCGGCGGCGGC***TCAGAAGAACTCGTCAAGAAGG***	
M70HA*wt*-Rev1 ●	AGAACGCCGCGGGGGCGGCGGACGAGGCGATGCTGTAC GGCGGCGGCGGC**TACGACGAAGACGACGACCC ***	
M70 Suboptimal Primers		
M70ExtLargeF	TTTCCAGTTGGGATGAGAGG	60
M70ExtSmallF	CGGAAATGTGGCTAGGTGTT	60
M70SubOpt For2	GCTCCTCCTCGTAATCCGACG	64
M70SubOpt For3	AGGGAATAATGACCCGGAG	60
M70SubOpt For4	CGCGTCTCGTCTTCTTTTTC	60
M70SubOpt For5	AACATTTCGTGTAATCCCGC	58
M70SubOpt Rev1	CGTAATGTGGAGGACGACCT	59.9
M70SubOpt Rev2	TCTCGTTGAACATCTCGTGC	60
M70SubOpt Rev3	ATCTTGGCGTCGACGTATTC	60.1
M70SubOpt Rev4	TTGAGATACCGGAGAATGGC	60

● The common sequence between *wt* and the suboptimal synthesised sequence is shown in bold and underlined.

Table 2.9 : Other primers used in the study

Primer name	Forward sequence	Tm (°C)
RpsL-For	GGGTGGAGAGGCTATTCGG	69.0
RpsL-Rev	GCCGAATAGCCTCTCCACCC	69.0
6530R	TCCGTTAGCCTTAGATTCCACCG	68.7
m06F	TTGGAGCGATACGTTGACAATG	66.8
ARK25 15204 R	AATACATCGTCACCTGGGACA	64.1
ARK25 14832 F	CATATCCCACGGCTGTTCAAT	65.7
Other ORFs sequencing primers		
m20 F1	TCGACGGCCATCTGTTCCTC	64
M20 R1	GCACGGATGTTCGGAATCGAC	66
m21 F1	AGGGCGTGCGACTTTAAGCC	64
m21 R1	AGGGTGTCGGGACTCTTCTC	64
m132.1 F1	CCCATAGGCAGTAACGACACC	66
m132.1 R1	TGCCTGCTGAAGACGGACAAC	66

Table 2.10: HCMV Merlin strain primers

Primer name	Forward sequence	Tm (°C)
UL70 Fwd	GTAATCCAGACAGAAGCGCC	62^{o}C
UL70 Rev	ATCTACCACCATGGACGCTC	62^{o}C
UL70 inter Fwd	GCGTGTAGTTGAGACAGCGA	62^{o}C
UL70 inter Rev	ATCTACCACCATGGACGCTC	62^{o}C
External UL70 Fwd	TCTTCCGCGAGATGGTATTC	60^{o}C
External UL70 Rev	GTACACCCCAGCGTTTTACG	60^{o}C
UL70mut Fwd ■	CGCTTGACGGCCACG**T**AGCAGGCGCCGTGGG	50
UL70mut Rev ■	CCCACGGCGCCTGCT**A**CGTGGCCGTCAAGCG	50
UL70 RpsL-Fwd*	TTTATCGAGACGCGCTCGCTTAACGTGACGCGTTATCG ACGCCGCGGTCT***CCTGGTGATGATGGCGGGATC***	
UL70 RpsL-Rev*	TCAGACGGCGGTCGCCGGCGGCATGGGCGCGTCGGG CGGTCTGATTTTGA***TCAGAAGAACTCGTCAAGAAGG***	

■ The 30 base primers used in the site-direected mutagenesis containing one base substation in the middle of the pirmer.

* 3’ end of the primer annealing to the RpsL-neo cassette is shown in bold and italics.

2.5.7 Polymerase chain reaction (PCR)

PCR for screening of bacterial recombinants, checking the excision of the BAC cassette from BAC-derived virus DNA or amplifying viral sequences for sequencing, was performed using the 2 x ReddyMix™ PCR Master Mix (Abgene, Surrey, UK). The PCR reaction was performed in a 25 µl final volume comprising 12.5 µl 2x ReddyMix PCR Master Mix [1.25 units of *Thermus aquaticus* (Taq) Thermoprime Plus DNA polymerase, 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% (v/v) Tween 20, 0.2 mM each of dATP, dCTP, dGTP and dTTP, precipitant and red dye for electrophoresis], 10-300 ng of DNA template or 0.5-2 µl of DNA prepared from bacterial overnight culture as described in section 2.5.3.3, 0.6 µM of each forward and reverse primer (Sigma-Genosys, Suffolk, UK or Eurogentec, Southampton, UK), and the appropriate volume of sterile distilled water. Standard PCR conditions were as follows:

	Temperature	Duration	Number of cycles
Initial denaturation	95ºC	2 minutes	1 cycle
Denaturation	95ºC	30 seconds	29 cycles
Annealing	XºC1	30 seconds	
Extension	72ºC	Y minutes2	
Final extension	72ºC	7 minutes	1 cycle

1- The annealing temperature depends on the melting temperatures (Tm) of the primers (Tables 2.3 – 2.10); in this case it is 3-5°C below the lowest primer Tm.

2- Extension time depends on the length of sequence to be amplified; in this case it is 45-60 seconds per 1kb.

Linear DNA fragments used for mutagenesis (Section 2.4.4.2) were amplified using the Extensor Hi-Fidelity PCR Master Mix (Abgene). The PCR reaction was carried out in a total volume of 50 µl, and contained 100-300 ng of DNA template, 0.3 µM of each forward and reverse primer and 25 µl Extensor Hi-Fidelity PCR Master Mix [1.25 units total DNA polymerase (Taq Thermoprime Plus DNA polymerase with proprietary thermostable

proofreading enzyme), 2.25 mM $MgCl_2$, 350 µM of each dNTP, and red dye for electrophoresis] in distilled autoclaved water to a final volume of 50 µl. The PCR protocol was as described above.

2.5.8 Restriction enzyme digestion

For restriction fragment length polymorphism (RFLP) analysis , BAC DNA isolated as described in section 2.5.3.2, was digested with *Eco*RI, *Ase*I, *Dra*I, *Hpa*I (Fermentas, York, UK) fast digest enzymes. The BAC DNA (1.5-3.5 µg) was digested with 1 µl of enzyme in a final volume of 50 µl containing 5 µl of appropriate buffer. The reaction mixture was incubated at 37°C for 10-20 minutes. Loading buffer [0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% ficoll type 400 (Pharmacia) in water] was added and the digest visualised by agarose gel electrophoresis as described in section 2.5.9.

Samples of linear PCR products, amplified from plasmid DNA (e.g. pRPSL-neo plasmid) for the purpose of mutagenesis, were treated with the restriction enzyme *DpnI* to destroy any residual parental plasmid DNA. The digestion reaction was carried out in a final volume of 50 µl with 1 µl of *Dpn*I enzyme (New England Biolab, Hitchin, UK), 5 µl of 10 x NE Buffer (New England Biolab) added to 40 µl of PCR product. The reaction was left to proceed at 37°C for one hour. The product was then purified (Section 2.5.10) and used for electroporation of electrocompetent cells (Section 2.4.4.2).

2.5.9 Agarose gel electrophoresis

Products of PCR and plasmid digests were separated and visualised on 0.8% agarose gels. Agarose gels were prepared by dissolving an appropriate amount of agarose (Sigma-Aldrich) by heating in 0.5 x TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM

EDTA, pH 8.0). GelRed™ (Biotium, Heyward, USA) was added to the agarose gel solution at a final dilution of 1:10,000 (i.e. 10 µl of the GelRed™ 10,000 x stock reagent added to 100 ml of the gel solution). Gels were run in horizontal tanks containing 0.5 x TBE buffer. 3-10 µl of the DNA sample was mixed with 6 x loading buffer before loading onto the gel and a voltage of 100 volts was applied to separate DNA samples. A 1kb DNA ladder (Fermentas) was used as a marker.

To get an enhanced separation of RFLP digest fragments, the digest products were run on 0.4% agarose gels dissolved in 1% TAE (40 mM Tris acetate and 1 mM EDTA, pH 8.0). GelRed™ was added to the agarose gel solution at a final dilution of 1:10,000 (50 µl in 500 ml gel). 50 µl of digest samples were mixed with 6 x loading buffer and loaded onto a 30 cm length gel. 2.5 µl of a 1 kb DNA ladder was used as a marker. Electrophoresis was carried out in 1% TAE buffer at a voltage of 40 volts for 16-20 hours. DNA bands were visualized under UV light in a transilluminator, and gel images were recorded using a UVP digital camera.

2.5.10 PCR product purification from PCR reactions and agarose gels

A QIAquick PCR Purification Kit (Qiagen) was used to purify DNA from PCR samples and QIAquick Gel Extraction Kit (Qiagen) was use to extract DNA from bands in the agarose. All centrifuging steps were carried out in an Eppendorf bench centrifuge at 17,900 x g at RT. One volume of PCR sample was mixed with 5 volumes of Buffer PBI and loaded onto the QIAquick spin column. The column was centrifuged for 30 seconds and the flow-through was discarded. 0.75 ml of Buffer PE was added to the column for washing and centrifuged for 30 seconds. The flow-through was discarded and the column centrifuged for 1 more minute to ensure removal of all residues of washing buffer. The

PCR product was eluted form the column with 50 µl of TE applied to the centre of the QIAquick membrane and incubation for 1 minute. Then the column was centrifuged for 1 minute to collect the eluted DNA.

For extraction of DNA from agarose gels, the gel slice (containing the DNA band) was weighed and 3 volumes of Buffer QG were added to 1 volume of gel (100 mg ~ 100 µl). The sample was incubated at 56°C until the gel slice had completely dissolved (~ 10 minutes). One volume of isopropanol was added to the sample and the mixture was applied to the QIAquick column and centrifuged for 1 minute. The column was washed and DNA was eluted as described above. After purification DNA was stored at 4°C for use or at -20°C for long-term storage.

2.5.11 Sequencing

PCR and plasmid DNA were sequenced using the ABI PRISM™ BigDye™ Terminator Cycle Ready reaction Kit (Perkin Elmer Applied Biosystems Division, Foster City, CA) on an automated ABI 377 sequencer in the University of Birmingham Functional Genomics Laboratory, Plasmid to Profile sequencing (Birmingham, UK). The sequencing reaction was carried out in a 10 µl reaction volume, consisting of 0.6 µmole primer and template DNA in concentrations as shown below.

	Size in bp	**Quantity in ng**
PCR product	100-200	1-3
	200-500	3-10
	500-1000	5-20
	1000-2000	10-40
	> 2000	40-100
Plasmid DNA	-	200-500

The sequences obtained from the ABI Prism 377 DNA Analyser were analysed with Chromas software (version 1.45, Griffith University, Queensland, Australia). The NCBI BLAST network service was used to compare obtained sequences with that of the published MCMV K181 (Perth) genome sequence (accession number AM886412).

2.6 Cloning

2.6.1 Preparation of UL70 insert and vector

HCMV Merlin was cloned as a BAC in *E. coli* SW102 and designated as PAL1111 (Stanton *et al.*, 2010). UL70 gene fragment was synthesised from the *wt* Merlin BAC genome using the UL70 Fwd and UL70 Rev primers (Table 2.10). The PCR was performed using the high fidelity polymerase (Section 2.5.4) and the product was quantified using the NanoDrop (Section 2.5.4).

The UL70 gene fragment was clonec into pGEM®-T Easy Vector (Promega, Madison, USA). pGEM –T Easy vector and the insert were combined in a 0.5 ml centrifuge tube at a ratio of 1:3. 5 µl of 2x rapid ligation buffer and 1 ml of T4 DNA ligase were added and scaled up to 10 µl using distilled water. The ligation reaction was carried out at RT for 1 hour.

Two µl of ligation reaction was transformed into 100 µl of *XL1-blue* competent cells (Stratagene, La Jolla, California, USA) (Section 2.4.5). Fifty µl of cells were plated on an agar plate containing 20 µg/ml X-Gal, 0.1mM IPTG and 100 µg/ml Ampicillin (Table 2.2) and incubated at 37°C overnight. White colonies (carrying recombinant plasmids) were screened by PCR and the presence of the inserted gene was confirmed by sequencing.

2.6.2 Site-directed mutagenesis

The QuikChange Site-directed Mutagenesis Kit (Stratagene, Cambridge, UK) was used to generate a nucleotide substitution in UL70 gene of HCMV Merlin strain at nt positions 102,094 of leading to the amino acid change cysteine to tyrosine at the position 890 of the UL70 encoded primase. The UL70 gene fragment was cloned in the pGEM –T Easy vector (Section 2.6.1) and two synthetic oligonucleotide primers containing a base substitution (Table 2.10) were designed, according to Stratagene's guidelines and obtained from Eurogentec. The strategy used for site-directed mutagenesis is shown in Figure 2.2.

The mutant strands synthesis reaction was set as follows: 5 µl of 10 x reaction buffer, 60 ng of dsDNA template, 125 ng of each of oligonucleotide primer, 1 µl of dNTP mix, 1 µl of *PfuTurbo* DNA polymerase (2.5 U/µl) and sterile water to a final volume of 50 µl. Cycling conditions were as follows:

	Temperature	**Duration**	**Number of cycles**
Initial denaturation	95ºC	30 seconds	1 cycle
Denaturation	95ºC	30 seconds	18 cycles
Annealing	55ºC	1 minute	
Extension	68ºC	X minutes*	
Final extension	68ºC	7 minutes	1 cycle

* Extension time depends on the length of sequence to be amplified (1 minute per 1 kb of plasmid length).

The oligonucleotide primers, each complementary to opposite strands of the vector, were extended during temperature cycling generating a mutated plasmid with staggered nicks. The parent *wt* DNA was digested with *Dpn*I (Section 2.5.7) and the mutated plasmid was purified (Section 2.5.10) and transformed into *XL1-blue* competent cells (Section 2.4.5). Plasmids were extracted from *XL1-blue* and sequenced to confirm the presence of the mutation.

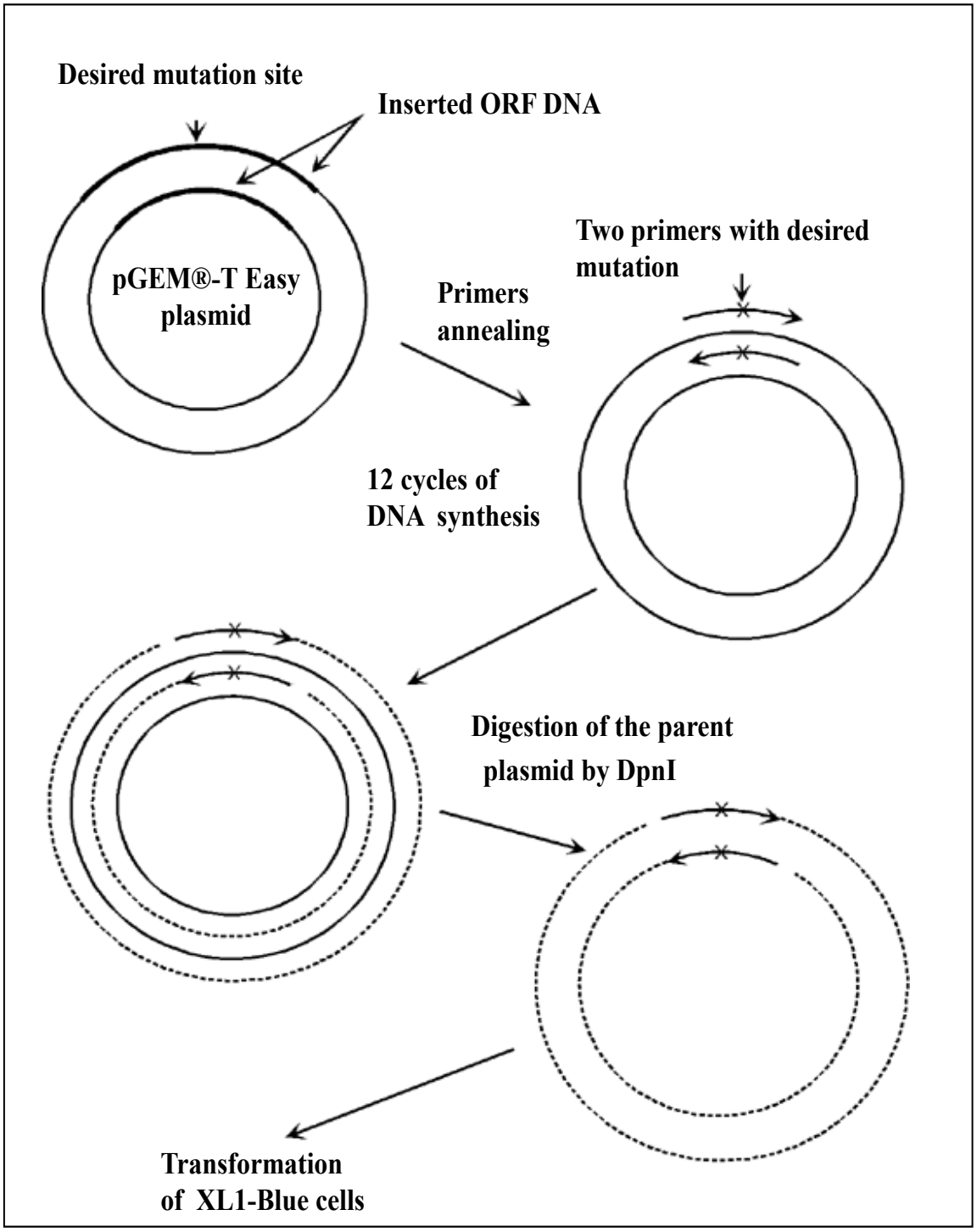


Figure 2.2: Strategy used for the site-directed mutagenesis. Two primers containing the desired mutation anneal to the opposite strands of the vector and are extended by DNA polymerase to produce a mutated plasmid. Parent plasmid is digested with *Dpn*I and mutated plasmids were used for transformation of XL-1 Blue cells.

2.7 BAC mutagenesis using ET homologous recombination

To introduce a mutation into the K181 (Perth variant) of MCMV cloned as a BAC (Redwood *et al.*, 2005), a two-step mutagenesis strategy involving homologous recombination was used as shown in Figure 2.4.

2.7.1 Insertion of the RpsL-neo cassette into the MCMV BAC

A linear RpsL-neo PCR product flanked by homology arms was used in the first step. Using the pRpsL-neo plasmid (Figure 2.3) as template, the RpsL-neo cassette was amplified by PCR (Section 2.5.7) using a primer designed to contain a 51-54 nucleotide homology arm from the 5' end of the gene of interest plus 21-24 nucleotides homologous to the 5' end of the pRpsL-neo plasmid and a similar primer designed to contain 21-24 nucleotides and 51-54 nucleotides of the 3' end of pRpsL-neo plasmid and gene of interest resepectively (Table 2.3). After amplification, the PCR products were run on an agarose gel to confirm that the products had been amplified. To remove any residual template, PCR samples were purified (Section 2.5.10) and digested for one hour with the *Dpn*I restriction enzyme to digest the methylated RpsL-neo plasmid. The PCR products were purified once again and used to transform pARK25 electrocompetent cells (300 ng DNA). 100µl of culture were spread on an LB agar plate containing 12 µg/ml chloramphenicol, 20 µg/ml kanamycin and 50 µg/ml carbanacillin. After an overnight incubation at 30°C, colonies grown on the kanamycin/chloramphenicol plate were streaked on an LB agar plate containing kanamycin (20 µg/ml), streptomycin (80µg/ml) plus chloramphenicol (12µg/ml). The plates were incubated at 30°C overnight to confirm that the RpsL-neo cassette had been inserted. The plate was examined and the colonies which didn't grow on

this plate were identified. The recombinant BAC plasmid DNA was isolated (Section 2.5.3.1) and used for PCR screening to confirm MCMV BAC-RpsL-neo clones.

2.7.2 Replacing the RpsL-neo cassette by linear DNA of the gene of interest carrying the point mutation

A linear DNA fragment derived by PCR from the gene of interest and containing the desired point mutation was prepared by PCR as described above. The product was visualized on an agarose gel, purified and then used to transform cells containing the MCMV K181(Perth) BAC in which the region of interest had been replaced by the RpsL-neo cassette. Electroporated cells were then incubated for at least one hour at 37°C without any antibiotic pressure to give time for cells to recover and allow time for homologous recombination to occur. One hundred µl of culture were spread on an LB agar plate containing streptomycin (80µg/ml), carbanacillin (50 µg/ml) plus chloramphenicol (12µg/ml). After overnight incubation at 30°C, colonies grown were streaked on an LB agar plate containing kanamycin (20µg/ml), streptomycin (80µg/ml) plus chloramphenicol (12µg/ml). The plates were incubated at 30°C overnight. The kanamycin plate was checked and the colonies which didn't grow on this plate were identified. The recombinant BAC plasmid DNA was isolated and used for PCR screening and sequencing in order to confirm that the RpsL-neo cassette had been replaced with the mutated MCMV gene. Revertants were producend by the same two-step process, initially replacing the mutated region of ORF with the RpsL-neo cassette then the cassette was replaced with the wild type linear DNA.

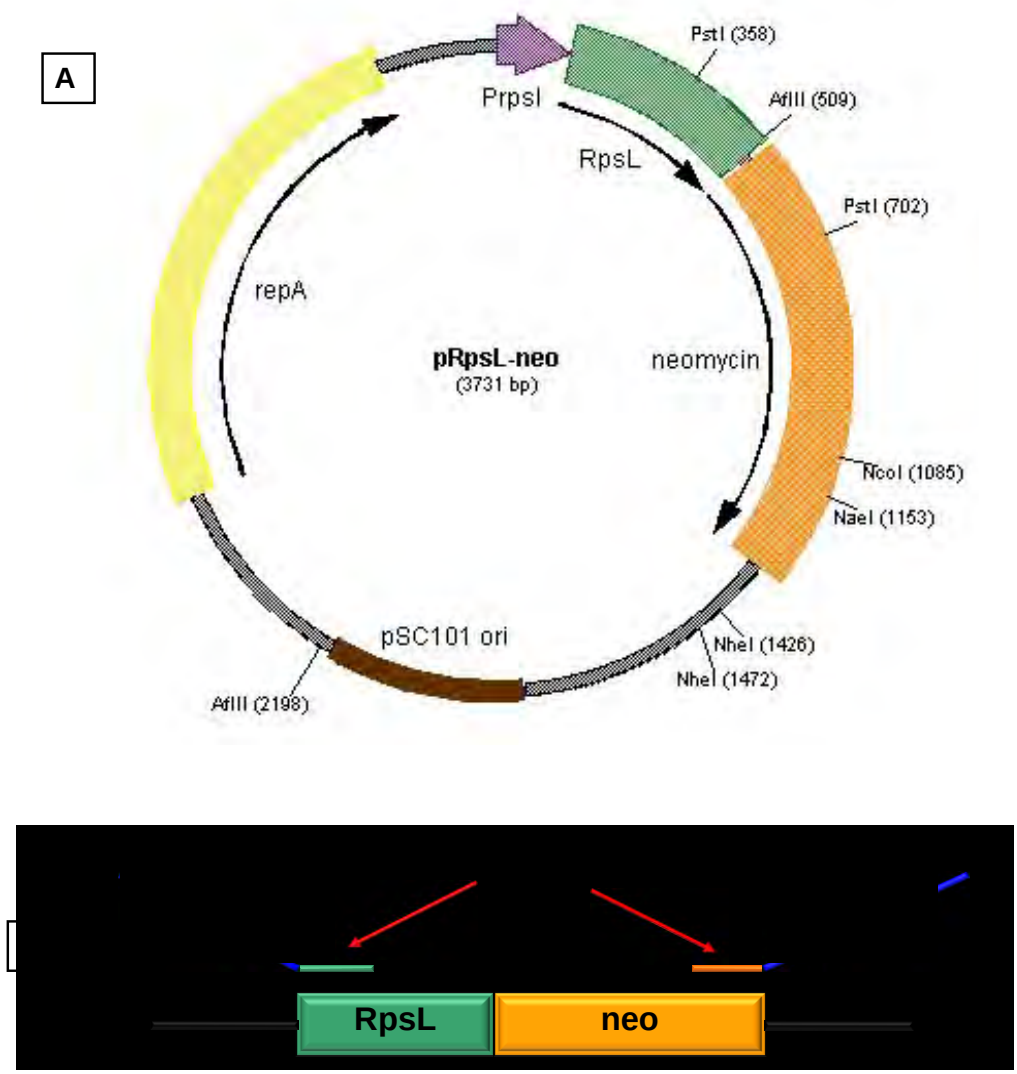


Figure 2.3. Diagram showing: A: pRpsL-neo plasmid containing both the wild-type RpsL gene (green colour) and neomycin resistance gene (orange colour). B: Use of primers with 50 base homology arms (HA of *wt* K181 (Perth) BAC sequence) at the 5'ends and 20 bases of RpsL-neo sequence to generate the RpsL-neo cassette to replace the wild-type sequence of the gene of interest in K181 (Perth) BAC.

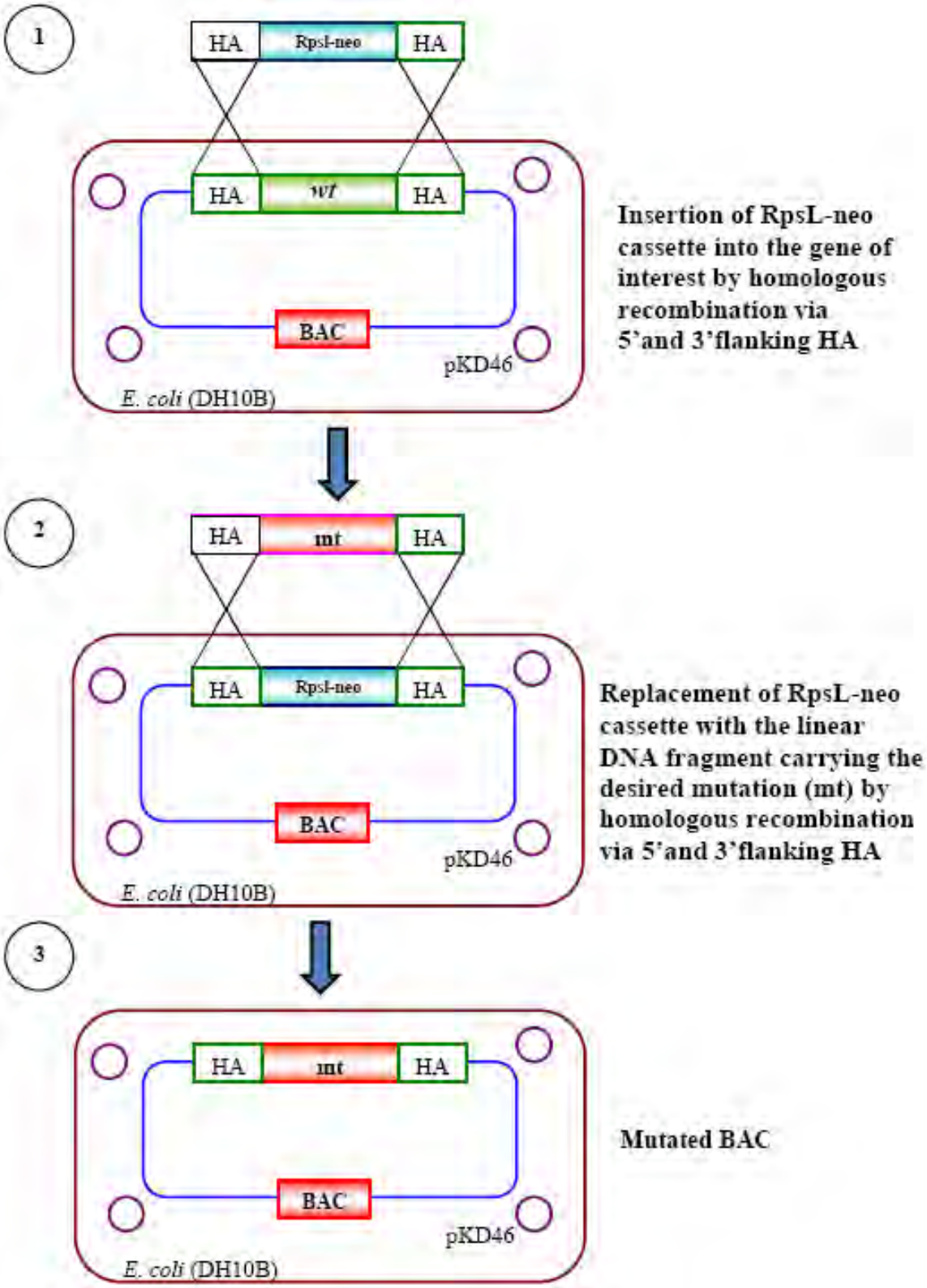


Figure 2.4 Schematic representation of the two steps BAC mutagenesis.

2.8 Comparative genome sequencing

Comparative genome sequencing (CGS) provided by Nimblegen Systems Inc. is a hybridisation-based method of screening whole genomes for changes against a reference genome (Wong *et al*., 2004; Albert *et al*., 2005; Waldmuller *et al*., 2008). In this method, putative mutations are first mapped approximately within the genome. Then, to identify the mutation precisely, each base within the mutated loci is exhaustively screened. Apart from the production of viral DNA, this procedure was essentially performed by Roche NimbleGen as described below.

2.8.1 DNA labelling

MCMV K181 (Birmingham), K181 (Perth), and *tsm5* DNA was extracted from viral particles as described in section 2.5.1. Viral genomic DNA was cleaved to pools of low molecular weight fragments by sonication and labelled with a random prime reaction. One μg of DNA was mixed with 1 O.D. of 5'-Cy5 or 5'-Cy3 labelled random nonamer (TriLink Biotechnologies) in 62.5 mM Tris-HCl, 6.25 mM $MgCl_2$ and 0.0875% β mercaptoethanol, denatured at 98°C for 5 min, chilled on ice and incubated with 100 units of Klenow fragment and dNTP mix (6 mM each in TE buffer) for 2 h at 37°C. Reactions were terminated with 0.5 M EDTA (pH 8.0), precipitated with isopropanol and re-suspended in water.

2.8.2 Mutation mapping microarray design

Based on the published annotated sequence of the K181 (Perth) MCMV variant (Smith *et al.*, 2008); EMBL accession number AM886412), mutation mapping microarrays were designed and produced by NimbleGen (Albert *et al.*, 2005). The microarrays

contained 385,000 29-mer "tiled" oligonucleotides spaced every 7 bases on each strand of the genome sequence (Figure 2.5. A). All oligonucleotides were synthesised in parallel on a two-array set using a Digital Light Processor (Texas Instruments, Plano, Texas, USA) and photoprotected phosphoramidite chemistry (markless array synthesis) (NimbleGen Systems, Madison, WI, USA) in a random probe layout.

2.8.3 Microarray hybridisation

Labelled genomic DNA from the reference virus (*labelled* with Cy5) and from test virus (*labelled* with Cy3) were hybridised in parallel to arrays in 1 x NimbleGen Hybridization Buffer (NimbleGen) for 16 hours at 42°C and washed with non-stringent buffer, centrifuged in a NimbleGen custom centrifuge to remove buffer and then stored until scanned.

2.8.4 Analysis of mapping array data and hybridisation of re-sequencing arrays

Microarrays were scanned at 5 μm resolution using a Genepix® 4000b scanner (Axon Instruments, Union City, CA, USA) and pixel intensities were extracted using NimbleScan™ software (NimbleGen). Hybridisation intensity ratios (reference/test) plotted versus genome position mapped possible mutations sites.

Probes that spanned potential mutations were selected for re-sequencing. Each nucleotide of both the sense and anti-sense DNA strands was interrogated with eight probes (29-39 bases, depending on sequence and calculated T_m), four for each strand of DNA, that differed only with respect to the central position (A, C, G and T) (Figure 2.5B). When target DNA was hybridised to these arrays the perfect match probe will hybridise more strongly than the three corresponding mismatch probes for each strand. This differential

signal intensity between the perfect probe and mismatch probes allows the base to be determined precisely.

These re-sequencing arrays were synthesised, hybridised with labelled genomic DNA from each test line and scanned as above. Sequence base assignments were made using a machine-learning algorithm (Albert *et al.*, 2005). Segments containing mutations in *tsm5* identified by CGS were PCR amplified and verified by conventional sequencing (Sections 2.5.7 and 2.5.11).

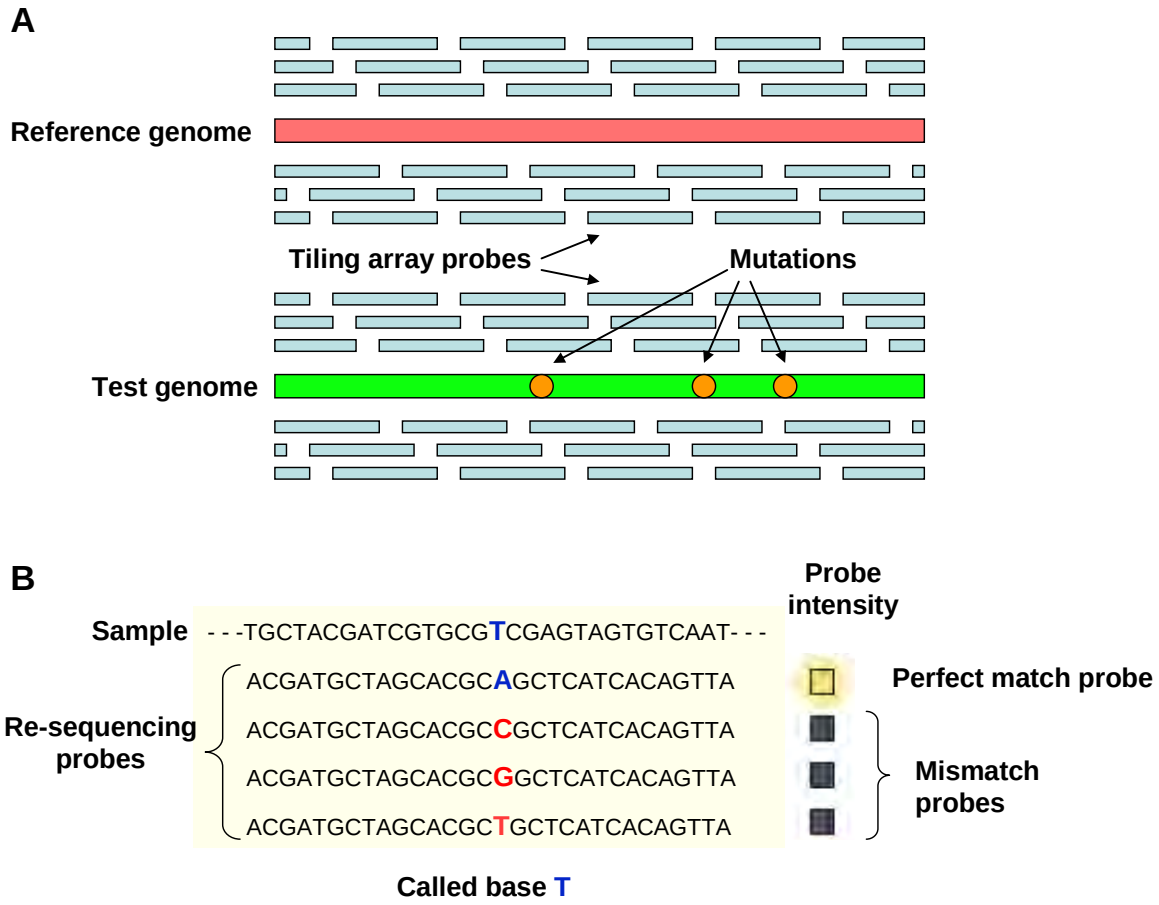


Figure 2.5: Diagram of the CGS process. A. Labelled reference (red) and test (green) genomic DNA were hybridised in parallel to microarrays composed of 29-base-long oligonucleotides (blue) tiled with 7-base spacing. B. Re-sequencing probes are short oligonucleotides in which every possible variant is represented at the central position of the probe (coloured font). Four probes for each strand are used to interrogate each nucleotide position. Hybridisation of the sample with perfect match probe will result in the highest intensity (yellow coloured box) (Modified from Albert *et al.*, 2005 and Gresham *et al.*, 2008).

Chapter 3: **Results**

3.1 Sequence analysis of M27 and M36 Mutations in *tsm5*

Tsm5 was generated in our laboratory from the MCMV K181 (Birmingham) variant by chemical mutagenesis (Sammons & Sweet, 1989). Partial sequencing of the *tsm5* genome revealed point mutations in several genes (Sweet *et al.*, 2007). Among them there were two mutations in immune evasion genes: a C to A mutation in the M27 gene (the IFN signalling inhibitor) and a C to T mutation in the M36 gene (the apoptosis inhibitor) resulting in A658S and V54I residue changes in the two genes respectively. Other mutations were also identified in genes involved in DNA replication, processing and packaging; a C to T mutation (G439R) in the large terminase subunit (M56), a C to T mutation (C890Y) in the primase component (M70) of the helicase-primase complex and a C to T mutation (P324S) in the alkaline nuclease (M98) (Sweet *et al.*, 2007; Timoshenko *et al.*, 2009b).

The aim of this study is firstly to introduce the mutations identified in the M27 and M36 genes, individually and together, into the *wt* virus using the BAC containing the MCMV genome and RecE/T homologous recombination to study their potential role in the phenotype of *tsm5*. The second aim is to identify any other possible mutations in the *tsm5* genome that might be contributing to its phenotype using CGS in which its genome will be compared to its parent K181 (Birmingham) variant and the published MCMV K181 (Perth) variant sequence.

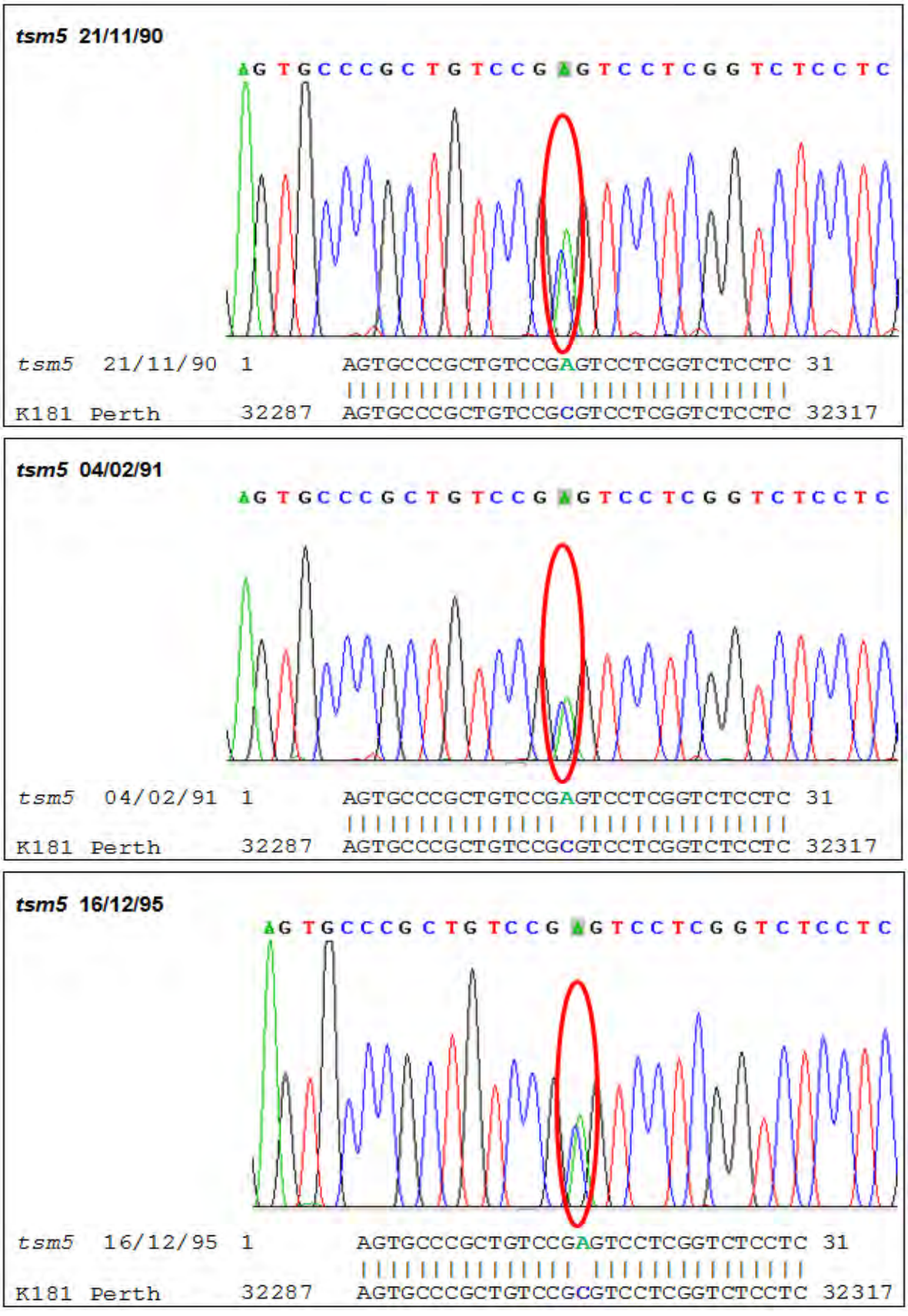
3.1.1 Confirmation of the M27 and M36 mutations in *tsm5* virus stocks

To study the role of the M27 (A658S) and M36 (V54I) mutations identified in *tsm5* in its phenotype they need to be introduced into the *wt* virus and the phenotype of the mutated viruses assessed *in vitro*. Since *tsm5* was to be used as the template for generating PCR products containing the mutations it was important to confirm that stocks of *tsm5* still contained the mutations. As described below it was necessary to sequence the M27 and M36 ORFs in all currently available stocks of *tsm5*.

3.1.1.1 Sequencing of the M27 gene

The M27 gene mutation in *tsm5* is a C to A nucleotide change at base position 32,302 compared to the parent K181 (Birmingham) and the published K181 (Perth) (accession number AM886412) variants of MCMV. The mutation results in an amino acid alanine substitution for serine at position 658 (Sweet *et al.*, 2007). The region spanning the M27 mutation was sequenced in all available stocks (Figure 3.1).

As with *tsm5* virus stock prepared on the 27/07/2004, the sequence of all other available *tsm5* virus stocks prepared at various dates from 21/11/1990, 04/02/1991, 16/12/1995, 22/09/1998 to 22/08/2002 were heterogeneous at base position 32,302 revealing double peaks showing both the mutant A and the *wt* C nucleotides (Figure 3.1).


tsm5 21/11/90
AGTGCCCGCTGTCCGAGTCCTCGGTCTCCTC
tsm5 21/11/90 1 AGTGCCCGCTGTCCGAGTCCTCGGTCTCCTC 31
K181 Perth 32287 AGTGCCCGCTGTCCGCGTCCTCGGTCTCCTC 32317
tsm5 04/02/91
AGTGCCCGCTGTCCGAGTCCTCGGTCTCCTC
tsm5 04/02/91 1 AGTGCCCGCTGTCCGAGTCCTCGGTCTCCTC 31
K181 Perth 32287 AGTGCCCGCTGTCCGCGTCCTCGGTCTCCTC 32317
tsm5 16/12/95
AGTGCCCGCTGTCCGAGTCCTCGGTCTCCTC
tsm5 16/12/95 1 AGTGCCCGCTGTCCGAGTCCTCGGTCTCCTC 31
K181 Perth 32287 AGTGCCCGCTGTCCGCGTCCTCGGTCTCCTC 32317

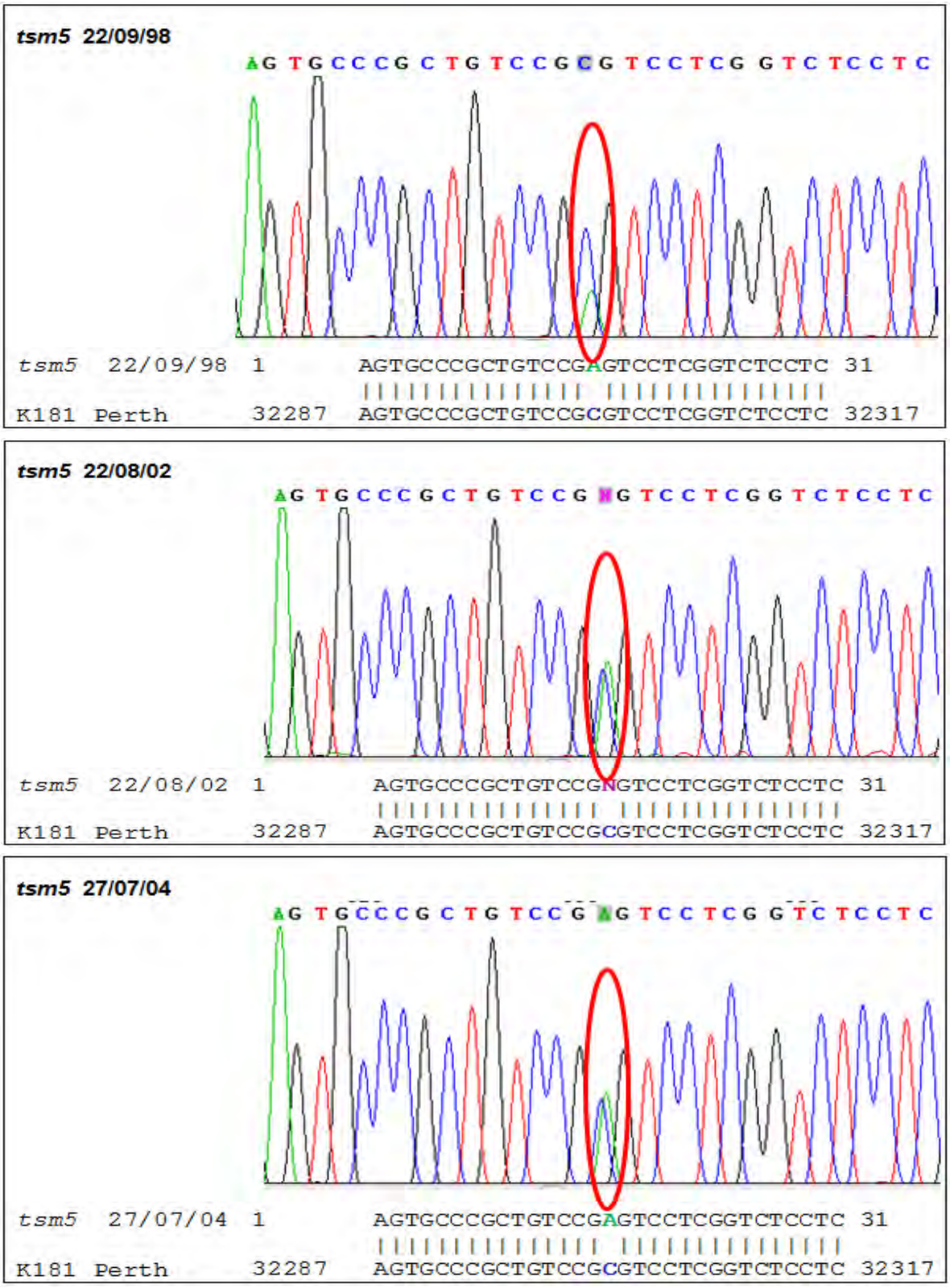
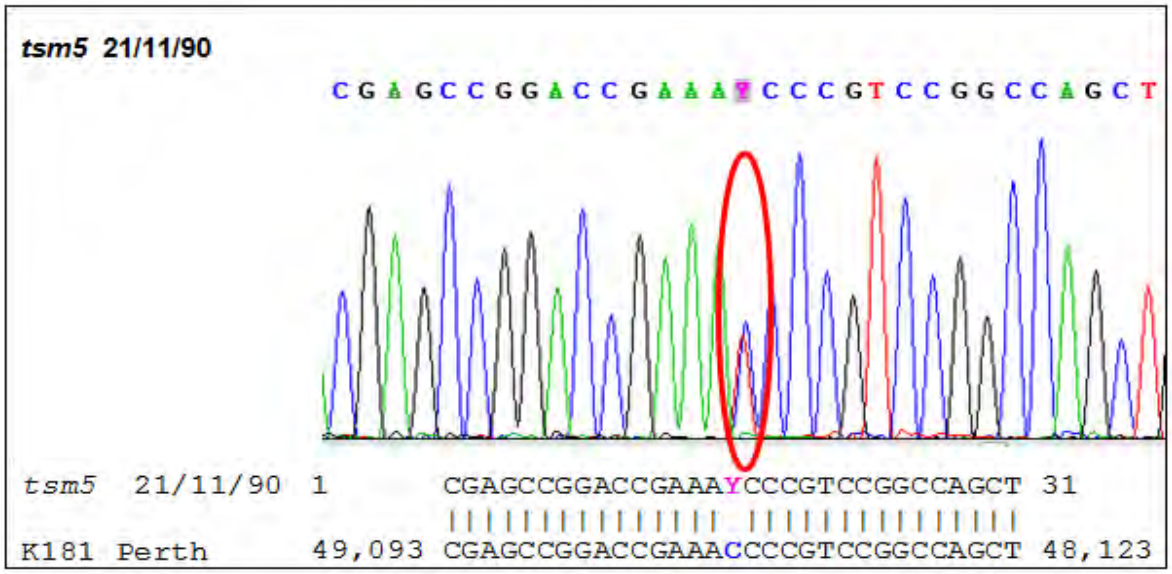


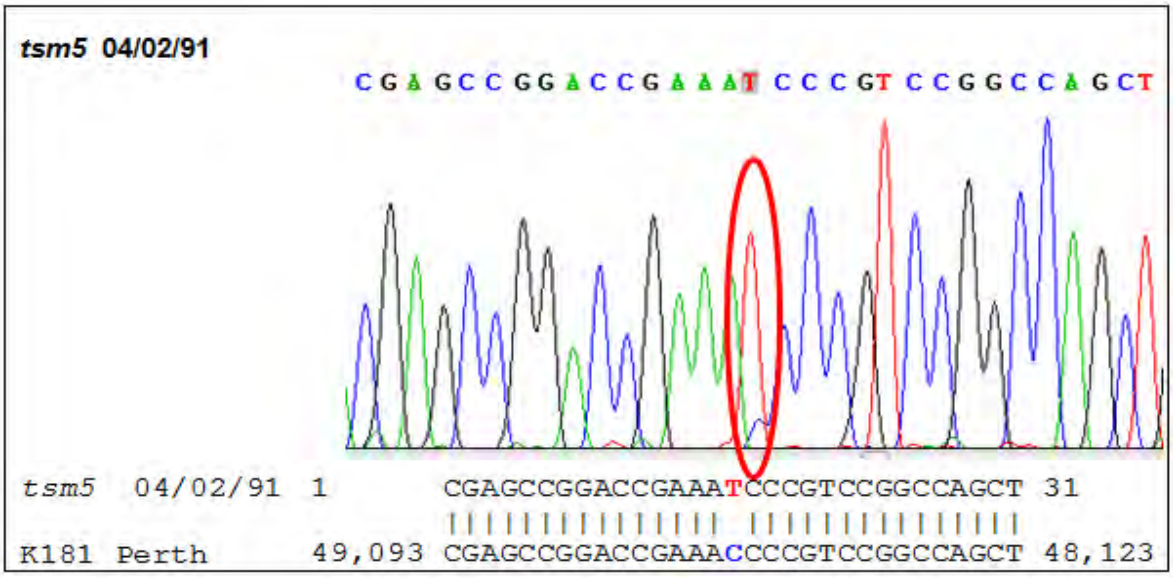
Figure 3.1: Virus stocks produce in 1990, 1991, 1995, 1998, 2002 and 2004 were examined and showed double peaks for the mutated (A) and *wt* (C) nucleotides.

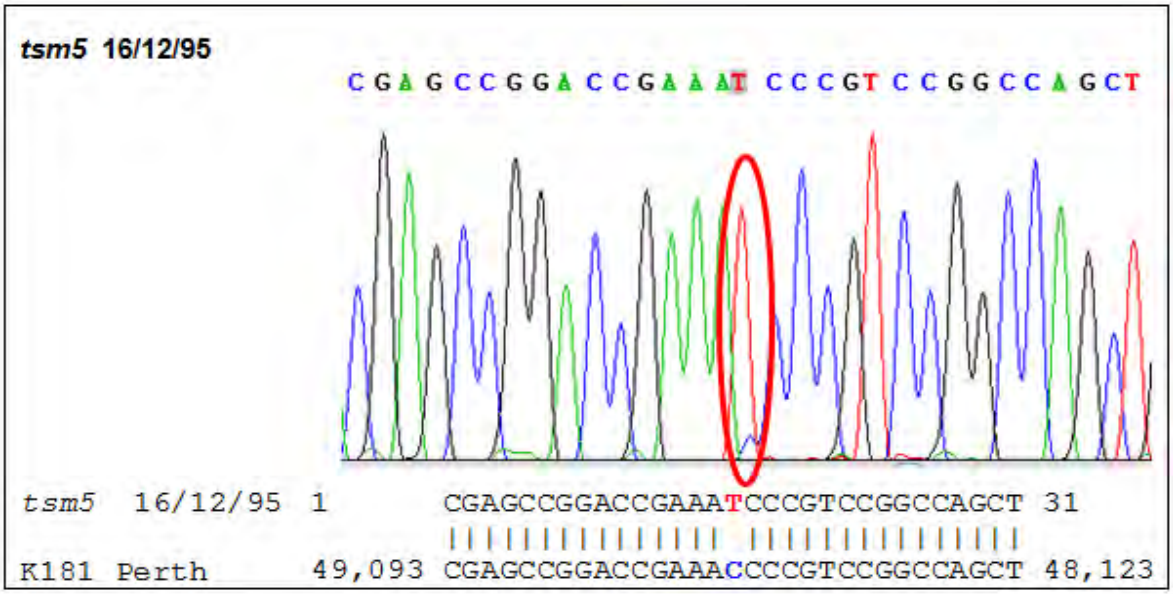
3.1.1.2 Sequencing of the M36 gene

In *tsm5*, the M36 gene mutation is a C to T change at base position 49,108 compared to the parent K181 (Birmingham) and the published K181 (Perth) variants of MCMV. This mutation result in an amino acid valine substitution for isoleucine at residue 54 of the M36 gene (Sweet *et al.*, 2007).

As with the M27 gene, the M36 gene was sequenced in all available *tsm5* stocks 21/11/1990, 04/02/1991, 16/12/1995, 22.09/1998, 22/08/2002 and 27/07/2004. Again the M36 gene showed double peaks at base position 49,108 in most stocks but the 1990 and 2004 stocks contained the mutated nucleotide only (Figure 3.2). The 1990 stock thus was passaged for production of seed and working stocks. DNA extracted from cells infected with this virus stock was re-sequenced, but unfortunately contained a mixed population showing a double peak for M36.


tsm5 21/11/90
CGAGCCGGACCGAAAYCCCGTCCGGCCAGCT
tsm5 21/11/90 1 CGAGCCGGACCGAAAYCCCGTCCGGCCAGCT 31
K181 Perth 49,093 CGAGCCGGACCGAAACCCCGTCCGGCCAGCT 48,123


tsm5 04/02/91
CGAGCCGGACCGAAATCCCGTCCGGCCAGCT
tsm5 04/02/91 1 CGAGCCGGACCGAAATCCCGTCCGGCCAGCT 31
K181 Perth 49,093 CGAGCCGGACCGAAACCCCGTCCGGCCAGCT 48,123


tsm5 16/12/95
CGAGCCGGACCGAAATCCCGTCCGGCCAGCT
tsm5 16/12/95 1 CGAGCCGGACCGAAATCCCGTCCGGCCAGCT 31
K181 Perth 49,093 CGAGCCGGACCGAAACCCCGTCCGGCCAGCT 48,123

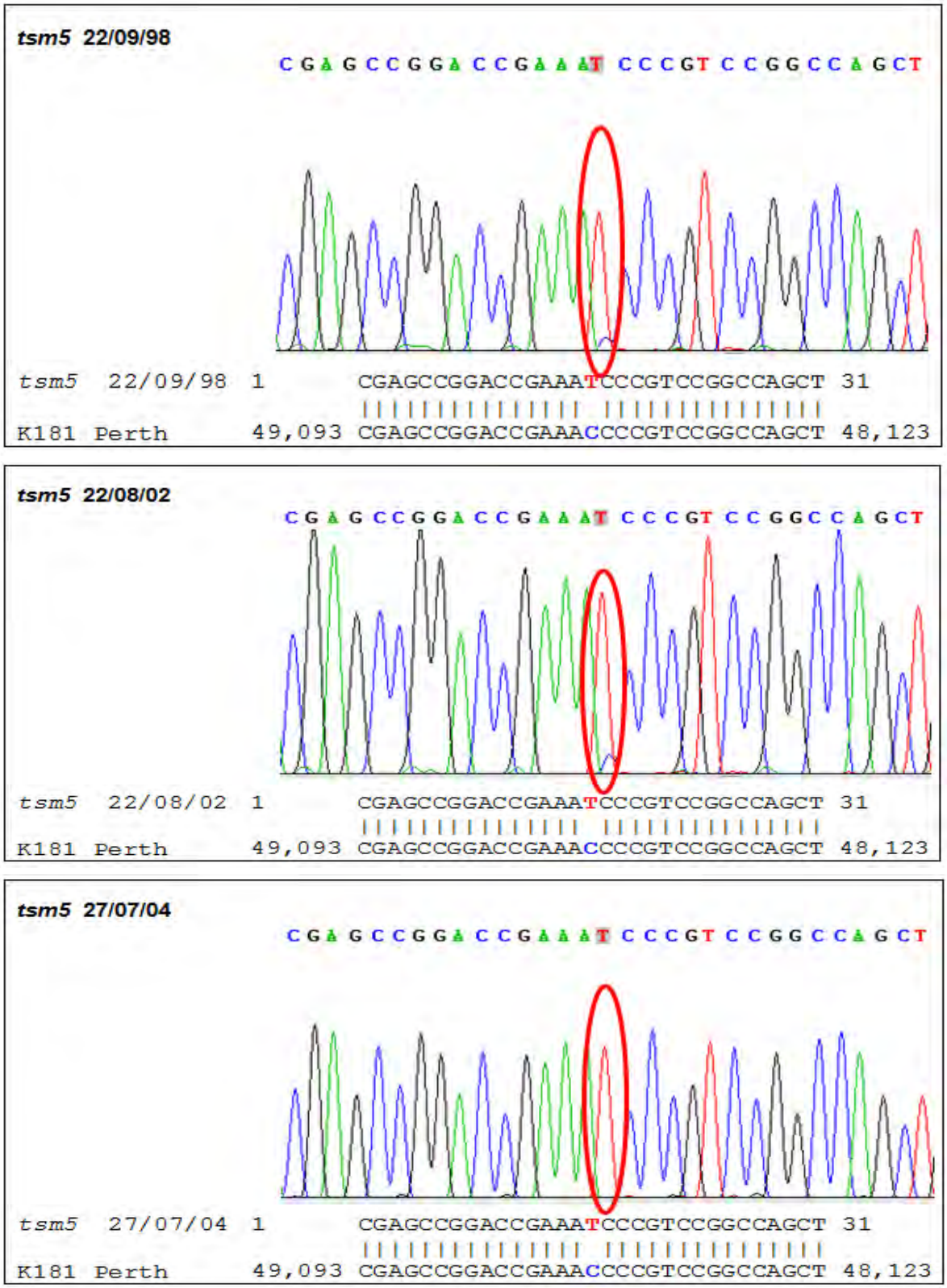


Figure 3.2: Sequence profile of the M36 ORF region of *tsm5* containing the mutation at nucleotide position 49,108 compared to the *wt* K181 (Perth) published sequence. *Tsm5* virus stocks produced in1990, 1991, 1995, 1998, and 2002 were examined and showed double peaks for the mutated (T) and *wt* (C) nucleotides, whereas the stock produced in 2004 contains the mutated (T) only.

Sequence analysis of other genes including M56, M70 and M98 in *tsm5* stocks carried out by Dr. Olga Timoshenko showed that these were also heterogeneous in some stocks while other stocks contained only the mutant or *wt* nucleotide. *Tsm5* contains a C to T nucleotide change in its M56 gene at base position 86,783. This was confirmed in *tsm5* stocks produced in 1990, 1991, and 2004 whereas in the 1995, 1998 and 2002 stocks the sequence showed double peaks.

A nucleotide substitution (C to T) at base position 99,286 of the M70 gene of *tsm5* was initially confirmed in the 1990, 1991 and 2004 stocks but later, after only one passage for preparation of working stocks, showed a mixture of mutant (T) and *wt* (C) nucleotides (Table 3.1). A C to T nucleotide change at base position 143,114 of the M98 gene of *tsm5* was *wt* in stocks prepared in 1990 and 1991 but all other examined stocks were a mixed population of mutant and *wt* nucleotides.

Table 3.1: Summary of sequencing results obtained for *tsm5* virus stocks.

Gene	M27	M36	M56*	M70*	M98*
Base position	32,302	49,108	86,783	99,286	143,114
tsm5 21/11/90	Hetero**	Hetero	Mutant	Hetero	Wild type
tsm5 04/02/91	Hetero	Hetero	Mutant	Hetero	Wild type
tsm5 16/12/95	Hetero	Hetero	Hetero	Hetero	Hetero
tsm5 22/09/98	Hetero	Hetero	Hetero	Hetero	Hetero
tsm5 22/08/02	Hetero	Hetero	Hetero	Hetero	Hetero
tsm5 27/07/04	Hetero	Mutant	Mutant	Hetero	Hetero

* The M56, M70 and M98 genes of the *tsm5* stocks produced in 1990, 1991, 1995, 1998, 2002 and 2004 were sequenced by Dr. Olga Timoshenko.

** **Hetero** – heterogeneous population of viruses containing mutant and *wt* copies of the gene

3.2 Sequence analysis of K181 (Birmingham) and *tsm5* using comparative genome sequencing

Sequencing of some targeted *tsm5* genes identified single point mutations in some of the genes involved in DNA packaging and cleavage (M98, the alkaline nuclease, [P324S]; M56, the large terminase subunit [G439R]; and M70, the primase [C890Y]) as well as some immune evasion genes (M27 [A658S] and M36 [V54I]) (Sweet *et al.*, 2007). To determine if mutations in other genes may also be involved in the attenuation of virus growth at 40^{o}C and *in vivo*, the whole genome (≈ 230kb) of *tsm5* has to be sequenced. Sequencing technology at the time of this work was too expensive as it required sequencing of both *tsm5* and the parent virus K181 (Birmingham) variant to detect any variation of both genomes from the published K181 (Perth) sequence. Instead we considered another comparatively inexpensive and rapid method in which the genomes are screened against a reference sequence [the published K181 (Perth) sequence] called Comparative Genome Sequencing (CGS) provided as a service by NimbleGen Systems Inc (Albert *et al.*, 2005). This technique is a microarray method in which sequence variations are detected as hybridisation signal intensities. It enables the screening of the whole genome and detects any single nucleotide polymorphisms (SNPs) including mutations, insertions or deletions. In this method the SNP loci are first identified then the bases within the identified loci are carefully analysed for any base changes. Recent publication of the K181 (Perth) MCMV genomic sequence (Smith *et al.*, 2008) made this method suitable to be used for re-sequencing of K181 (Birmingham) and *tsm5*, because CGS requires synthesis of a reference oligonucleotide array based on an already established genome sequence.

K181 (Perth), K181 (Birmingham) and *tsm5* (27/07/2004 stock) viruses were provided by Dr. Olga Timoshenko. Viral genomic DNA was prepared by Abdulaziz Alali. Viral genomic DNA was then submitted to NimbleGen Systems Inc.

3.2.1 MCMV K181 (Birmingham) mutation mapping

CGS is a two-step approach in which mutations are mapped approximately as Regions of Interest (ROIs) followed by determination of mutations precisely (Albert *et al.*, 2005). Based on the K181 (Perth) variant published sequence, microarrays containing 385,000 29-mer "tiled" oligonucleotides spaced every 7 nucleotides along the genome on both strands were synthesized by NimbleGen Systems Inc.

DNA from MCMV K181 (Perth), the reference strain, and K181 (Birmingham), the tested strain, was fragmented by sonication then labelled with Cy5 and Cy3 respectively. Both reference and tested labelled DNAs were hybridised in parallel to the designed microarrays. Microarrays were scanned at 5 μm resolution using a Genepix 4000b scanner (Axon Instrument, Union City, CA) and the pixel intensities were extracted using NimbleGen^{TM} software (NimbleGen Systems Inc.). The software is very sensitive and can detect the slightest reduction in hybridisation intensity. The hybridisation intensity ratio (reference/test) for each pair of probes was plotted versus genome position. ROIs were determined by a significant difference in the probe signal intensity.

Based on the determined ROIs, a re-sequencing microarray was designed to investigate all possible sequence differences at single-base pair resolution. Eight probes per base position (four for each strand) were designed to contain all the possible nucleotides at a centrally located position. One of the four probes hybridises with the genomic DNA more strongly than the other corresponding mismatch probes for each strand that allows the

precise determination of the base difference as single nucleotide polymorphisms (SNPs). CGS identified three SNPs in the K181 (Birmingham) at base positions 10,078 (T to G), 40,250 (A to G) and 45,607 (C to G) compared to the published K181 (Perth) sequence (Table 3.2).

PCR products of regions containing these SNPs were sequenced by conventional ABI3799 capillary sequencing and confirmed two of the putative SNPs, the T to G at the base position 10,078 (m10 gene), and the A to G at the base position 40,250 (M32 gene) whereas the third SNP, C to G at the base position 45,607 was not confirmed.

Other genes of the K181 (Birmingham) variant of MCMV had been sequenced in this laboratory previously (Sweet *et al.*, 2007). Eleven mutations were identified in the K181 (Birmingham) variant compared to the published K181 (Perth) variant of MCMV, not all of which were detected by the CGS analysis. The mutations are an A to C polymorphism at position 41,958 (M107L) in the M33 gene; a G to A at position 76,843 (D123D) in M51; a T to C at position 82,062 (D309G) in M54; a G to A at position 89,124 (A919V) in M57; a C to A at 202,998 (Q314H), a C to T at 203,055 (R295R), an A to G at 203,070 (G290G), a TT to CC at 203,182 and 203,183 (K253G), a G to A at 203,238 (Y234Y) and a C to G at position 203,515 (S142T), all in m144 (Table 3.3).

3.2.2 *Tsm5* mutation mapping

For identification of *tsm5* mutations, MCMV K181 (Birmingham), the reference strain, was labelled with Cy5 and *tsm5*, the tested strain, was labelled with Cy3 then both labelled DNAs were hybridised in parallel to the K181 (Perth) designed microarrays. CGS was able to identify fifteen ROIs in *tsm5*, which revealed a total of 10 synonymous and 15 non-synonymous SNPs and 1 mutation characterized as intergenic (Table 3.2). Identified

non-synonymous changes were then examined by conventional ABI3700 capillary sequencing.

Conventional sequencing confirmed 14 out of the 15 non-synonymous SNPs in *tsm5*, while the T to G base change at position 44,715 leading to the amino acid substitution V548G in the M34 ORF is considered false positive. Importantly, three previously observed *tsm5* mutations from sequencing were confirmed by CGS: the C to T mutations at positions 49,108 (V54I) in M36, 99,286 (C890Y) in M70 and 143,114 (P324S) in M98 (Sweet *et al.*, 2007). However, previous sequencing studies of *tsm5* in the laboratory revealed five mutations that were not detected by CGS. Two false negative non-synonymous mutations were observed, the C to A mutation at position 32,302 (A658S) in M27 and the A to G mutation at position 86,783 (G439R) in M56 and three synonymous mutations were not confirmed by CGS: an A to G at position 76,843 (D123D) in M51; an A to G at positions 77,752 (R281R) and 77,791 (L294L) in M52.

Table 3.2: Polymorphisms identified in the K181 (Birmingham) variant compared to the K181 (Perth) variant and mutations identified in *tsm5* by CGS.

Gene	ORF position (nt)[a]		Position of mutation	Nucleotide change	Amino acid change[b]
	From:	To:			
K181 Birmingham polymorphisms (also present in *tsm5*)					
m10	9,624	10,499	10,078	T to G	V152G
M32	39,261	41,426	40,250	A to G	F393L
M34	43,073	45,646	45,607	C to G	A846A[c]
m34.2	45,512	45,844	45,607	C to G	G80R[c]
tsm5 mutations[e]					
m20	20,575	23,028	20,949	C to T	A694T
			22,698	C to T	E111K
m21	22,628	23,317	22,698	C to T	S24F
M25	25,995	28,793	27,523	G to A	R510K
			28,697	G to A	Q920Q[d]
M34	43,073	45,646	44,687	C to T	P539S
			44,715	T to G	V548G[c]
m34.2	45,512	45,844	45,740	G to A	P35P[d]
M36 exon 1	49,031	49,267	49,108	C to T	V54I
M45	59,515	63,039	61,705	G to A	I445I[d]
M47	63,924	67,046	65,612	G to A	Q563Q[d]
			66,658	C to T	A912V
M53	78,456	79,454	79,272	G to A	V273M[f]
M54	79,694	82,987	82,379	G to A	S203S[d]
M69	96,243	98,771	98,442	C to T	R110R[d]
M70	99,060	101,954	99,286	C to T	C890Y[f]
M82	115,757	117,556	115,946	C to T	G537G[d]
M87	127,433	130,213	127,756	C to T	V108V[d]
M97	140,185	142,116	141,258	C to T	G358G[d]
intergenic			142,121	C to T	Promoter of M98?
M98	142,145	143,830	143,114	C to T	P324S
m127	185,267	185,668	185,594	C to T	R25R[d]
m132.1	188,672	189,499	188,683	C to A	G273C
m139	194,162	196,096	194,402	G to T	Y565X[f]
m141	197,787	199,313	198,731	C to T	V195M
m143	201,008	202,639	201,944	C to T	M232I

[a] Gene positions are based on the K181 (Perth) sequence (EMBL Acc. No. AM886412)

[b] Non-synonymous mutations in red font

[c] These mutations were not confirmed by conventional capillary sequencing

[d] These synonymous mutations were not sequenced

[e] The K181 (Birmingham) SNPs were also present in *tsm5*

[f] Conventional capillary sequencing revealed heterogeneous nucleotide

Table 3.3 K181 (Birmingham) variant polymorphisms compared to K181 (Perth) and mutations in *tsm5* compared to K181 (Birmingham) not identified by CGS.

Gene	ORF position (nt)[a]		Position of mutation	Nucleotide change	Amino acid change[b]
	From:	To:			
K181 Birmingham polymorphisms					
M33	41,476	42,770	41,958	A to C	M107L
M51	76,510	77,211	76,843	G to A	D123D
M54	79,694	82,987	82,062	T to C	D309G
M57	88,304	91,879	89,124	G to A	A919V
m144	202,788	203,939	202,998	C to A	Q314H
m144	202,788	203,939	203,055	C to T	R295R
m144	202,788	203,939	203,070	A to G	G290G
m144	202,788	203,939	203,182-203,183	TT to CC	K253G
m144	202,788	203,939	203,238	G to A	Y234Y
m144	202,788	203,939	203,515	C to G	S142T
tsm5 mutations[c]					
M27	32,225	34,273	32,302	C to A	A658S
M52	76,910	78,463	77,752	A to G	R281R
			77,791	A to G	L294L
M56	85,701	88,097	86,783	C to T	G439R

[a] ORF positions are based on the K181 (Perth) sequence (EMBL Acc. No. AM886412)

[b] Non-synonymous mutations highlighted in red

[c] The K181 (Birmingham) SNPs were also present in *tsm5*

3.3 Construction of recombinant viruses

The BAC cloned MCMV K181 Perth variant genome was used for construction of recombinant MCMVs by targeted mutagenesis (Redwood *et al.*, 2005). The homologous recombination technique was used for introduction of mutations into the MCMV K181 (Perth) BAC (Figure 2.3) (Lee *et al.*, 2001; Yu *et al.*, 2000a). The recombinant viruses were then reconstituted by transfection of the corresponding BAC plasmids into mouse fibroblasts as described (Section 2.3.1). The recombination-deficient *E. coli* DH10B strain was used for propagation of the MCMV K181 Perth variant genome BAC used for homologous recombination following transformation with the plasmid pKD46 that expresses lambda Red recombination proteins that are induced by an L-arabinose-inducible promoter (Datsenko & Wanner, 2000). Because of a mutation in the RpsL gene, *E. coli* strain DH10B is resistant to streptomycin, so it can be grown selectively on media containing streptomycin. However, the effect of the mutated RpsL gene can be overridden by the wild-type RpsL gene (introduced on a plasmid) resulting in susceptibility of the bacteria to streptomycin.

In the beginning of this study our intention was to study the role of the identified *tsm5* M27 (C to A at nt 32,302) and M36 (C to T at nt 49,108) mutations in its phenotype by introducing these two mutations separately and together into the wild-type MCMV K181 (Perth) BAC and reconstitution of the mutated recombinant viruses (Figure 2.4 & Figure 3.3) (Sweet *et al.*, 2007). However, following the determination of other mutations present in the *tsm5* using CGS, we decided to include three other immunoevasion genes m139, m141 and m143, in our study. A G to T substitution at base position 194,402 in m139 results in the introduction of a stop codon instead of tyrosine that lead to a truncation of the m139 protein product at residue 565. In m141, a C to T base change at base position

198,731 resulted in a valine substitution with methionine at residue 195. Whereas in the m143 gene a C to T mutation at base position 201,944 resulted in a methionine substitution with isoleucine at residue 232 (Timoshenko *et al.*, 2009a).

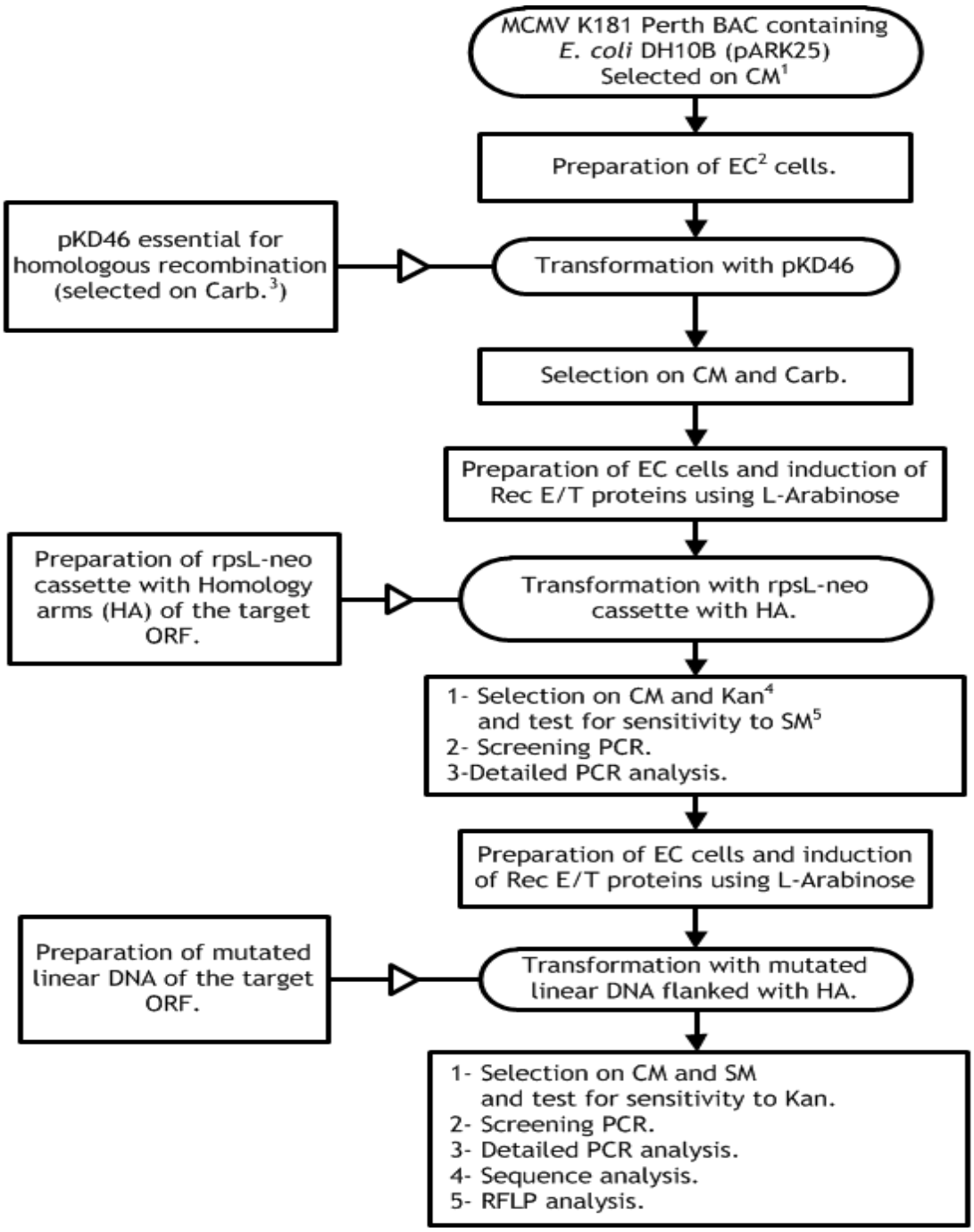


Figure 3.3 : Flowchart showing the strategy followed for construction and analysis of MCMV mutants starting with pARK25 *E.coli* containing the MCMV BAC. Mutants and revertants were constructed in a similar manner starting with the constructed mutants.

1- CM: chloramphenicol. 3- Carb: Carbenicillin. 5- SM: Streptomycin.

2- EC: Electrocompetent. 4- Kan: Kanamycin.

3.3.1 Construction of mutant and revertant BACs

To overcome the problem of heterogeneity of the M27 and M36 ORFs in all examined *tsm5* stocks and the presence of both mutant and *wt* bases represented with double peaks at base position 32,302 and 49,108 respectively, a cosmid library of *tsm5* that had been generated previously in our labo (Sweet *et al.*, 2007) was examined. Sequencing of cosmid H that represents the region nt 29970-nt71089 confirmed that both mutations of interest for the M27 and M36 ORFs were present (Figure 3.4 & Figure 3.5). Cosmid H was then used as a template for generation of mutated linear DNA for each of these two genes to be used for construction of Mt[M27^{A658S}], Mt[M36^{V54I}] and Mt[M27^{A658S} M36^{V54I}] mutant BACs.

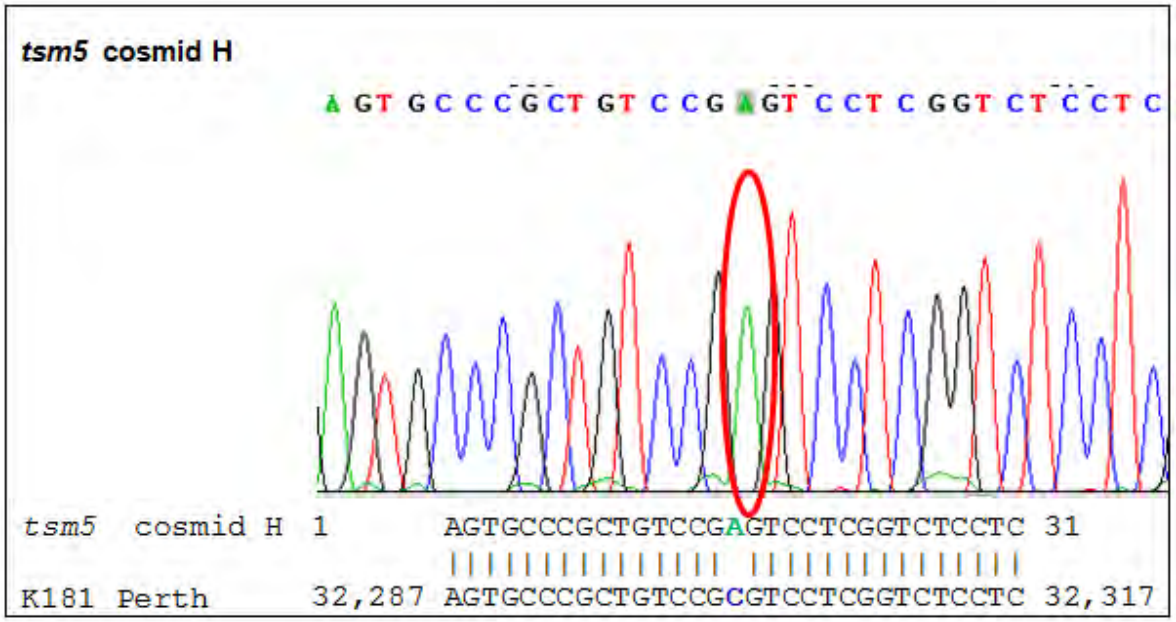


Figure 3.4: The sequence profile of region of interest in the M27 gene in Cosmid H showing the presence of the mutation.

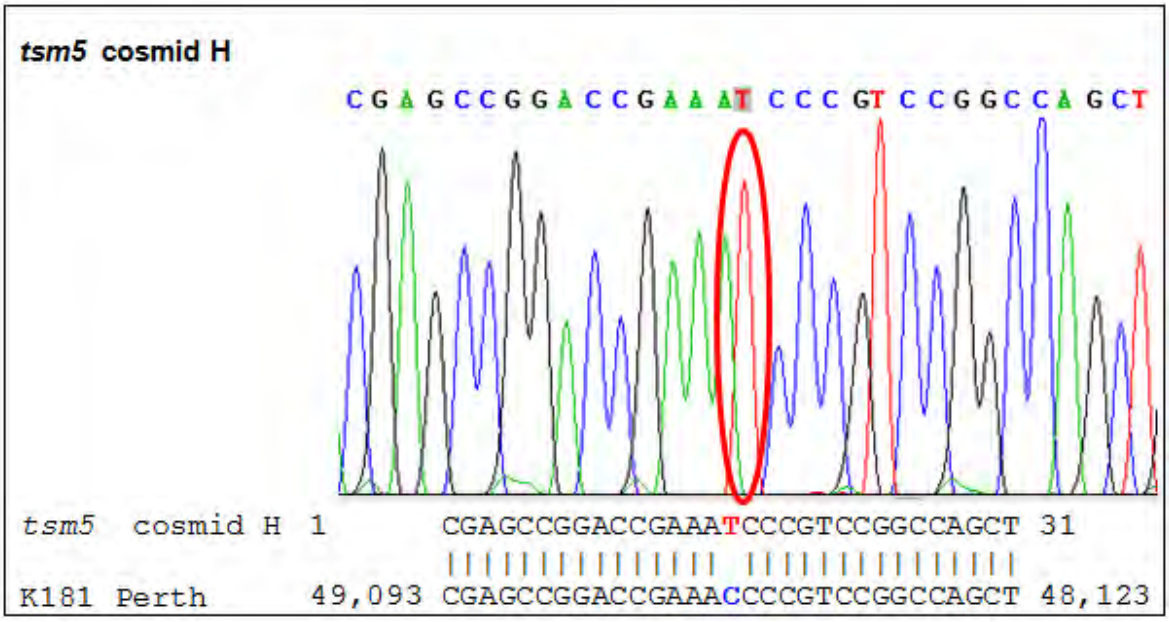


Figure 3.5: The sequence profile of region of interest in the M36 gene in Cosmid H showing the presence of the mutation.

Mutated linear DNA fragments for the m139, m141, and m143 genes were generated from the *tsm5* (27/07/2004) stock as the stock contained the mutated nucleotide for all 3 genes (Figure 3.6). The generated mutated linear DNA for each of these three genes was then used to construct Mt[m139^{Y565X}], Mt[m141^{V195M}] and Mt[m143^{M232I}] mutant BACs.

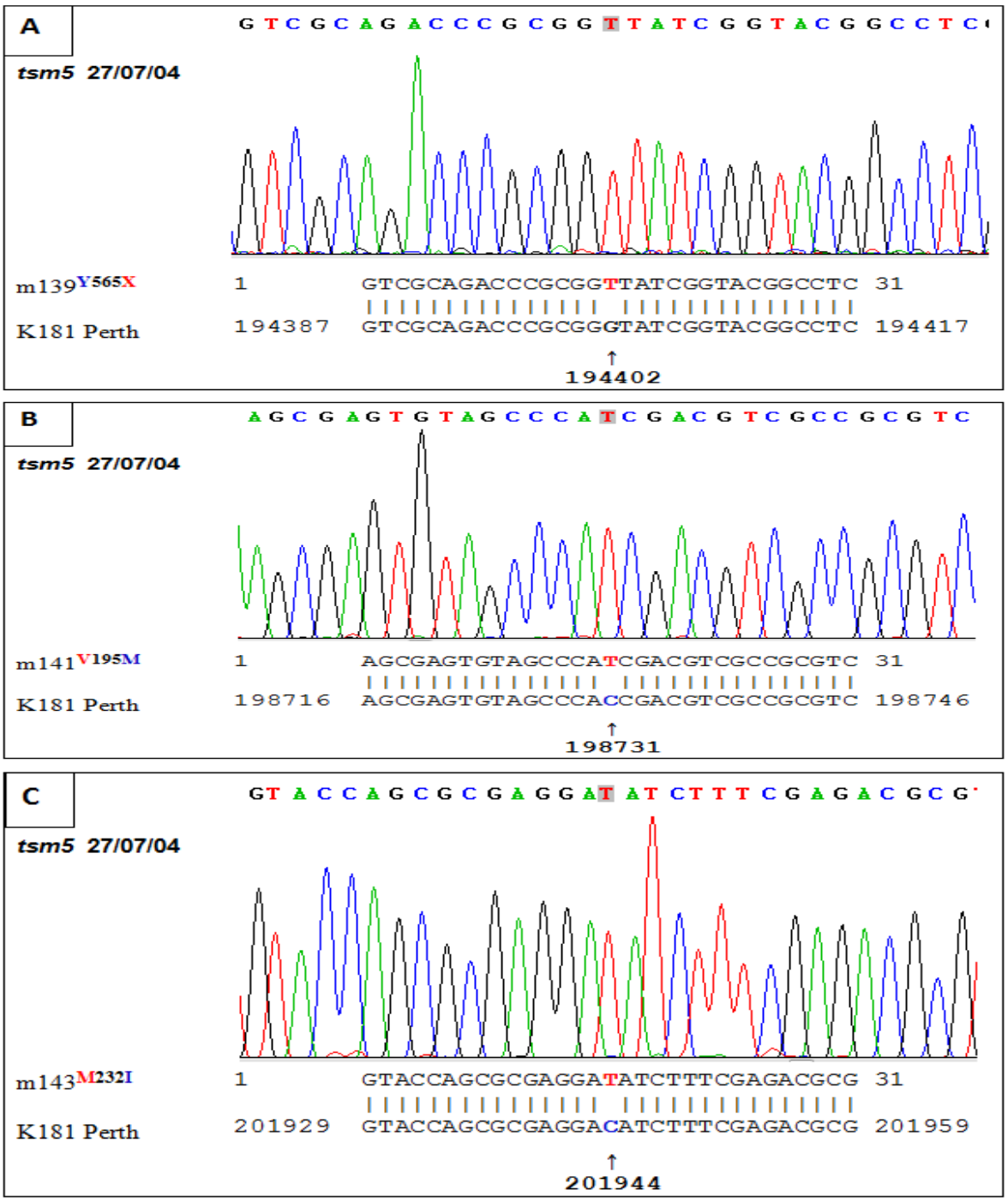


Figure 3.6 Sequence profile of A; m139, B; m141and C; m143 mutations in the *tsm5* (27/07/2004) stock showing: a G to T; base substitution in m139 and a C to T base substitution in both the m141 and m143 genes.

In the first mutagenesis step, a fragment of about 500 bp of the gene of interest spanning the position of the potential mutation was replaced with a linear DNA fragment (1,320 bp) derived from the RpsL-neo cassette by PCR; this fragment contains a kanamycin resistance and streptomycin sensitivity cassette flanked with sequences of 51-52 nucleotides homologous to each end of the target gene locus of the K181 (Perth) BAC (Section 2.7). This cassette was PCR amplified using ET M27F+R, ET M36F+R, ET m139F+R, ET m141F+R and ET m143F+R primers with the flanking 52 bases (Table 3.4) for M27, M36, m139, m141 and m143 respectively.

Table 3.4: Primers used for amplification of RpsL-neo cassette.

ORF	RpsL-neo cassette amplification primers (1,320 bp)		RpsL-neo cassette replacing the segment at position		Replaced segment size (bp)	Construct designated name
	Forward primer	Reverse primer	Start	End		
M27*	ET M27F	ET M27R	31989	32559	570	M27 RpsL-neo
M36*	ET M36F	ET M36R	48819	49359	540	M36 RpsL-neo
m139	ET m139F	ET m139R	194089	194641	552	m139 RpsL-neo
m141	ET m141F	ET m141R	198459	198961	502	m141 RpsL-neo
m143	ET m143F	ET m143R	201671	202151	480	m143 RpsL-neo

* M27 and M36 mutations were introduced into constructs both individually and together.

Following insertion of RpsL-neo cassette into the target genes of the wild type BACs, bacterial isolates with BACs containing the RpsL-neo cassette were then selected based on their resistance to kanamycin and streptomycin sensitivity phenotype. The isolates were then PCR screened for RpsL-neo cassette presence using viral genome primers just outside the flanking arms at both ends. Because the RpsL-neo cassette is ~800 bp bigger than the replaced viral gene fragment, a larger PCR product obtained using primers flanking the insertion site would indicate an RpsL-neo cassette insertion (Figure 3.7).

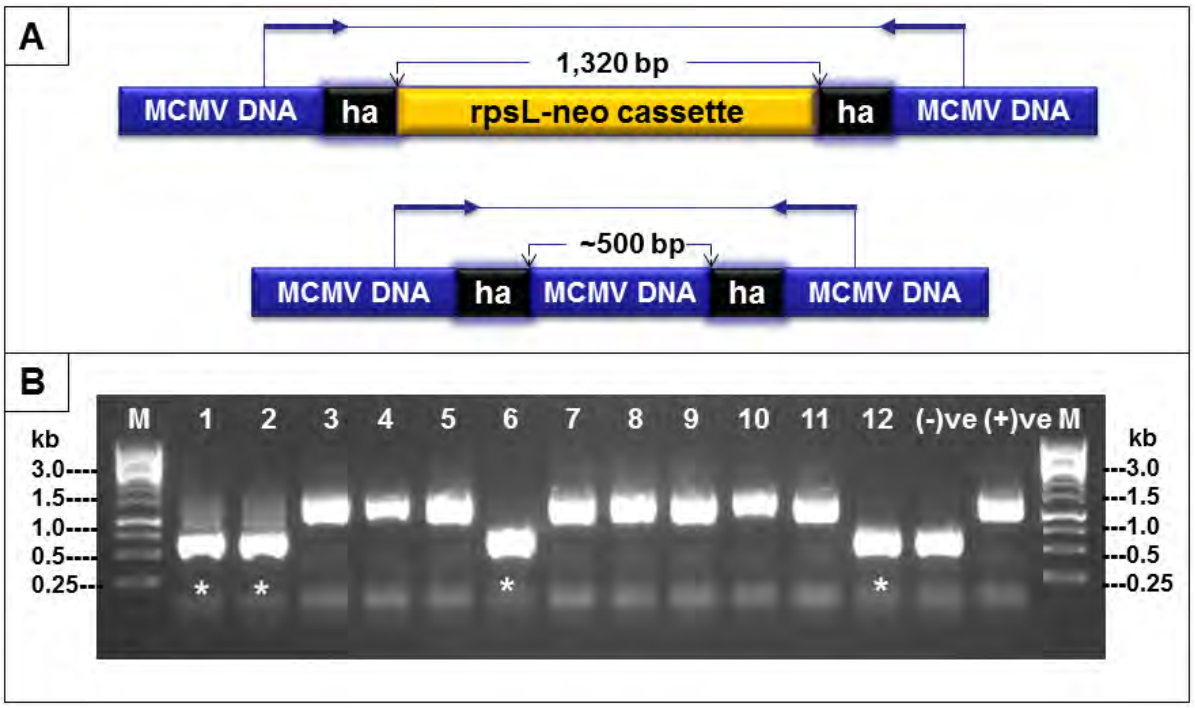


Figure 3.7 PCR screening of the rpsL-neo cassette insertion into the MCMV BAC using primers outside the recombination region. A. schematic diagram showing the difference in the expected product size before and after insertion or replacement of RpsL-neo cassette using primers outside the recombination region. B; PCR screening of 12 different isolates for replacement of the *wild-type* region of m141 gene segment with the RpsL-neo cassette in the gene of interest giving PCR products that are about 800 bp larger than the PCR products from the *wt* K181 BAC [(-)ve]. Isolates in which the RpsL-neo cassette insertion was unsuccessful are indicated by the stars. Lane (+)ve is the RpsL-neo positive control. Molecular size markers (lanes M), in kilobases are included.

A thorough PCR examination was then performed for isolates with apparent successful RpsL-neo cassette insertion (showing larger bands in the screening PCR) in the target gene using four pairs of primers. To confirm the insertion of the RpsL-neo cassette into the target gene, two pairs of primers are used where in each pair one primer binds to the RpsL-neo cassette and the other to the viral DNA sequence outside the recombination region. Another two pairs of primers are used to confirm the absence of the *wt* K181 BAC sequence where in each pair one primer binds to the replaced *wt* segment sequence and the other to the viral DNA sequence outside the recombination region. Positive and negative controls were routinely used for each PCR (Figure 3.9 – Figure 3.14).

In the second round of mutant construction, the rpsL-neo cassette was replaced with a PCR amplified linear fragment containing the desired mutation. The M27 and M36 gene linear fragments were amplified from the *tsm5* virus cosmid H DNA whereas m139, m141 and m143 gene fragments were amplified from *tsm5* virus stock 27/07/2004 DNA. Bacterial isolates showing a streptomycin resistant and kanamycin sensitive phenotype (as a result of losing the RpsL-neo cassette) were selected and BACs extracted for PCR screening and analysis. Constructs with the desired mutations were designated as mt[M27^{A658S}], mt[M36^{V54I}] , mt[M27^{A658S}M36^{V54I}] mt[m139^{Y565X}], mt[m141^{V195M}] and mt[m143^{M232I}] BACs.

3.3.2 PCR analysis and sequence confirmation of mutant constructs

PCR screening of recombinant BACs with the rpsL-neo cassette replaced by the M27, M36, m139, m141, or m143 mutated gene fragments, respectively, was performed using primer sets outside the flanking arm of the RpsL-neo cassette (Figure 3.8A). Primers M27F5+RD, M36F2+R, m139F3+R1, m141 F1+R1 and m143 F1+R1 were used for generation of the linear mutated segments of viral DNA giving a PCR products of 819, 1153, 904, 761, and 727 bp respectively. Following proper recombination, the sizes of the PCR products indicated the replacement of the rpsL-neo cassette (~1500 bp) with the correct viral sequence (Figure 3.8B).

To confirm the replacement of the RpsL-neo cassette in the target gene with the linear mutated segments of viral DNA, two pairs of primers are used where one primer of each pair binds to the inserted linear mutated viral DNA segment and the other to the viral DNA sequence outside the recombination region. Another two pairs of primers are used to confirm the absence of the RpsL-neo cassette sequence where one primer of each pair binds

to the replaced RpsL-neo cassette sequence and the other to the viral DNA sequence outside the recombination region. Positive and negative controls were routinely used for each PCR (Figure 3.9 - Figure 3.14).

Furthermore, each gene manipulated by mutagenesis of these BACs was sequenced to ensure the presence of the desire mutation in each manipulated gene. Sequence studies of mt[M27^{A658S}], mt[M36^{V54I}], mt[m139^{Y565X}], mt[m141^{V195M}] and mt[m143^{M232I}] BACs confirmed the presence of mutations at base position 32,302 (C to A), 49,108 (C to T), 194,402 (G to T), 198,731 (C to T) and 201,944 (C to T) respectively (Figure 3.10 – Figure 3.14).

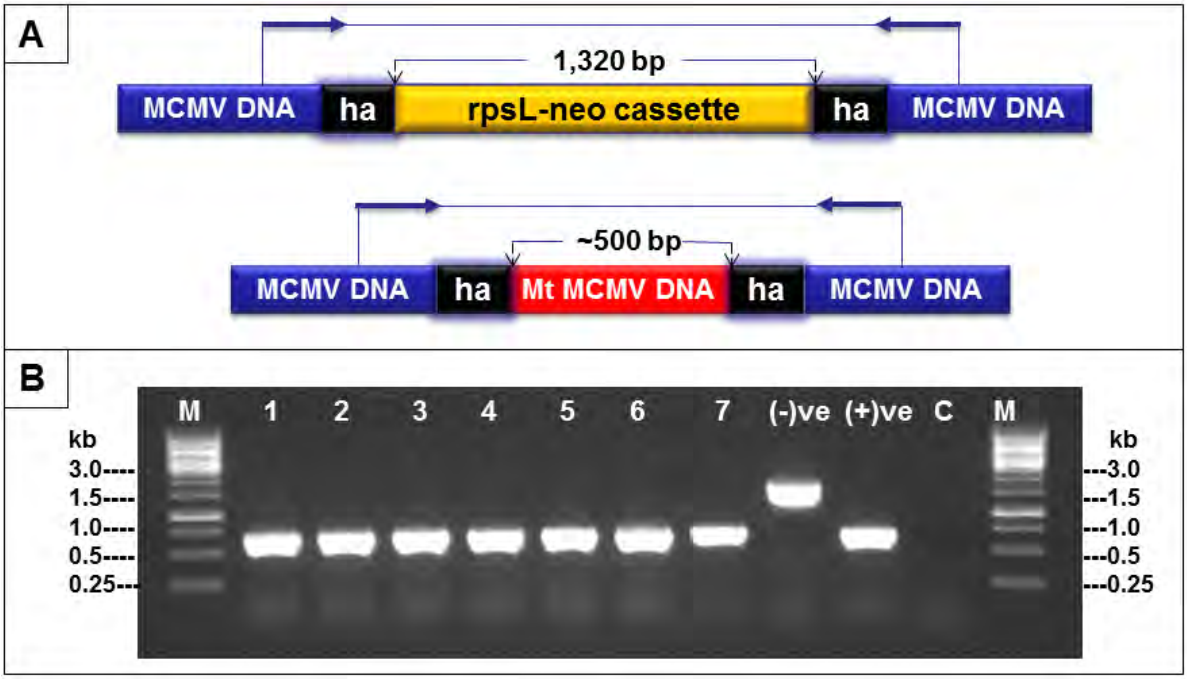


Figure 3.8. PCR screening for the RpsL-neo cassette replacement with the mutated linear DNA segment in the MCMV BAC using primers outside the recombination region. A. Schematic representation of the primer binding sites. ha – homology arms. B; PCR screening of 7 different isolates for replacement of RpsL-neo cassette in the m141 gene segment with MCMV sequence containing the desired mutation which gives PCR products that are about 800 bp smaller than the PCR products from the RpsL-neo cassette [(-)ve]. Lane (+)ve is MCMV segment positive control and (C) is a negative control (PCR with no DNA). Lanes M contain molecular size markers (in kilobases).

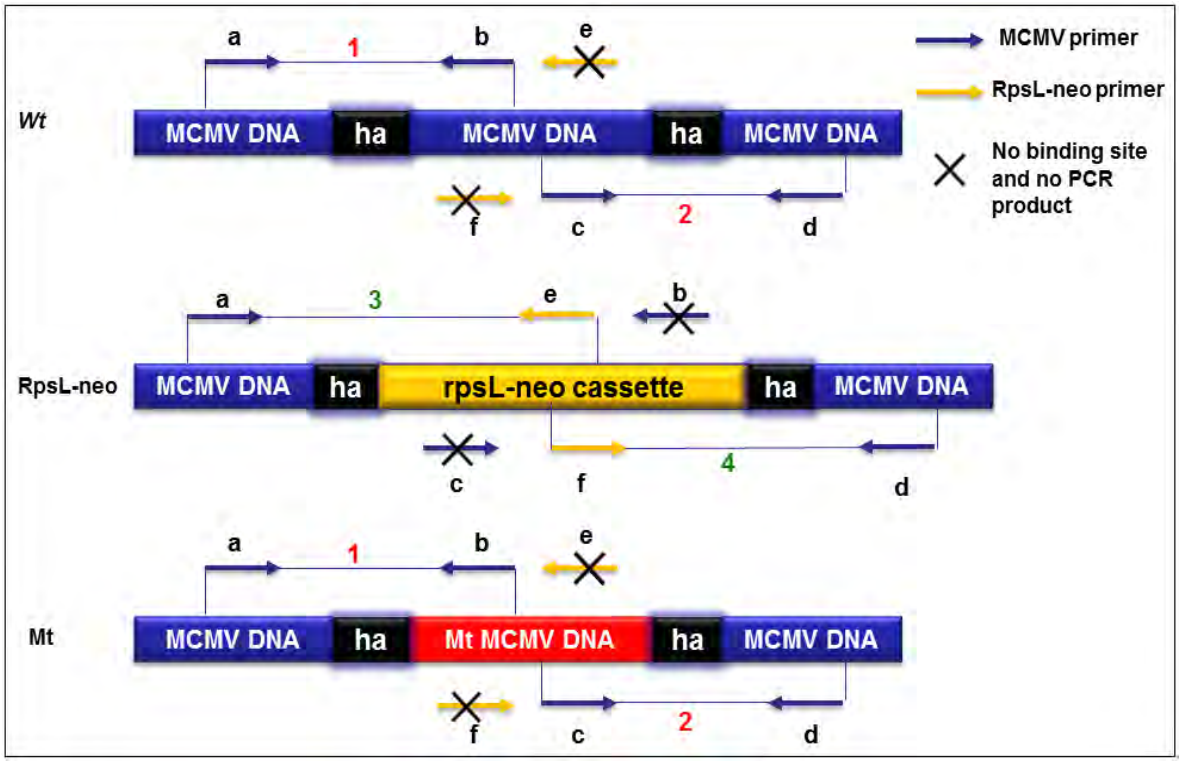


Figure 3.9 Schematic representation of the detailed PCR analysis and the primer binding sites in the manipulated gene. K181 Perth sequence primers (a+b and c+d) are used in the **1st** and **2nd** PCR reaction that will give products in the presence of *wt* K181 (Perth) or mutated sequence in the region of the gene of interest, whereas after replacement of the *wt* K181 (Perth) sequence with the RpsL-neo cassette these combinations of primers do not give any products. Primer combinations (a+e and f+d) are used in the **3rd** and **4th** PCR to verify the successful insertion of RpsL-neo cassette in the gene of interest (agarose gel images are shown in the subsequent figures).

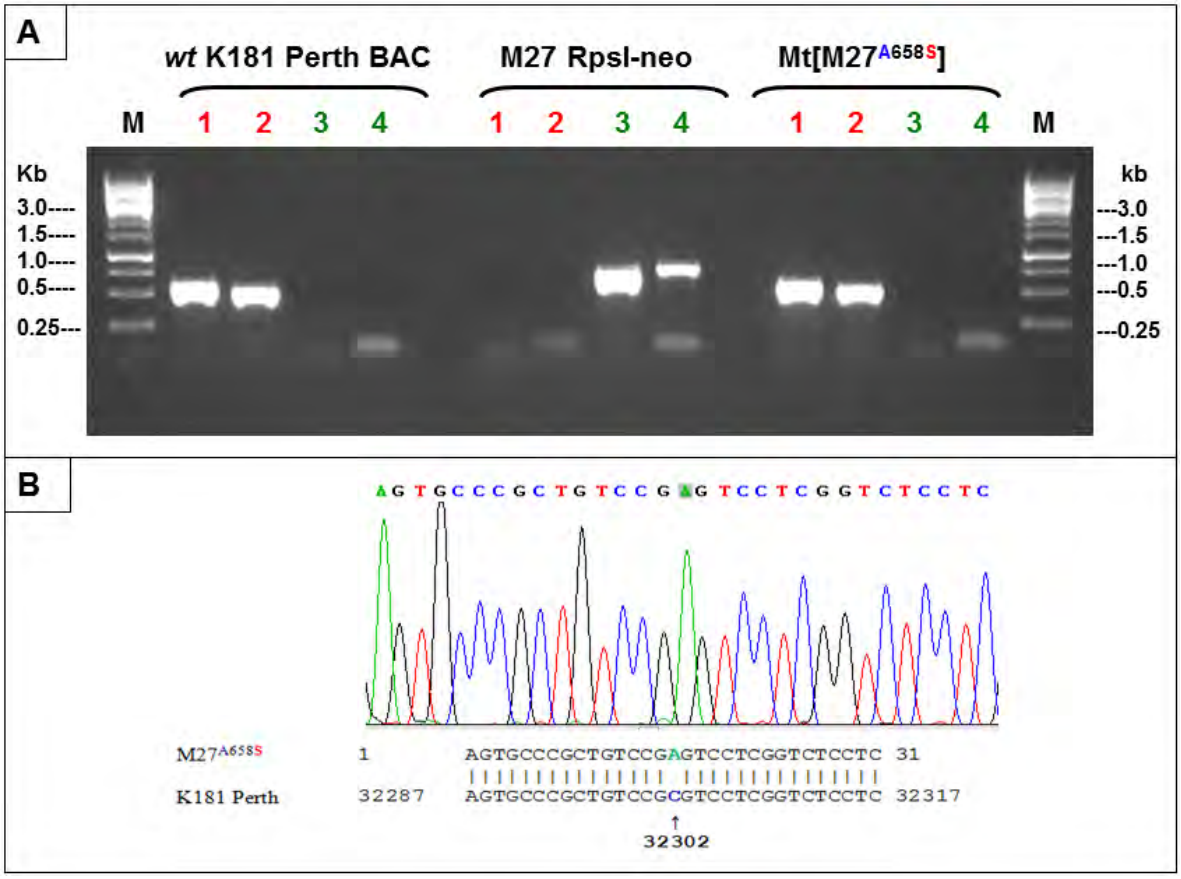


Figure 3.10 Mt[M27^{A658S}] construction. A. Shows the detailed PCR analysis (Figure 3.9) and the steps for the M27 mutant construction starting with the *wt* K181 (Perth) BAC, M27 RpsL-neo cassette (insertion of the RpsL-neo cassette), Mt[M27^{A658S}] (replacement of the RpsL-neo cassette with the mutated M27 ORF) using M27 specific primers in the **1st** and **2nd** PCR reactions, and RpsL-neo specific primers in the **3rd** and **4th** PCR reactions. Lanes M contain molecular size markers (in kilobases). B. The sequence profile of the M27 gene in Mt[M27^{A658S}]showing that the mutation of interest (C to A) has been inserted at base position 32,302 of the K181 (Perth) BAC.

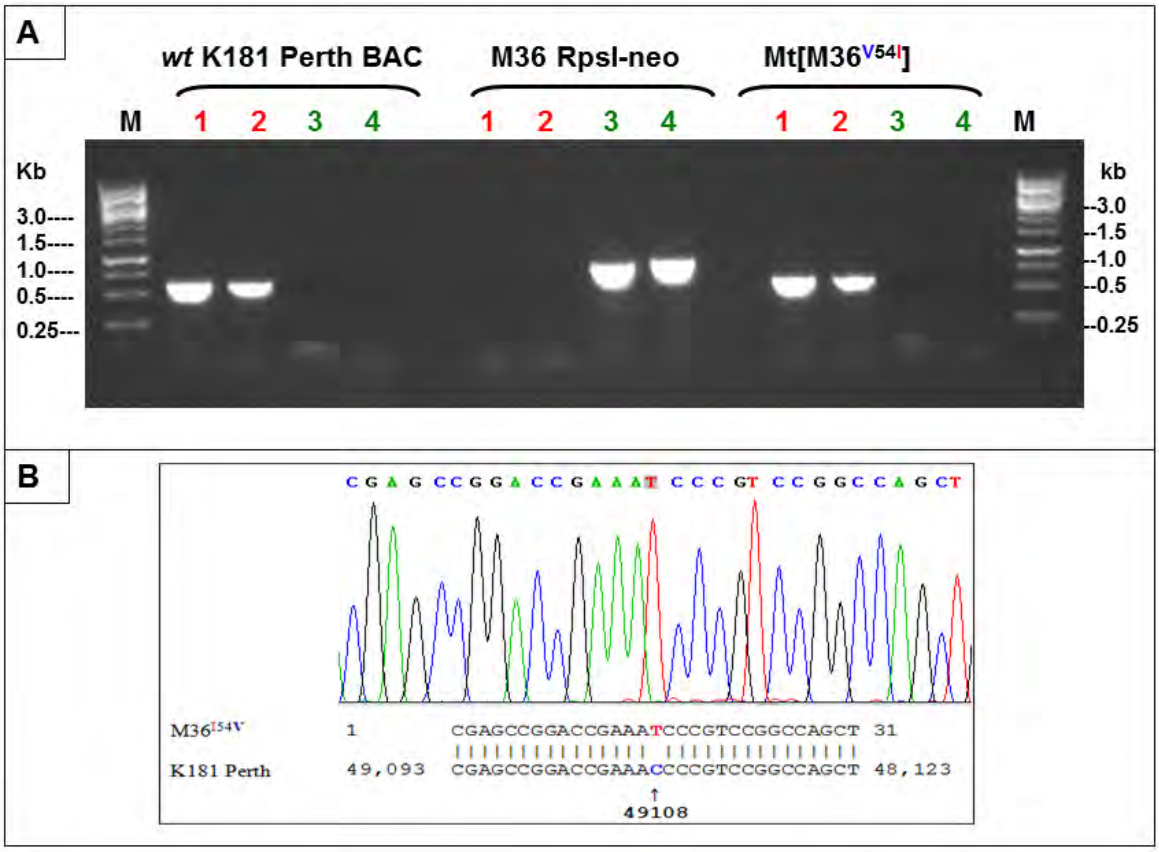


Figure 3.11 Mt[M36^{V54I}] construction. A. Shows the detailed PCR analysis (Figure 3.9) and the steps for the M36 mutant construction starting with the *wt* K181 (Perth) BAC, M36 RpsL-neo cassette (insertion of the RpsL-neo cassette), Mt[M36^{V54I}] (replacement of the RpsL-neo cassette with the mutated M36 ORF) using M36 specific primers in the **1st** and **2nd** PCR reactions, and RpsL-neo specific primers in the **3rd** and **4th** PCR reactions. Lanes M contain molecular size markers (in kilobases). B. The sequence profile of the M36 gene in Mt[M36^{V54I}] showing that the mutation of interest (C to T) has been inserted at base position 49,108 of the K181 (Perth) BAC.

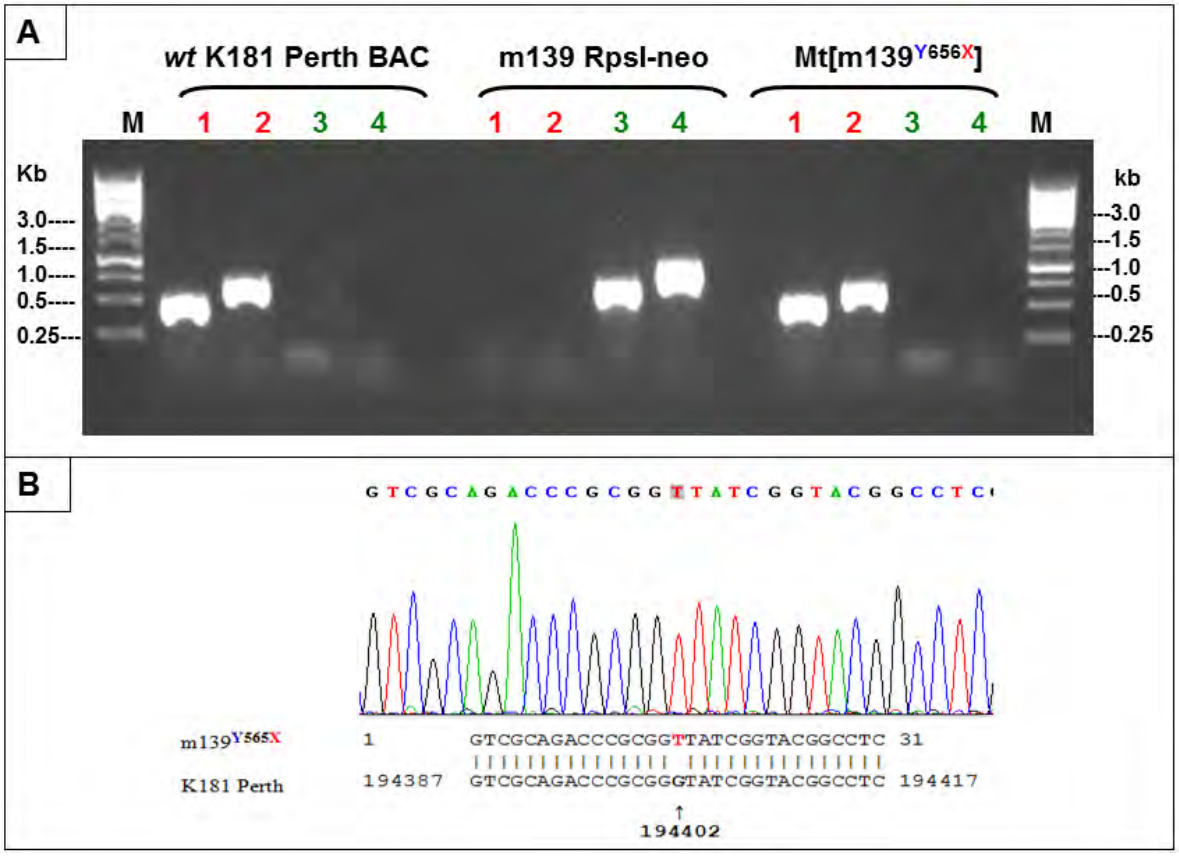


Figure 3.12 Mt[m139^{Y565X}] construction. A. Shows the detailed PCR analysis (Figure 3.9) and the steps for the m139 mutant construction starting with the *wt* K181 (Perth) BAC, m139 RpsL-neo cassette (insertion of the RpsL-neo cassette), Mt[m139^{Y565X}] (replacement of the RpsL-neo cassette with the mutated m139 ORF) using m139 specific primers in the 1st and 2nd PCR reactions, and RpsL-neo specific primers in the 3rd and 4th PCR reactions. Lanes M contain molecular size markers (in kilobases). B. The sequence profile of the m139 gene in Mt[m139^{Y565X}] showing that the mutation of interest (G to T) has been inserted at base position 194,402 of the K181 (Perth) BAC.

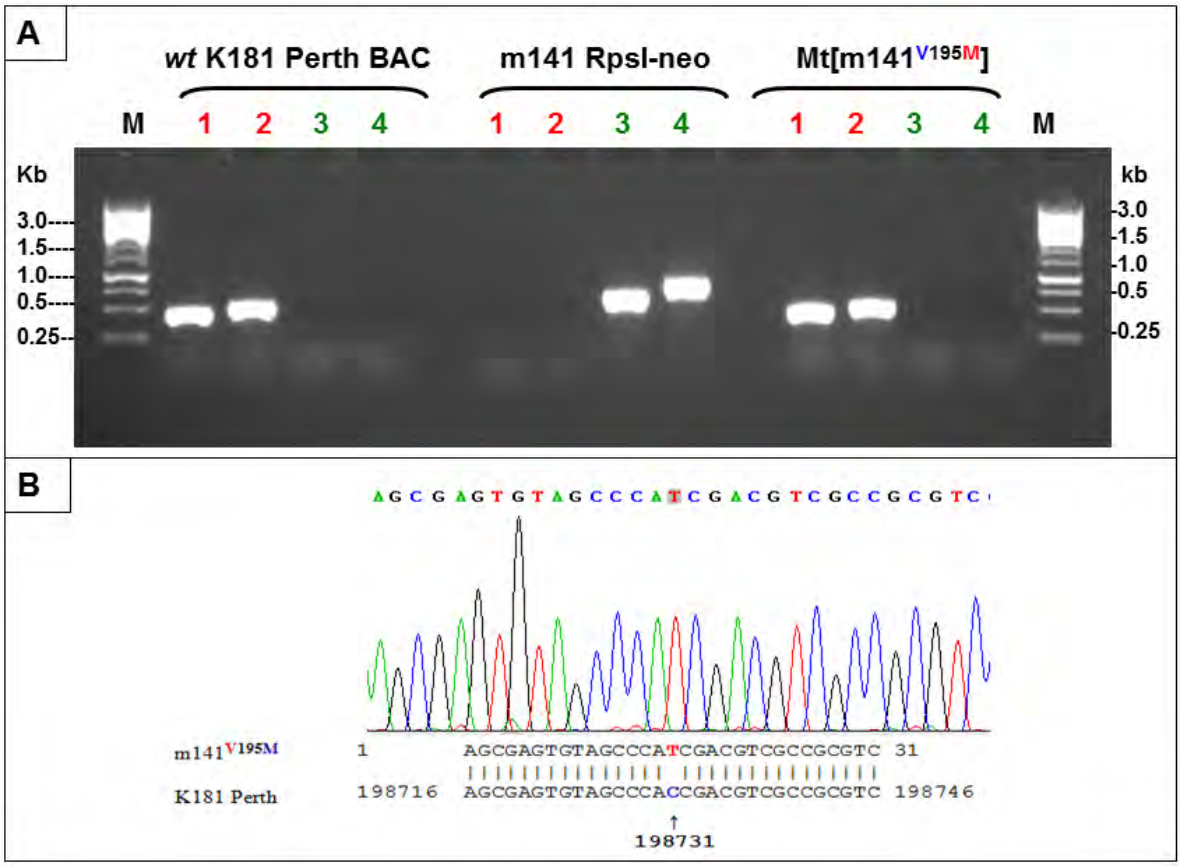


Figure 3.13 Mt[m141^{V195M}] construction. A. Shows the detailed PCR analysis (Figure 3.9) and the steps for the m141 mutant construction starting with the *wt* K181 (Perth) BAC, m141 RpsL-neo cassette (insertion of the RpsL-neo cassette), Mt[m141^{V195M}] (replacement of the RpsL-neo cassette with the mutated m141 ORF) using m141 specific primers in the 1st and 2nd PCR reactions, and RpsL-neo specific primers in the 3rd and 4th PCR reactions. Lanes M contain molecular size markers (in kilobases). B. The sequence profile of the m141 gene in Mt[m141^{V195M}] showing that the mutation of interest (C to T) has been inserted at base position 198,731 of the K181 (Perth) BAC.

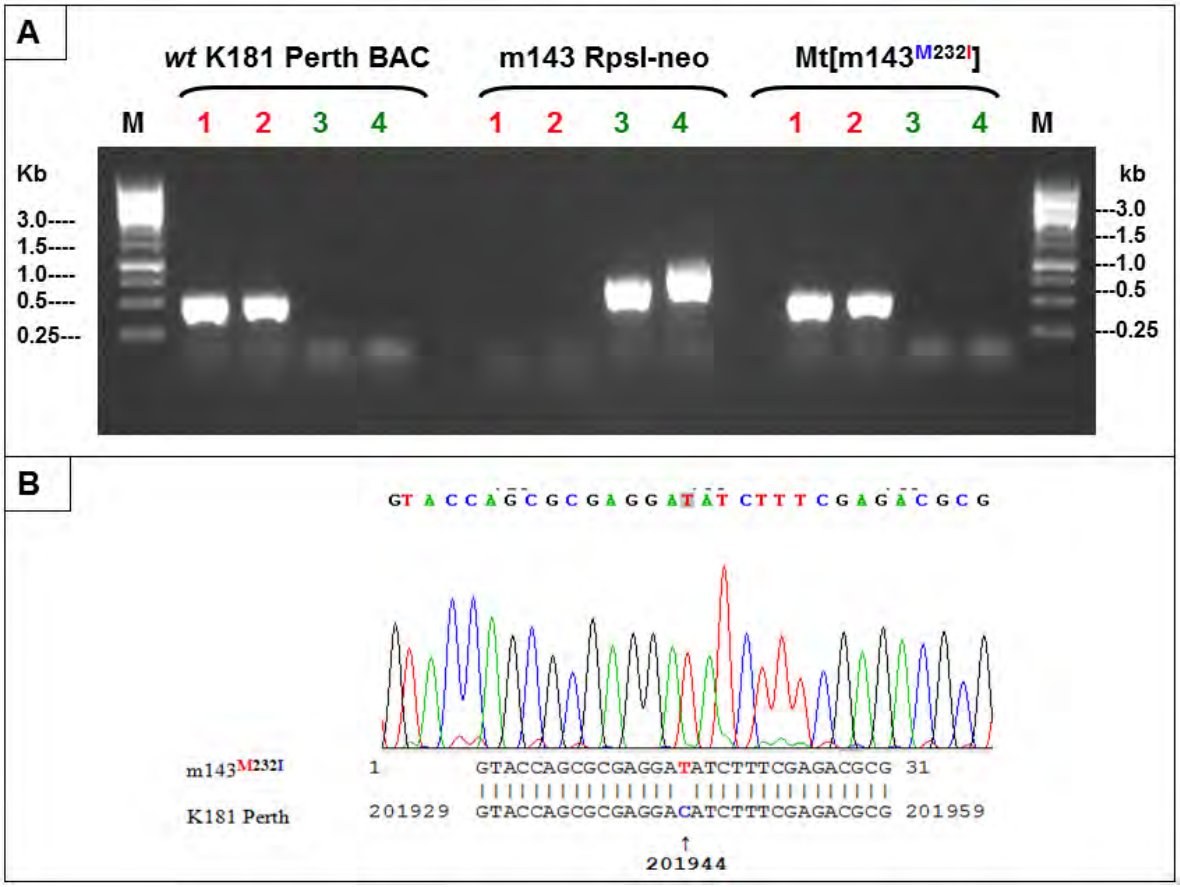


Figure 3.14 Mt[m143^{M232I}] construction. A. Shows the detailed PCR analysis (Figure 3.9) and the steps for the m143 mutant construction starting with the *wt* K181 (Perth) BAC, m143 RpsL-neo cassette (insertion of the RpsL-neo cassette), Mt[m143^{M232I}] (replacement of the RpsL-neo cassette with the mutated m143 ORF) using m143 specific primers in the 1st and 2nd PCR reactions, and RpsL-neo specific primers in the 3rd and 4th PCR reactions. Lanes M contain molecular size markers (in kilobases). B. The sequence profile of the m143 gene in Mt[m143^{M232I}] showing that the mutation of interest (C to T) has been inserted at base position 201,944 of the K181 (Perth) BAC.

3.3.3 Double mutant construction

Early in our study, we decided to study the impact of the presence of two mutations present in *tsm5* in two immune evasion genes M27^{A658S} and M36^{V54I}, in one construct. Starting from the construct in which the single mutation (C to A at nt 32,302) had been introduced into the M27 gene (mt[M27^{A658S}]), a C to T mutation at position 49,108 of the *wt* K181 (Perth) BAC was then introduced into the M36 gene by two-step homologous recombination as described above. The resultant double mutant BAC construct was designated as mt[M27^{A658S}M36^{V54I}]. The BAC construct was carefully checked by PCR and sequencing as described above (data not shown).

3.3.4 Revertants construction

Revertants of the MCMV mutants were constructed to ensure that the phenotypic properties of the constructed mutants were attributed to the mutations not to other factors or mistakes during their construction. In a similar way, the revertants were constructed using the two-step homologous recombination technique as described above, starting with the mutant construct in which the mutated segment was replaced with the RpsL-neo cassette which was in turn replaced with the *wt* linear DNA amplified form the *wt* K181(Perth) BAC. Revertants for each single mutation underwent four rounds of homologous recombination whereas the double revertant for the double mutant underwent six rounds. The BAC constructs of each stage of mutagenesis were thoroughly checked and analysed by PCR and sequencing as described above (data not shown). The revertant constructs were designated as Rv[M27^{S658A}], Rv[M36^{I54V}], Rv[M27^{S658A}M36^{I54V}] Rv[m139^{X565Y}], Rv[m141^{M195V}] and Rv[m143^{I232M}].

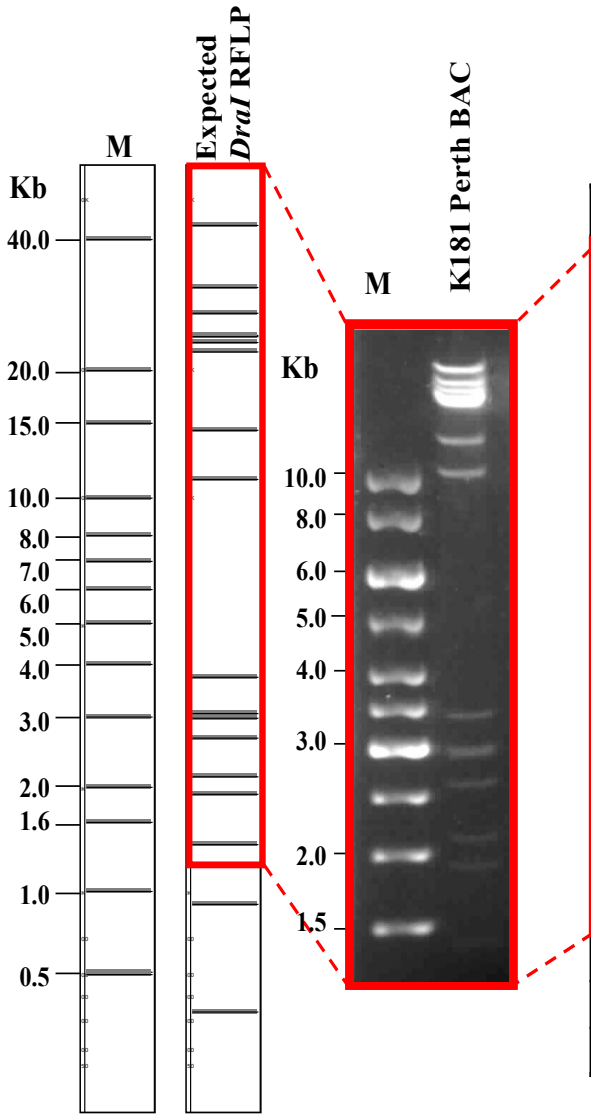
3.3.5 Restriction fragment length polymorphism (RFLP) analysis

During the construction of the mutants and their revertants it is possible that deletions, insertions or gene re-arrangements may occur. RFLP is widely used to exclude these possibilities during the recombineering process (Redwood *et al.*, 2005; Yu *et al.*, 2002).

Due to the difficulties in separating big fragments (>10 kbp) and visualisation of small fragments (<1 kbp) on the gel, a complete picture of the whole genome restriction fragment digestion pattern is not usually achievable using one restriction enzyme only. Thus, using several restriction enzymes gives a better coverage of the genome. In the present study, RFLP analyses were performed on the *wt* K181 (Perth) BAC as well as the constructed mutant and revertant BACs using two or more of six different restriction enzymes (*Hpa*I, *Eco*RI, *Ase*I *Dra*I, *Hind*III and *Bam*HI) to ensure that no rearrangements had occurred anywhere in the whole BAC.

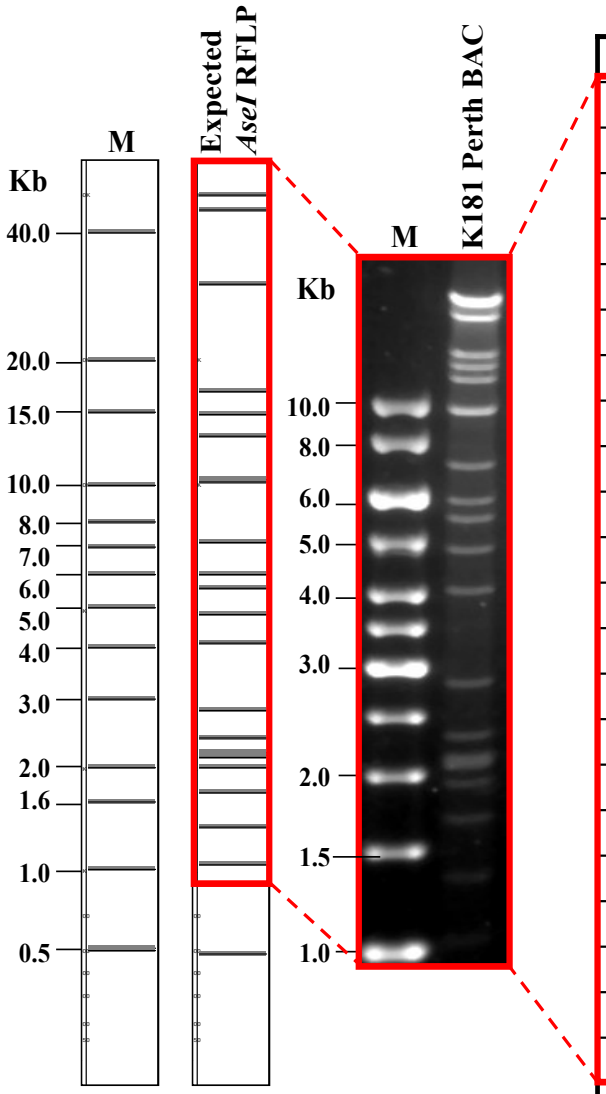
BAC DNA of *wt* K181 (Perth), mutants and revertants were purified from bacterial cultures (Section 2.5.3.2) and digested with *Hpa*I, *Eco*RI, *Ase*I and *Dra*I restriction enzymes (Section 2.5.8). The digests were then separated on a 0.4% agarose gel and analysed (Section 2.5.9). The K181 Perth BAC RFLP pattern was first compared to the theoretical RFLP pattern of the K181 Perth BAC (sequence provided by Dr Alec Redwood, University of Western Australia, Australia) generated by the Vector NTI Advance™ 11 programme (Invitrogen, Paisley, UK). The analysis showed that the size and number of the restriction fragments distinguishable on the gels were consistent with the theoretical digestion patterns (Figure 3.15- Figure 3.18).

Recombinant mutants and revertants RFLPs were then compared to that of the K181 (Perth) BAC where results showed that there were no genome rearrangements in either of the recombinant BACs compared to the *wt* BAC digested with *Hpa*I, *Ase*I, *Dra*I, *BamH*I or *EcoR*I enzyme (Figure 3.19- Figure 3.26). Therefore, the RFLP patterns obtained in this study for the constructed BACs were indistinguishable from that of both the experimental and theoretical RFLP patterns of the *wt* K181 (Perth) BAC.



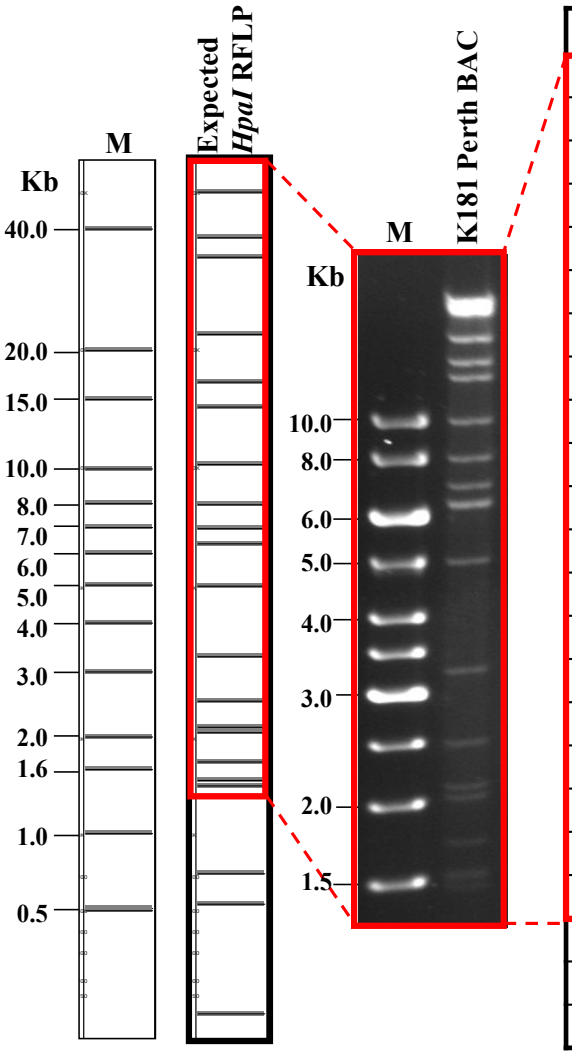
#	Ends	Coordinates	Length (bp)
1	DraI-DraI	20050-63395	43346
2	DraI-DraI	215279-6804	30970
3	DraI-DraI	87384-114429	27046
4	DraI-DraI	63396-87383	23988
5	DraI-DraI	140715-164586	23872
6	DraI-DraI	117540-140714	23175
7	DraI-DraI	192303-214348	22046
8	DraI-DraI	166748-181223	14476
9	DraI-DraI	181224-192302	11079
10	DraI-DraI	14303-18101	3799
11	DraI-DraI	114430-117539	3110
12	DraI-DraI	8220-11256	3037
13	DraI-DraI	11257-13963	2707
14	DraI-DraI	164587-166747	2161
15	DraI-DraI	18102-20049	1948
16	DraI-DraI	6805-8219	1415
17	DraI-DraI	214349-215278	930
18	DraI-DraI	13964-14302	339

Figure 3.15 Comparison of the experimental and the expected theoretical RFLP patterns of the K181 Perth BAC digested with *Dra*I. The experimental RFLP pattern was compared to the theoretical RFLP pattern generated by the Vector NTI Advance™ 11 programme. The exact coordinates of the fragments according to the K181 BAC genome sequence provided by Dr Alec Redwood and the fragment sizes are shown in the Figure. The restriction fragments present on the gel and the corresponding range of the theoretical restriction fragments are enclosed by the red boxes. Molecular size markers (lanes M), in kilobases, are indicated.



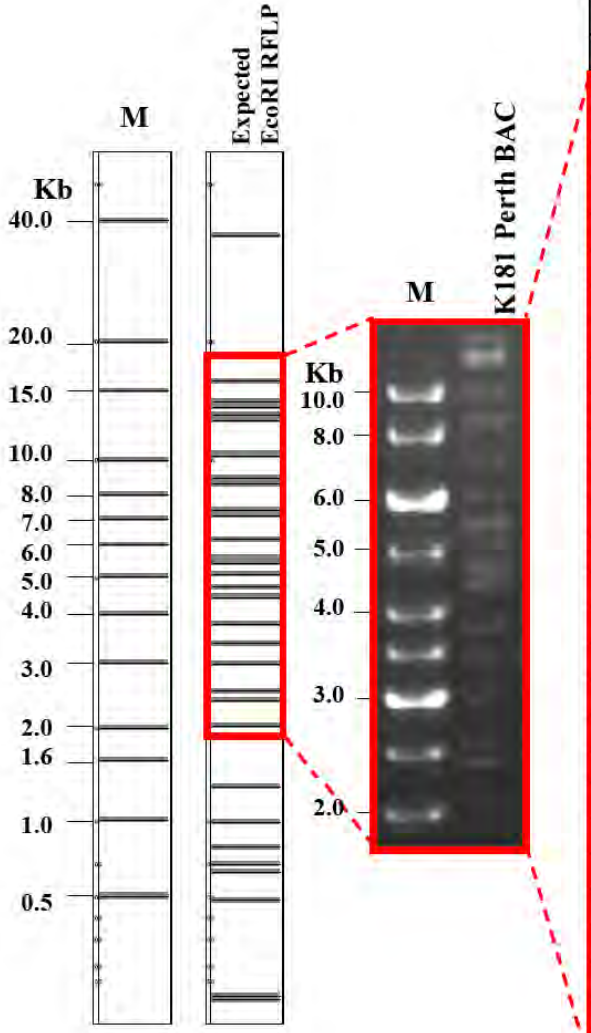
#	Ends	Coordinates	Length (bp)
1	AseI-AseI	50428-102017	51590
2	AseI-AseI	117473-163084	45612
3	AseI-AseI	18008-48265	30258
4	AseI-AseI	206767-223596	16830
5	AseI-AseI	226680-2080	14845
6	AseI-AseI	193653-206766	13114
7	AseI-AseI	174670-184988	10319
8	AseI-AseI	104865-115045	10181
9	AseI-AseI	184989-192276	7288
10	AseI-AseI	2081-8201	6121
11	AseI-AseI	168998-174669	5672
12	AseI-AseI	13115-18007	4893
13	AseI-AseI	164822-168997	4176
14	AseI-AseI	102018-104864	2847
15	AseI-AseI	115046-117472	2427
16	AseI-AseI	10889-13114	2226
17	AseI-AseI	8202-10396	2195
18	AseI-AseI	48266-50427	2162
19	AseI-AseI	223597-225620	2024
20	AseI-AseI	163085-164821	1737
21	AseI-AseI	192277-193652	1376
22	AseI-AseI	225621-226679	1059
23	AseI-AseI	10397-10888	492

Figure 3.16 Comparison of the experimental and the expected theoretical RFLP patterns of the K181 Perth BAC digested with *Ase*I. The experimental RFLP pattern was compared to the theoretical RFLP pattern generated by the Vector NTI Advance™ 11 programme. The exact coordinates of the fragments according to the K181 BAC genome sequence provided by Dr Alec Redwood and the fragment sizes are shown in the Figure. The restriction fragments present on the gel and the corresponding range of the theoretical restriction fragments are enclosed by the red boxes. Molecular size markers (lanes M), in kilobases, are indicated.



#	Ends	Coordinates	Length (bp)
1	HpaI-HpaI	112069-165394	53326
2	HpaI-HpaI	21317-59309	37993
3	HpaI-HpaI	61401-95428	34028
4	HpaI-HpaI	193536-215455	21920
5	HpaI-HpaI	95429-112068	16640
6	HpaI-HpaI	170219-184601	14383
7	HpaI-HpaI	228992-239231	10240
8	HpaI-HpaI	185331-193456	8126
9	HpaI-HpaI	221913-228991	7079
10	HpaI-HpaI	8261-14739	6479
11	HpaI-HpaI	215456-221912	6457
12	HpaI-HpaI	16260-21316	5057
13	HpaI-HpaI	239232-3133	3346
14	HpaI-HpaI	167663-170218	2556
15	HpaI-HpaI	4589-6743	2155
16	HpaI-HpaI	59310-61400	2091
17	HpaI-HpaI	165940-167662	1723
18	HpaI-HpaI	14740-16259	1520
19	HpaI-HpaI	6744-8260	1517
20	HpaI-HpaI	3134-4588	1455
21	HpaI-HpaI	184602-185330	729
22	HpaI-HpaI	165395-165939	545
23	HpaI-HpaI	193457-193535	79

Figure 3.17 Comparison of the experimental and the expected theoretical RFLP patterns of the K181 Perth BAC digested with *Hpa*I. The experimental RFLP pattern was compared to the theoretical RFLP pattern generated by the Vector NTI Advance™ 11 programme. The exact coordinates of the fragments according to the K181 BAC genome sequence provided by Dr Alec Redwood and the fragment sizes are shown in the Figure. The restriction fragments present on the gel and the corresponding range of the theoretical restriction fragments are enclosed by the red boxes. Molecular size markers (lanes M), in kilobases, are indicated.



#	Ends	Coordinates	Length (bp)
1	EcoRI-EcoRI	116454-153009	36556
2	EcoRI-EcoRI	227131-3539	15853
3	EcoRI-EcoRI	80027-94107	14081
4	EcoRI-EcoRI	32365-45981	13617
5	EcoRI-EcoRI	49416-62468	13053
6	EcoRI-EcoRI	186226-198894	12669
7	EcoRI-EcoRI	163280-173685	10406
8	EcoRI-EcoRI	153010-163279	10270
9	EcoRI-EcoRI	207528-216543	9016
10	EcoRI-EcoRI	100085-108905	8821
11	EcoRI-EcoRI	62469-71172	8704
12	EcoRI-EcoRI	6563-14043	7481
13	EcoRI-EcoRI	24104-31361	7258
14	EcoRI-EcoRI	73716-80026	6311
15	EcoRI-EcoRI	218949-224621	5673
16	EcoRI-EcoRI	14044-19615	5572
17	EcoRI-EcoRI	110967-116453	5487
18	EcoRI-EcoRI	94108-99266	5159
19	EcoRI-EcoRI	173686-178468	4783
20	EcoRI-EcoRI	202753-207527	4775
21	EcoRI-EcoRI	178469-183003	4535
22	EcoRI-EcoRI	19616-24103	4488
23	EcoRI-EcoRI	198895-202752	3858
24	EcoRI-EcoRI	45982-49415	3434
25	EcoRI-EcoRI	3540-6562	3023
26	EcoRI-EcoRI	183656-186225	2570
27	EcoRI-EcoRI	71173-73601	2429
28	EcoRI-EcoRI	216544-218948	2405
29	EcoRI-EcoRI	108906-110966	2061
30	EcoRI-EcoRI	224631-225943	1313
31	EcoRI-EcoRI	31362-32364	1003
32	EcoRI-EcoRI	99267-100084	818
33	EcoRI-EcoRI	225944-226641	698
34	EcoRI-EcoRI	183004-183655	652
35	EcoRI-EcoRI	226642-227130	489
36	EcoRI-EcoRI	73602-73715	114
37	EcoRI-EcoRI	224622-224630	9

Figure 3.18 Comparison of the experimental and the expected theoretical RFLP patterns of the K181 Perth BAC digested with *Eco*RI. The experimental RFLP pattern was compared to the theoretical RFLP pattern generated by the Vector NTI Advance™ 11 programme. The exact coordinates of the fragments according to the K181 BAC genome sequence provided by Dr Alec Redwood and the fragment sizes are shown in the Figure. The restriction fragments present on the gel and the corresponding range of the theoretical restriction fragments are enclosed by the red boxes. Molecular size markers (lanes M), in kilobases, are indicated.

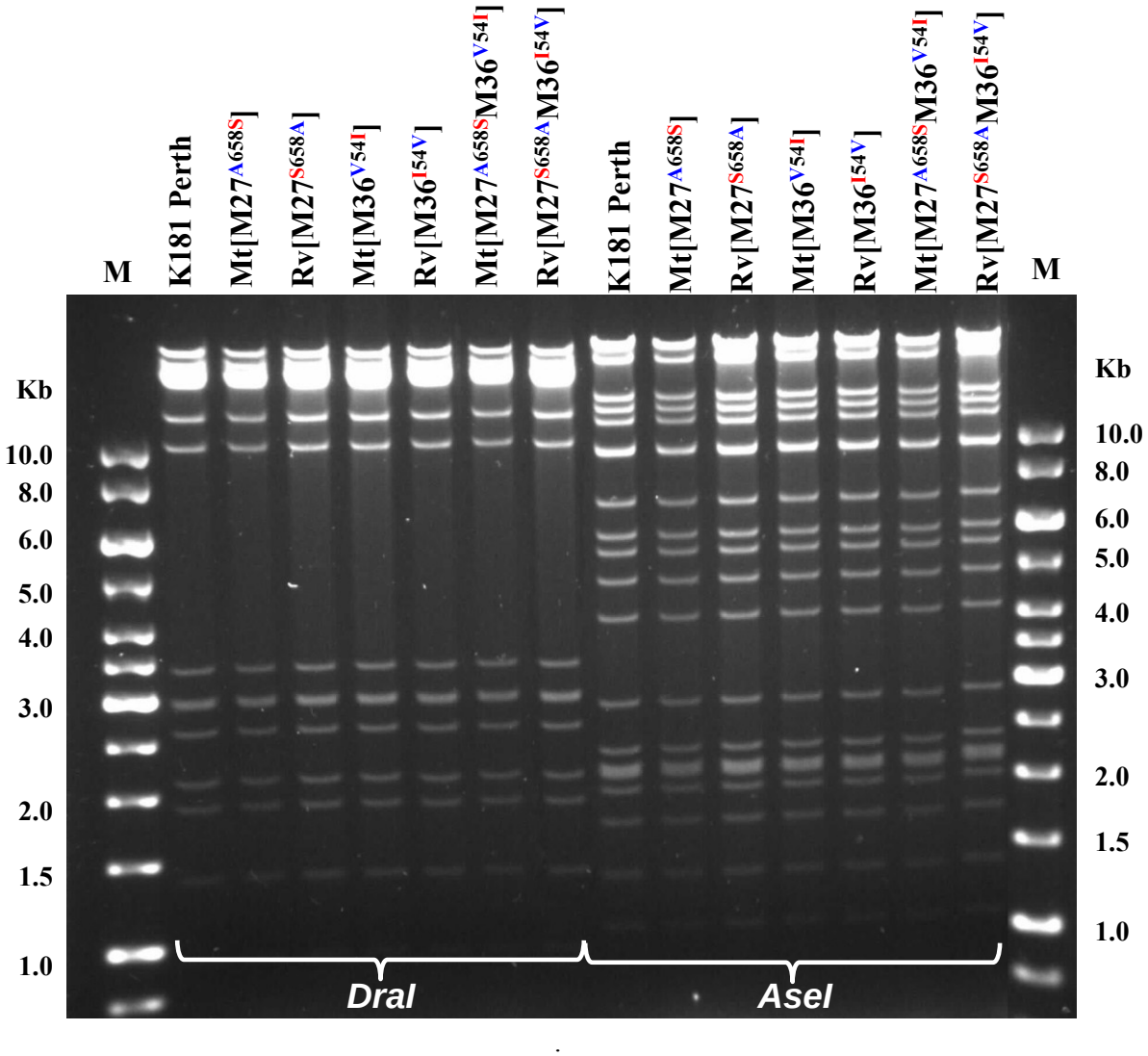


Figure 3.19: RFLP analysis of recombinant MCMV BAC genomes using *Dra*I and *Ase*I restriction enzyme digestion. wt K181 (Perth) and M27 and M36 mutant and revertant BAC DNA (~2 µg) was digested with 1 µl of *Dra*I or *Ase*I restriction enzyme and the resulting fragments were separated on a 0.4% agarose gel for ~19 hours at 2 V/cm. Molecular size markers (lanes M), in kilobases, are indicated.

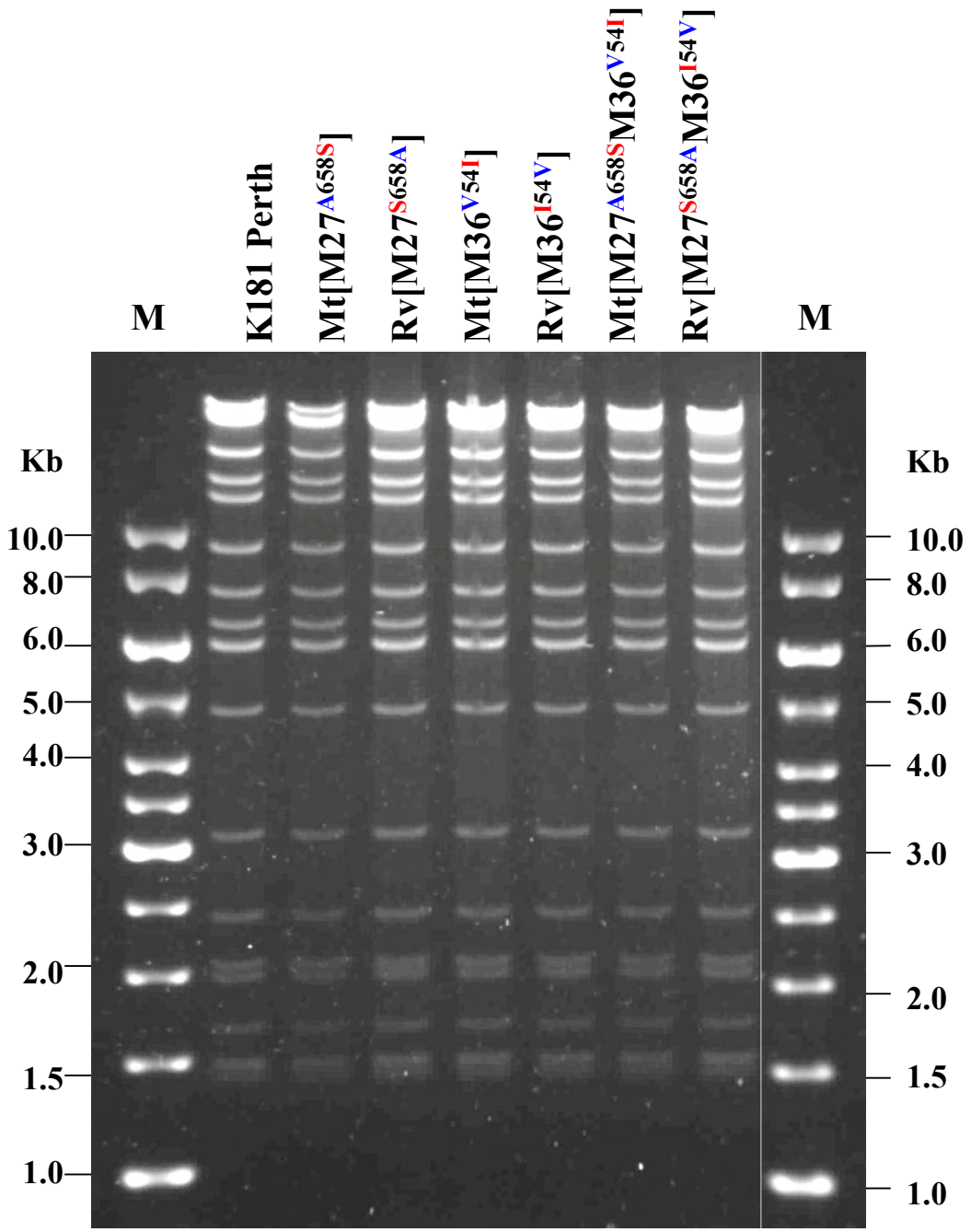


Figure 3.20: RFLP analysis of recombinant MCMV BAC genomes using *Hpa*I restriction enzyme digestion. *wt* K181 (Perth) and M27 and M36 mutant and revertant BAC DNA (~2 µg) was digested with 1 µl of *Hpa*I restriction enzyme and the resulting fragments were separated on a 0.4% agarose gel for ~19 hours at 2 V/cm. Molecular size markers (lanes M), in kilobases, are indicated.

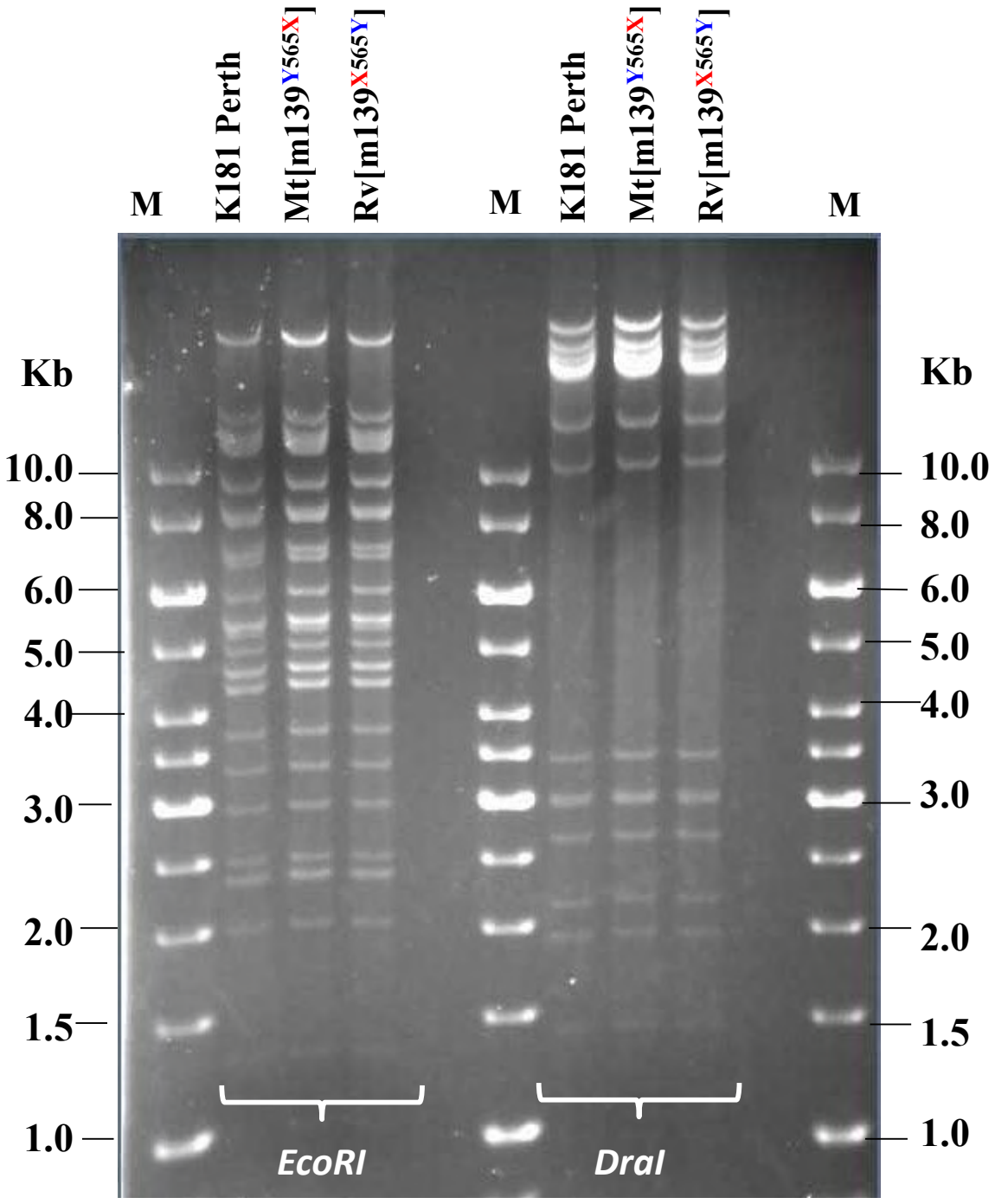


Figure 3.21: RFLP analysis of recombinant MCMV BAC genomes using *Eco*RI and *Dra*I restriction enzyme digestion. wt K181 (Perth) and m139 mutant and revertant BAC DNA (~2 µg) was digested with 1 µl of *Eco*RI or *Dra*I restriction enzyme and the resulting fragments were separated on a 0.4% agarose gel for ~19 hours at 2 V/cm. Molecular size markers (lanes M), in kilobases, are indicated.

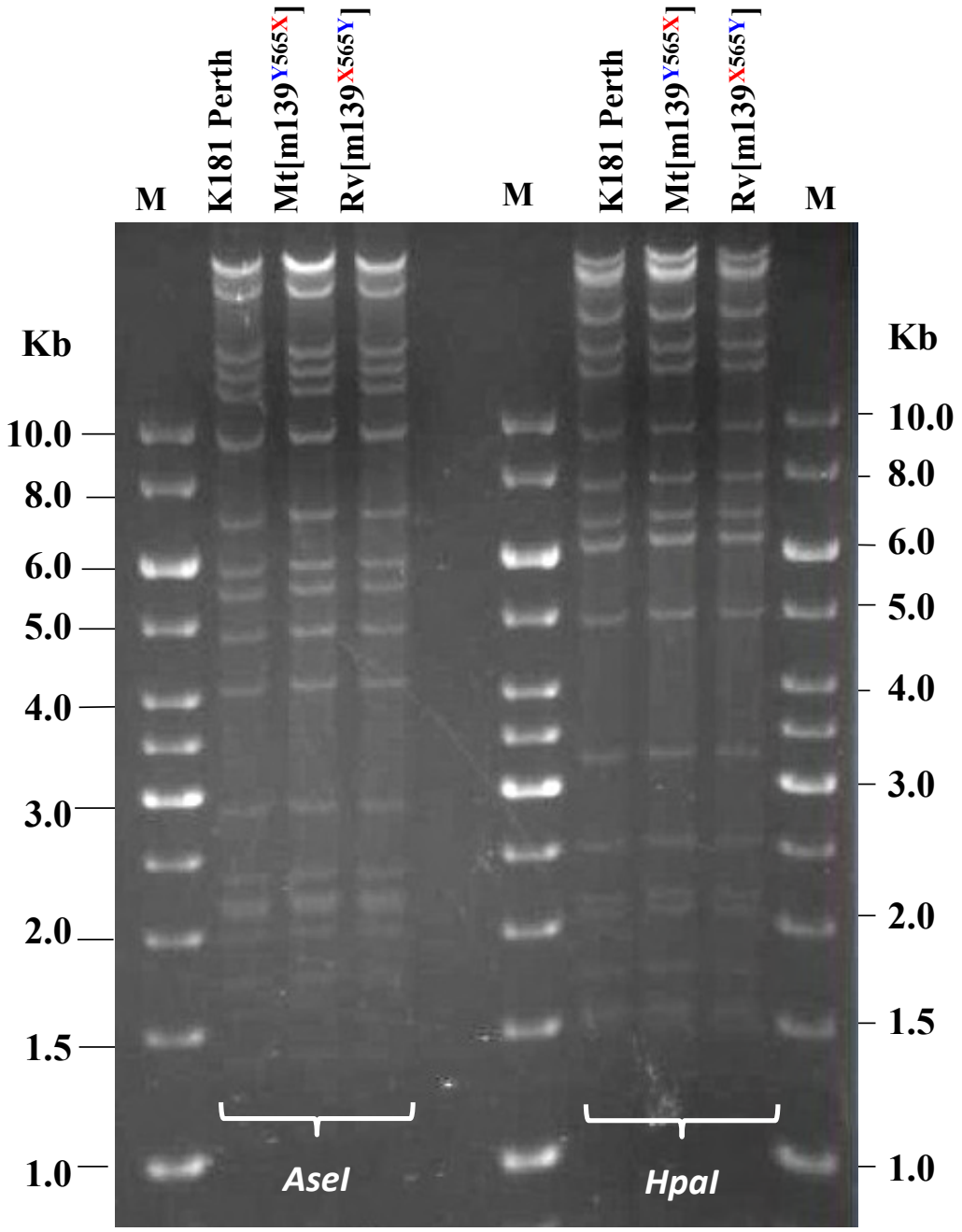


Figure 3.22: RFLP analysis of recombinant MCMV BAC genomes using *Ase*I and *Hpa*I restriction enzymes digestion. *wt* K181 (Perth) and m139 mutant and revertant BAC DNA (~2 µg) was digested with 1 µl of *Ase*I or *Hpa*I restriction enzyme and the resulting fragments were separated on a 0.4% agarose gel for ~19 hours at 2 V/cm. Molecular size markers (lanes M), in kilobases, are indicated.

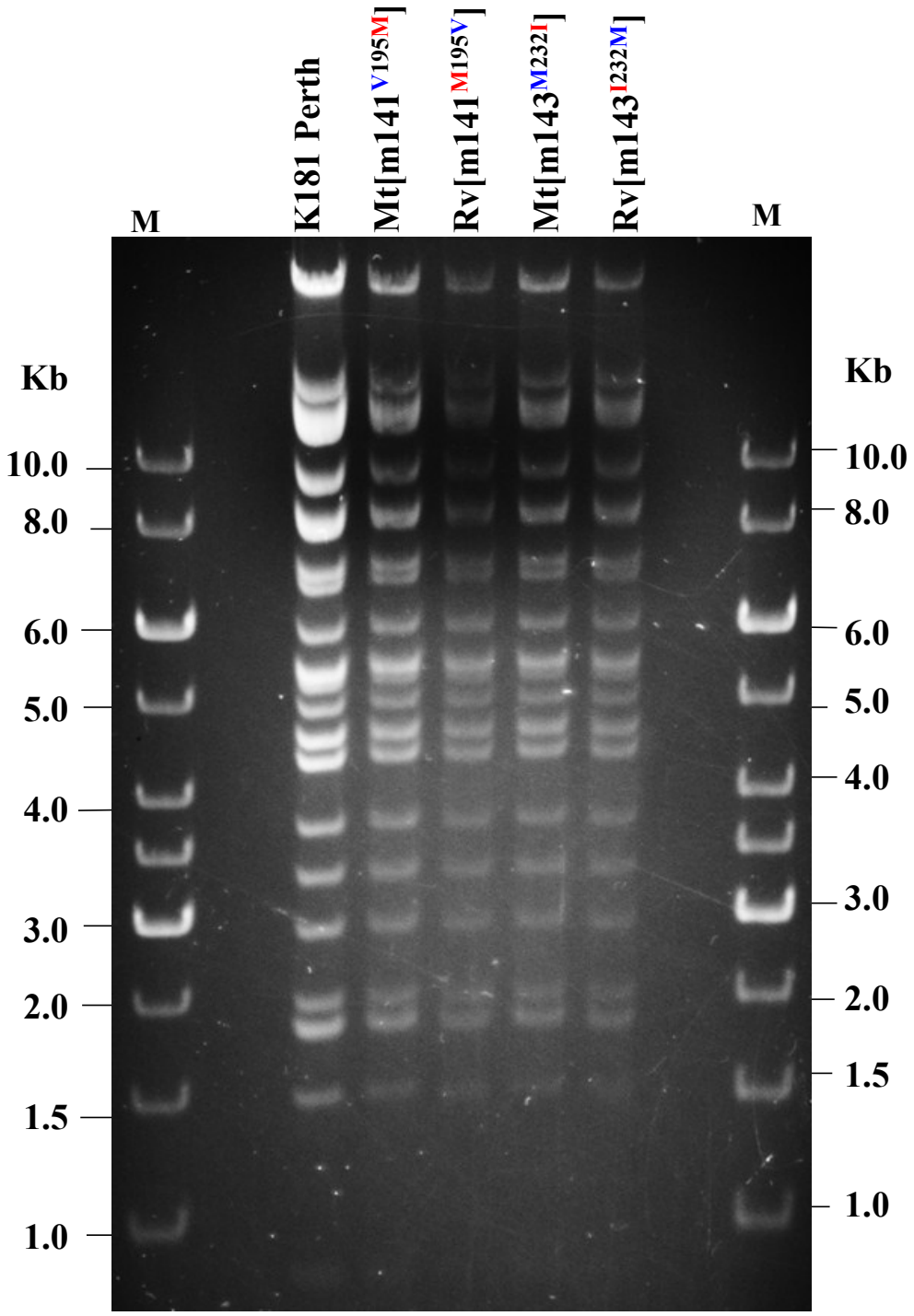


Figure 3.23: RFLP analysis of recombinant MCMV BAC genomes using *Eco*RI restriction enzyme digestion. wt K181 (Perth) and m141 and m143 mutant and revertant BAC DNA (~2 µg) was digested with 1 µl of *Eco*RI restriction enzyme and the resulting fragments were separated on a 0.4% agarose gel for ~19 hours at 2 V/cm. Molecular size markers (lanes M), in kilobases, are indicated.

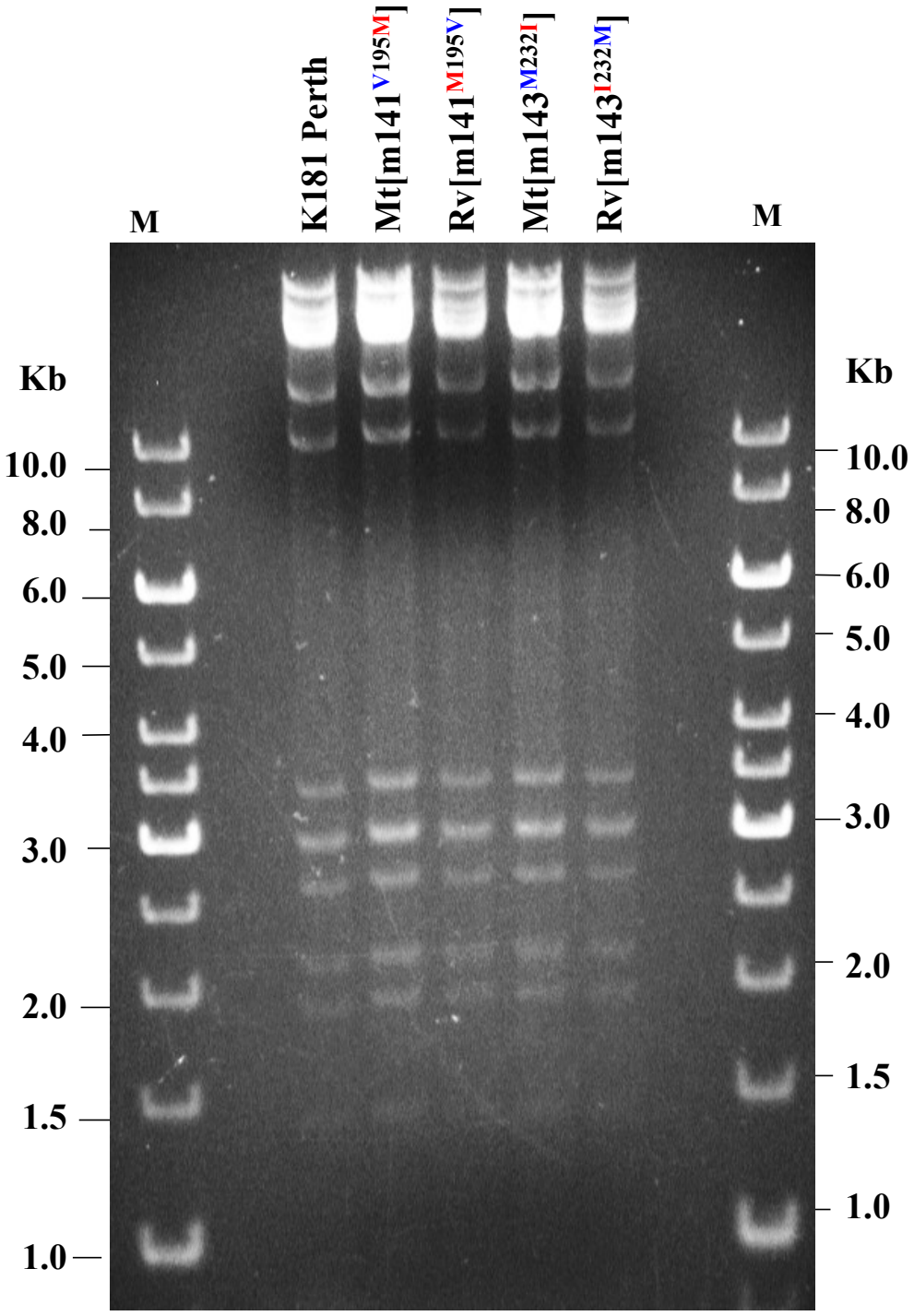


Figure 3.24: RFLP analysis of recombinant MCMV BAC genomes using *Dra*I restriction enzyme digestion. *wt* K181 (Perth) and mm141 and m143 mutant and revertant BAC DNA (~2 µg) was digested with 1 µl of *Dra*I restriction enzyme and the resulting fragments were separated on a 0.4% agarose gel for ~19 hours at 2 V/cm. Molecular size markers (lanes M), in kilobases, are indicated.

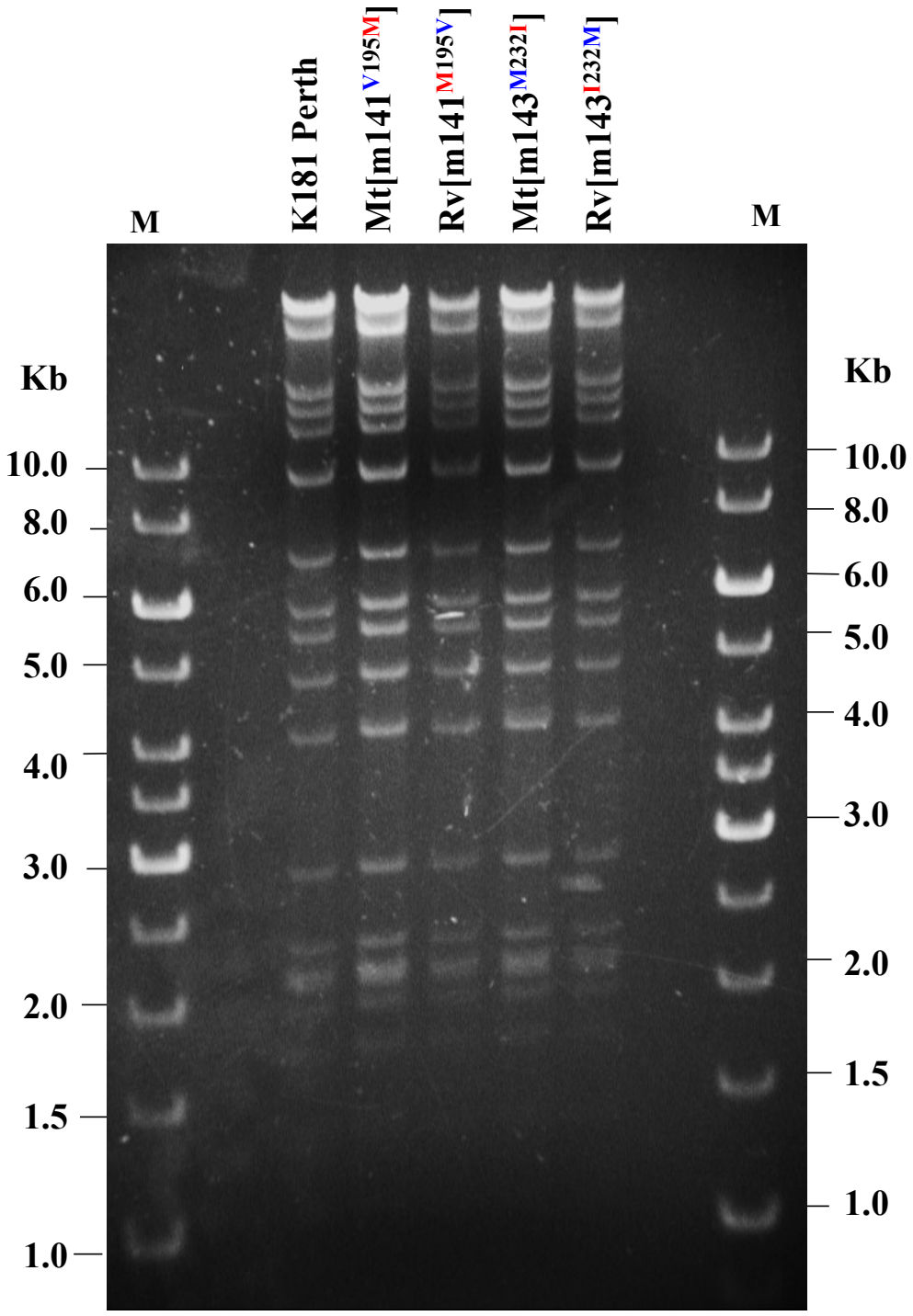


Figure 3.25: RFLP analysis of recombinant MCMV BAC genomes using *Ase*I restriction enzyme digestion. *wt* K181 (Perth) and m141 and m143 mutant and revertant BAC DNA (~2 µg) was digested with 1 µl of *AseI* restriction enzyme and the resulting fragments were separated on a 0.4% agarose gel for ~19 hours at 2 V/cm. Molecular size markers (lanes M), in kilobases, are indicated.

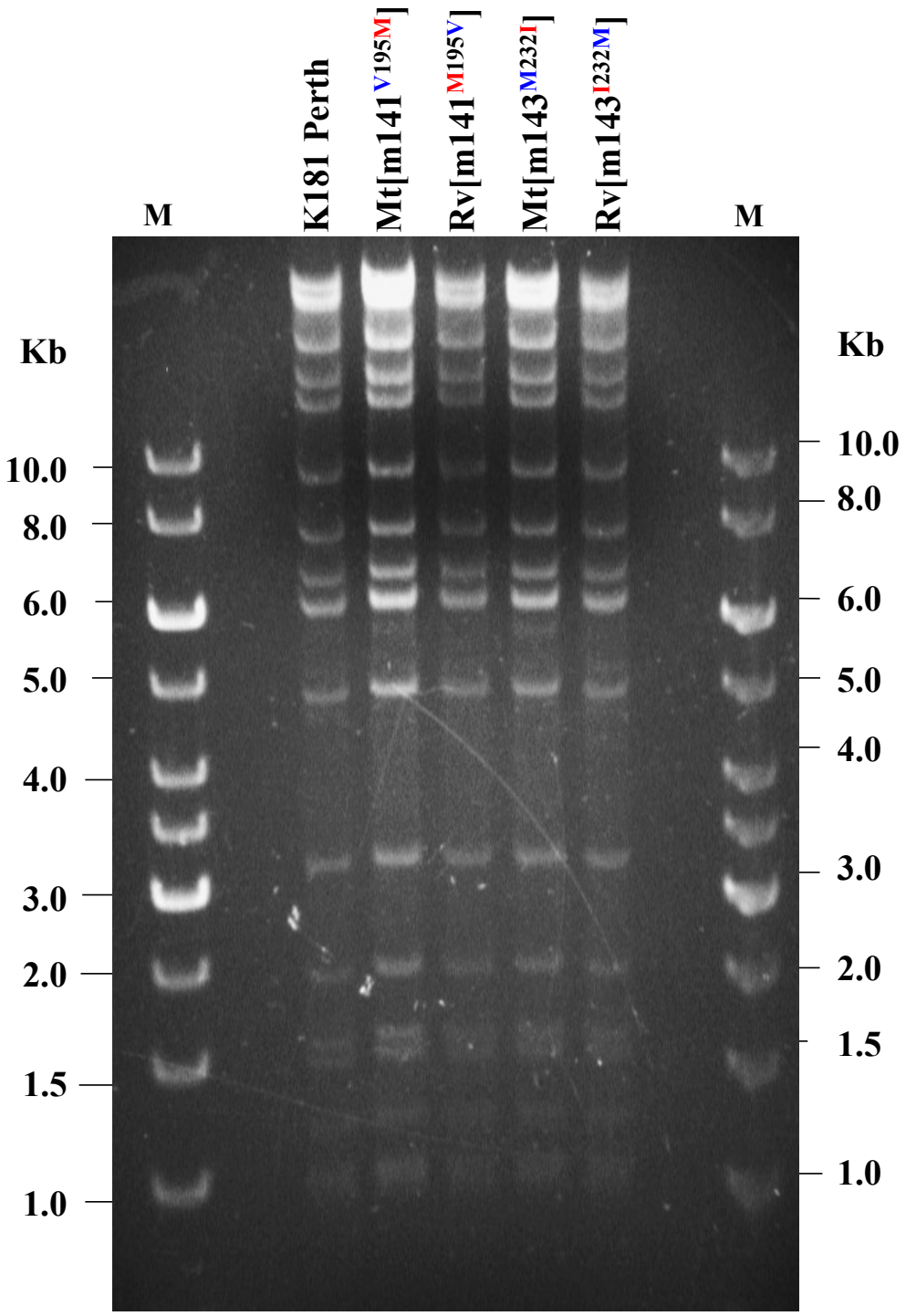


Figure 3.26: RFLP analysis of recombinant MCMV BAC genomes using *Hpa*I restriction enzyme digestion. *wt* K181 (Perth) and m141 and m143 mutant and revertant BAC DNA (~2 µg) was digested with 1 µl of *HpaI* restriction enzyme and the resulting fragments were separated on a 0.4% agarose gel for ~19 hours at 2 V/cm. Molecular size markers (lanes M), in kilobases, are indicated.

3.3.6 Virus reconstitution

Recombinant (mutant and revertant) as well as the *wt* MCMV viruses were reconstituted by transfection of the corresponding BACs into permissive eukaryotic cells (NIH 3T3). For BAC cassette excision, BAC-derived viruses were further passaged in cell culture to allow the recombination via repeated sequences flanking the BAC cassette of the viral genome. Excision of the BACs was confirmed by PCR. The mutations introduced into the targeted genes of the recombinant viruses were sequenced to confirm the presence of mutations by sequencing.

3.3.6.1 Transfection of recombinant BACs

NIH 3T3 cells were transfected with the recombinant mutants, revertants or *wt* BAC DNA [extracted as described in (Section 2.5.3.2)], using ExGen500 transfection reagent (Section 2.3.1). Successful transfection was detected and monitored by expression of green fluorescent protein (GFP) encoded by the *gfp* gene of the BAC cassette (Figure 3.50 on page 198) (Redwood *et al.*, 2005). Viral plaques were detected under the inverted microscope 3-6 days post infection. A 100% CPE was observed 4-5 days after detection of the first plaques, at which point virus was harvested (Section 2.3.3).

3.3.6.2 BAC cassette excision

The K181 (Perth) BAC is flanked with repeat sequences. Following transfection of the BAC into permissive eukaryotic cells, viral packaging restrictions preferentially lead to homologous recombination between the repeat sequences resulting in the exclusion of the BAC cassette (Redwood *et al.*, 2005; Wagner *et al.*, 1999).

Virus progeny obtained from the transfection were plaque purified on NIH 3T3 and/or MEF cells. After passage of the reconstituted virus in these cells, the excision of the BAC which can be monitored by loss of GFP expression and confirmed by PCR. Although GFP expression is usually lost within 3-4 passages where the green florescence can not be detected under the florescence microscope, BAC excision can be confirmed only by PCR. BAC excision needs up to 10 passages to be achieved.

The presence of the BAC cassette in the genome of the reconstituted viruses is detected using ARK2514832F and ARK2515204R primers (Table 3.2) which bind within the *gpt* gene of the BAC cassette (Figure 3.27A) to yield a PCR product of 413 bp. Primers m06F and 6530R bind to the viral sequence flanking the BAC cassette (Figure 3.27A). BAC cassette excision can be determined using m06F and 6530R primers which they will produce a PCR fragment of 1,245 bp. If the BAC is still preset, the PCR product of 10,391 bp would be amplified, but the PCR conditions used did not allow the amplification of 10,391 bp product (short extension time).

PCR analysis of the BAC cassette excision was performed using DNA isolated from cells collected after transfection and each passage of the virus. PCR analysis of the BAC cassette excision from the Mt[M27^{A658S}] virus genome is shown as a representative example in Figure 3.27B. Complete BAC cassette excision was usually achieved after at least five passages. Although a study in our lab (thesis of Dr. Olga Timoshenko) showed that presence of BAC in the reconstituted virus has no impact of the presence of the BAC on viral replication *in vitro*. In general, all viruses produced in this study were passaged until BAC cassette excisions were confirmed by PCR. Seed stocks of the reconstituted viruses were produced from the last passages showing the excision of BACs (Section 2.3.3).

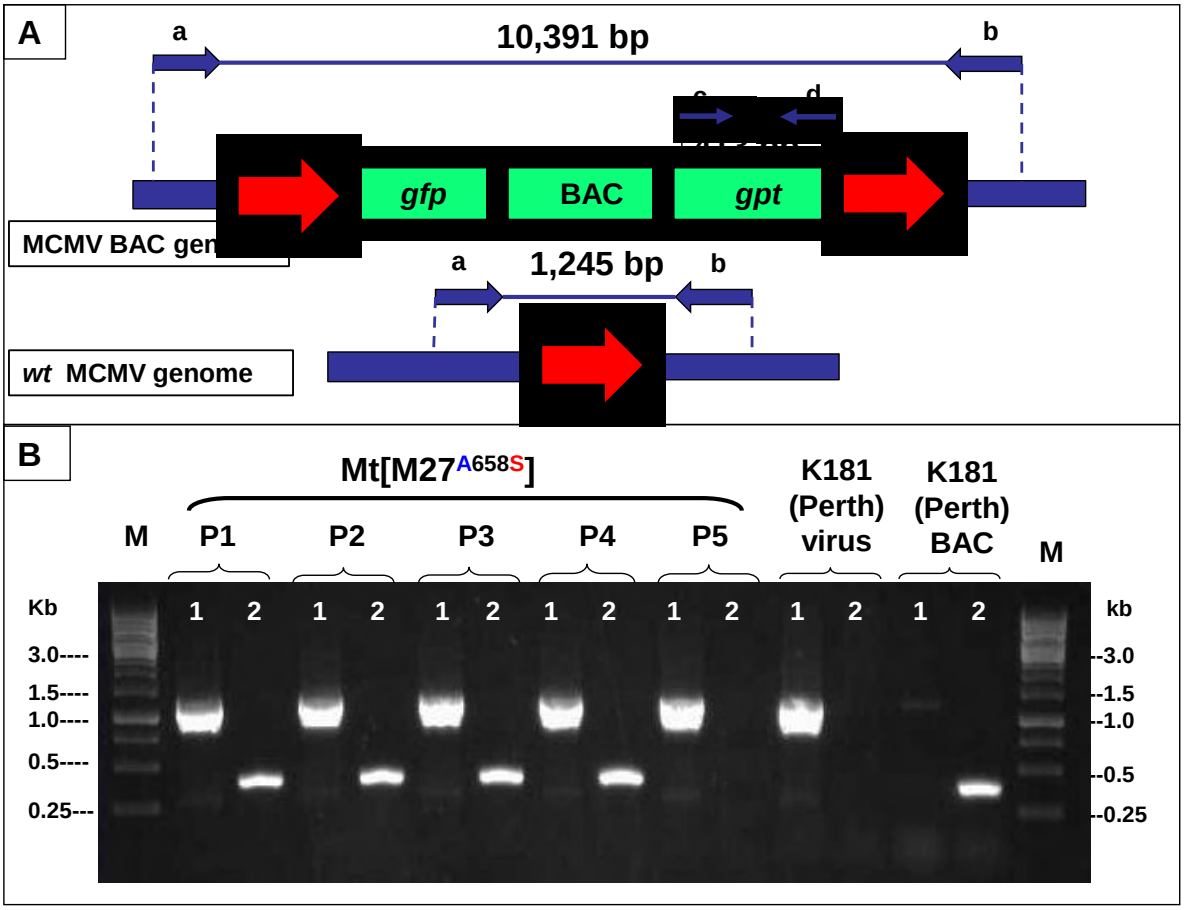


Figure 3.27: PCR screening of the BAC cassette excision from the viral genome reconstituted from the BAC plasmid. A. Schematic representation showing the structural organisation of the MCMV K181 (Perth) genomes before and following BAC cassette excision and primer binding sites. The red arrows indicates the repeated sequences flanking the BAC. B. PCR analysis of the BAC cassette excision from the Mt[M27A658S] BAC-derived virus. The PCR product of 1,245 bp (lanes 1) amplified with primers a (m06F) and b (6530R) indicated the excision of the BAC cassette from viral genomes apparent already in passage No. 1 (P1). The PCR product of 413 bp (lanes 2) amplified with primers c (ARK2514832F) and d (ARK2515204R) indicated the BAC cassette is still presence in some viral genomes up to P4. No product of 413 bp in P5 of the virus indicated the complete loss of the BAC cassette in all virus progeny. PCR conditions did not allow the amplification of a product of 10,391 bp using primers a and b from viral genome containing the BAC cassette. K181 Perth BAC and *wt* K181 (Perth) virus DNA were used as controls to amplify 413 and 1,245 bp products, respectively. Lanes M contain molecular size markers (in kilobases).

3.4 Characterisation of recombinant viruses phenotypes

To determine the effects of the various mutations inserted in the *wt* virus on the growth phenotype *in vitro*, each of the constructed mutant and revertant virus growth phenotypes was studied and compared to the wild-type MCMV-K181 strain (Birmingham and Perth variants) at both permissive (37º C) and non-permissive (40º C) temperatures. The growth rates of the constructed viruses as well as the *wt* virus were studied on MEF cells (Section 2.3.6) and on mouse macrophage cell line (Section 2.3.7). For growth characterization on MEF cells, the cells were seeded in three wells of the 24 well plates (0.5 x 10^5 cell/well) then infected with one of the viruses at an MOI of 0.05 (Section 2.3.6). Whereas for growth characterization on murine macrophage cells, the cells were seeded in three wells of the 24 well plates (3.5 x 10^5 cell/well) then infected with one of the viruses at an MOI of 0.1 (Section 2.3.7). One hour post infection, supernatants from each well of the infected MEF cells and macrophages were collected and assigned as day 0 and replaced with 1 ml GM1 or GM2 respectively. Every 24 hours the supernatants were collected and replaced with suitable fresh GM for up to seven days. Collected samples were stored at -80°C until titrated on MEF cells by plaque assay (Section 2.3.5). The growth rates of all viruses were performed and assayed in triplicate.

3.4.1 Characterisation of virus phenotype on MEF cells

3.4.1.1 Replication of the *wt* MCMV K181 strains

The MCMV K181 strain was developed from the Smith strain by serial passages in mouse salivary glands by Dr J. Osborn (Department of Medical Microbiology, University of Wisconsin, Madison, Wis., USA) (called Osborn strain) and described as a virulent variant of the Smith strain which later became designated as the K181 strain (Mims &

Gould, 1979; Smith *et al.*, 2008). The K181 strain of MCMV we are studying in our laboratory was obtained from Professor C. A. Mims (Department of Microbiology, United Medical and Dental Schools of Guy's and St. Thomas' Hospitals, Guy's Campus, London Bridge, UK) and designated as the MCMV K181 (Birmingham) variant. The K181 (Perth) variant was originally considered to be the Smith strain but later was shown to have a restriction pattern identical to that of the K181 strain (Hudson *et al.*, 1988). Both Birmingham and Perth variants of K181 may have originated from the same source, the Osborn strain, however passaging these K181 variants in different laboratories has resulted in a few nucleotide variations between the two variants as described in section 3.2.1 (Timoshenko *et al.*, 2009a).

The MCMV *tsm5* virus was obtained following chemical mutagenesis of the *wt* MCMV K181 (Birmingham) variant which has not been cloned as a BAC to be used for mutant construction (Sammons & Sweet, 1989). However, both the mutants and their corresponding revertants were to be constructed using the BAC cloned MCMV K181 (Perth) variant. Thus, it was important to show that their growth characteristics were similar.

The growth kinetics of both viruses was very similar at 37°C (Figure 3.28) where the viruses were first detected on day 2 post infection and their yields increased rapidly reaching the maximum yield of ~$10^{5.9}$ PFU/ml on day 6 and 7 post infection. No significant differences between K181 Perth and Birmingham virus yields were identified from 3 to 7 days post infection ($p>0.05$).

At 40°C, the growth kinetics of both viruses was also very similar (Figure 3.28). Virus yields were first detected on day 1 post infection, then they increased rapidly

reaching the maximum level of $10^{3.7}$ PFU/ml (K181 Birmingham) and $10^{3.9}$ PFU/ml (K181 Perth) on days 4-5 post infection. Statistical analysis showed that there were no differences between yields of the two viruses at any time point ($p>0.05$). The yields of both viruses were approximately 10-30 times lower at 40°C compared to those at 37°C ($p<0.05$).

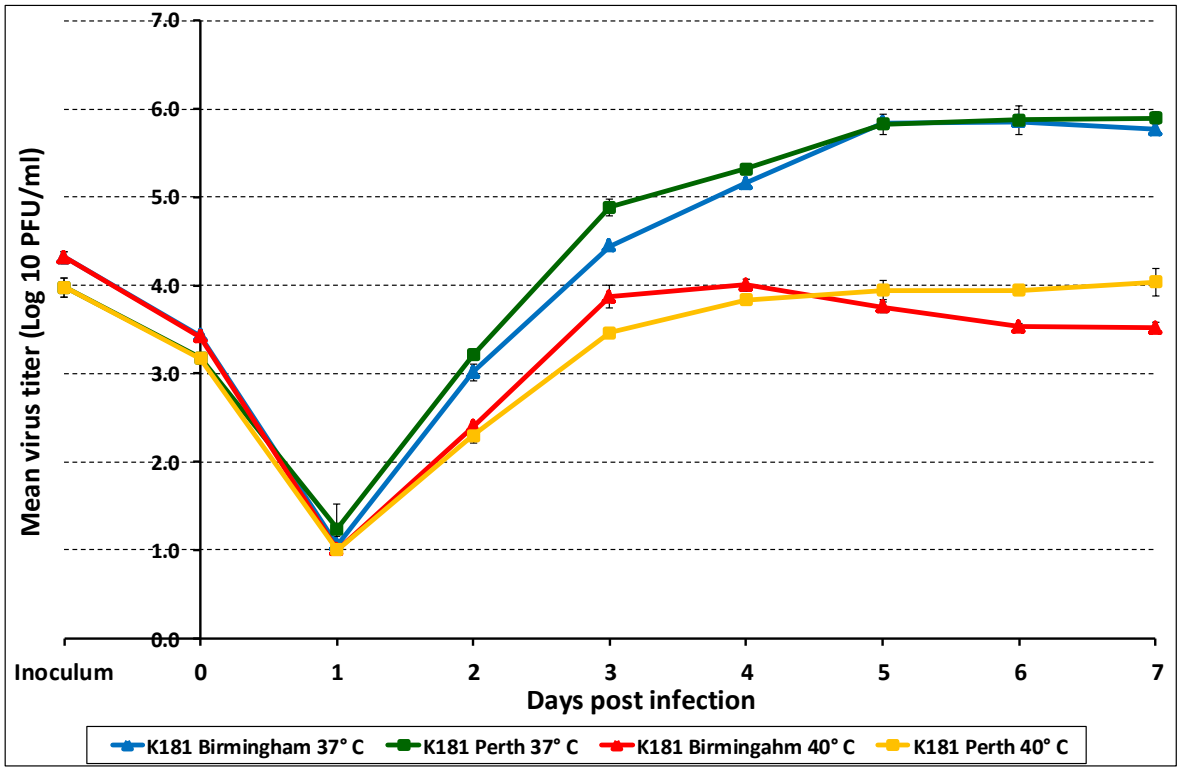


Figure 3.28: Replication of K181 (Birmingham) and K181 (Perth) variant viruses at the permissive (37°C) and non-permissive (40°C) temperatures. MEF cells were infected with virus at an MOI of 0.05 PFU per cell and incubated at either 37°C or 40°C. Virus titres (log10 pfu/ml) were determined in cell culture supernatants 1-7 days post infection by plaque assay. Results are the mean ± standard deviation (SD) of two separate experiments, each with three replicates. The limit of detection is 0.7 log10 PFU/ml.

Growth curves at both permissive and non-permissive temperature revealed that the growth kinetics of both K181 (Perth) and K181 (Birmingham) viruses were very similar. Therefore, introducing the *tsm5*-specific mutations into the K181 (Perth) genome and assaying their phenotype in the K181 (Perth) background, rather than in K181 (Birmingham) background, is justified.

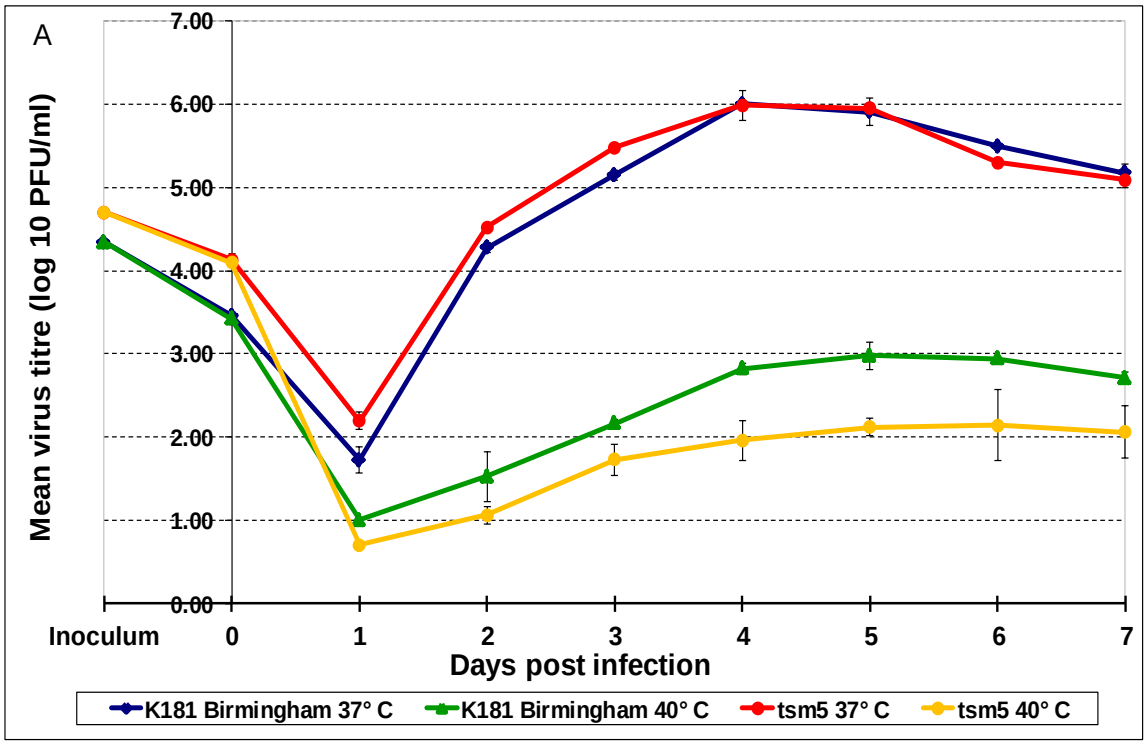
3.4.1.2 Characterisation of *tsm5* phenotype in MEF cells

The growth kinetics of *tsm5* was studied previously in our laboratory using one-step growth curves in which it was shown that *tsm5* yields in MEFs at the permissive (33°C or 37°C) and non-permissive (40°C) temperatures was reduced by approximately 10-fold and 1000-fold, respectively, on day 5 post infection compared to that of the parent K181 (Birmingham) virus (Karen Ball, PhD thesis, University of Birmingham, UK, 2005). Furthermore, a detailed study of the *tsm5* phenotype in MEFs using multi-step growth curves at 33°C and 41°C showed that virus was not detectable in cell culture supernatants until day 4 (33°C) or day 5 (41°C) post infection (Sweet *et al.*, 2007).

In the current study, replication of *tsm5* was examined under multi-step growth conditions at 37°C, 40°C, and 40.5°C in order to confirm its temperature-sensitive (*ts*) phenotype and possibly clarify the role of the M27, M36, m139, m141, and m143 ORFs, in this phenotype. At 37°C, the replication kinetics of *tsm5* virus was similar to that of K181 (Birmingham) variant (Figure 3.29). Both viruses were first detected in cell culture supernatants on day 2 post infection and reached maximum levels on days 4 and 5 post infection. Maximum yields of the K181 (Birmingham) variant and *tsm5* were ~$10^{6.00}$ PFU/ml and $10^{5.9}$ PFU/ml, respectively. No significant differences were identified between yields of the two viruses from days 2 to 8 post infection ($p>0.05$).

At 40°C, *tsm5* produced higher overall virus yields than those observed in previous studies (Figure 3.29A), (Sweet *et al.*, 2007). Virus was first detected on day 2 post infection (~$10^{1.0}$ PFU/ml). It increased rapidly to reach its maximum on day 5 post infection ($10^{2.2}$ PFU/ml). The yields of *tsm5* were about 2.6-7.5 times lower than that of *wt* virus from 3 to 7 days post infection ($p<0.05$).

Replication of the Perth variant was similar to that of the Birmingham variant at both 37^{o}C and 40.5^{o}C (Figure 3.29B). Again replication of *tsm*5 was impaired at 40.5°C where the virus could not be detected until day 6 compared to the *wt* virus which was first detectable at day 2 (Figure 3.29B). *Tsm5* only reached yields of ~$10^{1.0}$ PFU/ml by days 6 and 7 post infection compared to the *wt* K181 (Perth) variant that reached its maximum yields on day 6 post infection of ~$10^{3.0}$ PFU/ml. The yields of *tsm5* were about 25-60 times lower than that of *wt* virus from 3 to 7 days post infection ($p<0.05$). Although replication of mutant *tsm*5 was not identical to that observed previously, it is still clearly *ts* and the differences probably reflect the differences between stocks in the polymorphic viruses present within them (Section 3.1)



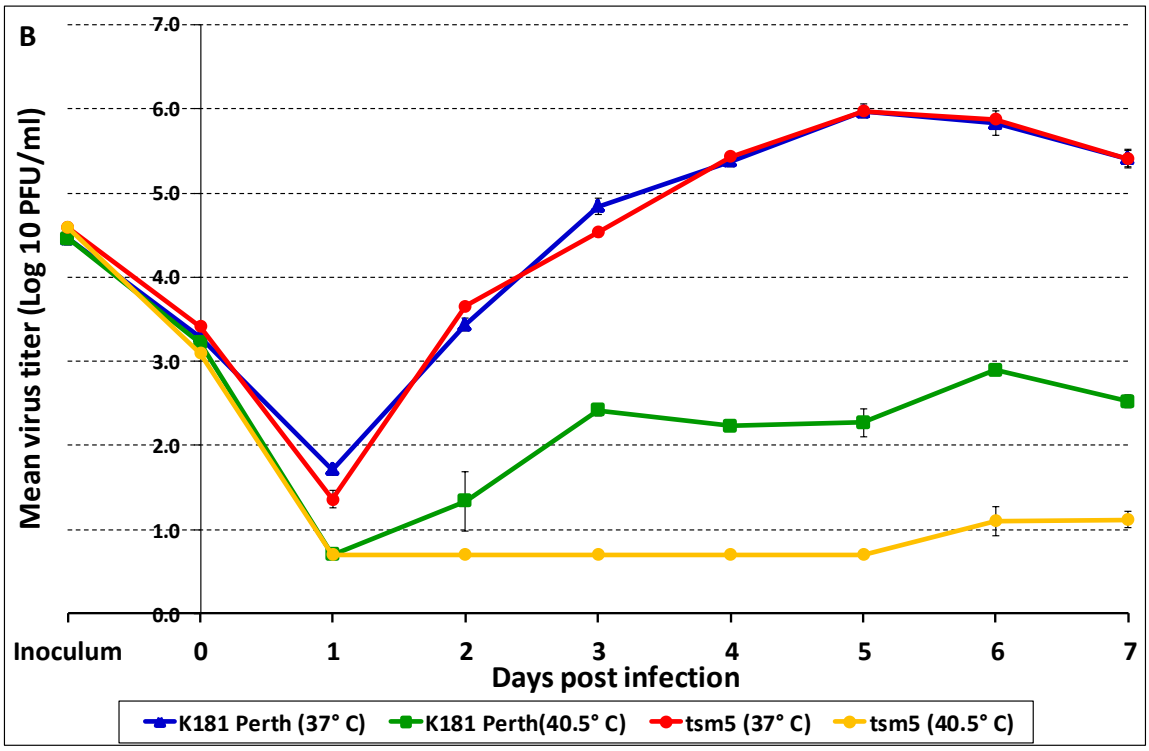


Figure 3.29: Replication of *tsm5* virus compared to *wt* K181 (Birmingham) and K181 (Perth) viruses at the permissive (37°) and non-permissive (40 or 40.5°C) temperatures. MEF cells were infected with virus at an MOI of 0.05 PFU per cell and incubated at 37°C and 40°C (A) or 37°C and 40.5°C (B). Viral titres were determined in cell culture supernatants 1-7 days post infection by plaque assay. Results are the mean ± SD of three replicates at each time point for the *wt* K181 (Birmingham) [A] or K181 (Perth) [B] and *tsm5* [A, B]. The limit of detection is 0.7 $\log_{10}$ PFU/ml.

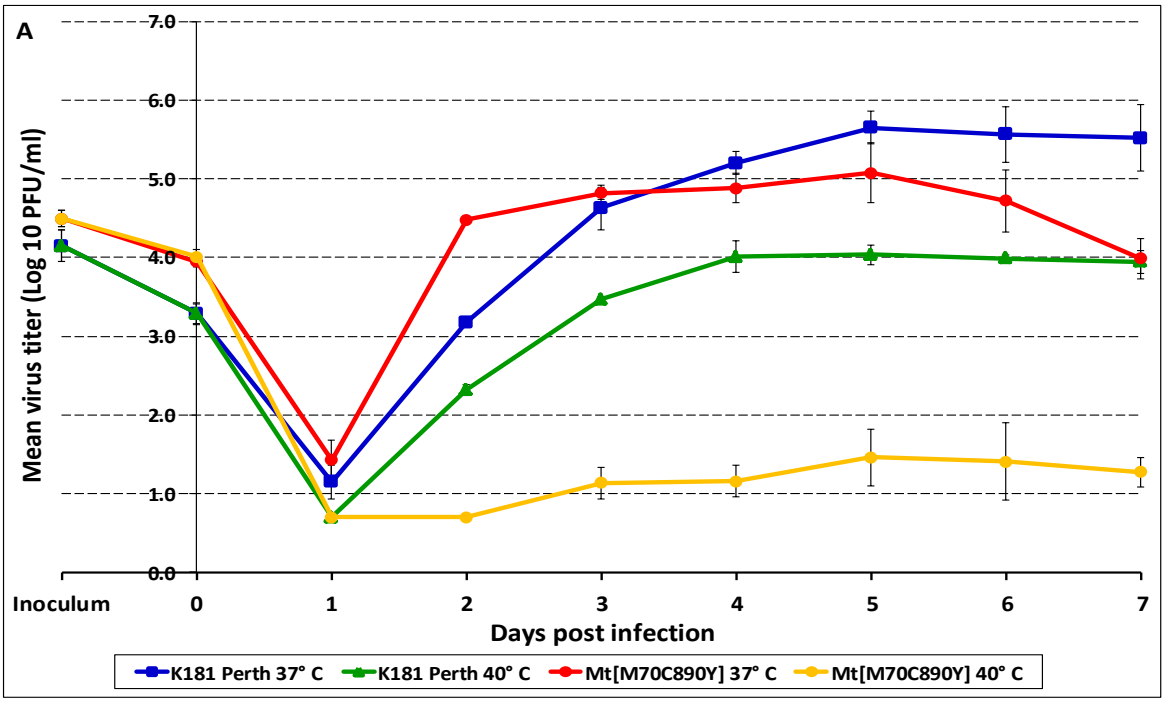
3.4.1.3 Replication of mutant Mt[M70^{C890Y}] virus

As previously shown in Section 3.1.1, the *tsm5* stock 27/07/2004 virus used in this study was heterogeneous in some genes including the aforementioned loci of the M27, M70 and M98 genes (Table 3.1) In addition, from the above it was clear that *tsm*5 no longer represented a good control virus for studies of temperature-sensitivity as virus stocks varied. A study in this laboratory, in which a mutation was introduced into the M70 gene (Timoshenko *et al.*, 2009b) and resulted in a temperature sensitive recombinant virus (Mt[M70^{C890Y}]), produced a cloned *ts* virus and this was used in the current study as the temperature sensitive control.

At 37°C, the replication kinetics of mutant Mt[M70^{C890Y}] was similar to that of K181 (Perth) (Figure 3.30). Both viruses were first detected in cell culture supernatants on day 2 post infection and reached maximum levels on day 5 post infection. Maximum yields of Mt[M70^{C890Y}] were ~$10^{5.00}$ PFU/ml. No significant differences were identified between yields of the two viruses from days 3 to 8 post infection ($p>0.05$).

Mutant Mt[M70^{C890Y}] produced significantly lower virus yields at 40°C (Figure 3.30A). Virus was first detected on day 3 post infection (~$10^{1.0}$ PFU/ml), yields increasing slowly to reach maximum on day 5 post infection ($10^{1.5}$ PFU/ml). Yields of Mt[M70^{C890Y}] were about 200-300 fold times lower than those of *wt* virus from 3 to 7 days post infection ($p<0.05$). The mutant Mt[M70^{C890Y}] virus was even more impaired for growth at 40.5°C where no virus could be detected from days 1-7 post infection compared to the *wt* virus, which was detectable at day 2 and reached maximum yields of $10^{2.9}$ PFU/ml by day 6 post infection (Figure 3.30B). In conclusion, the M70 mutant was more affected at the non-

permissive temperature than *tsm5*, which can be attributed to the presence of *wt* and mutated forms of the genes in *tsm5* virus stock populations.



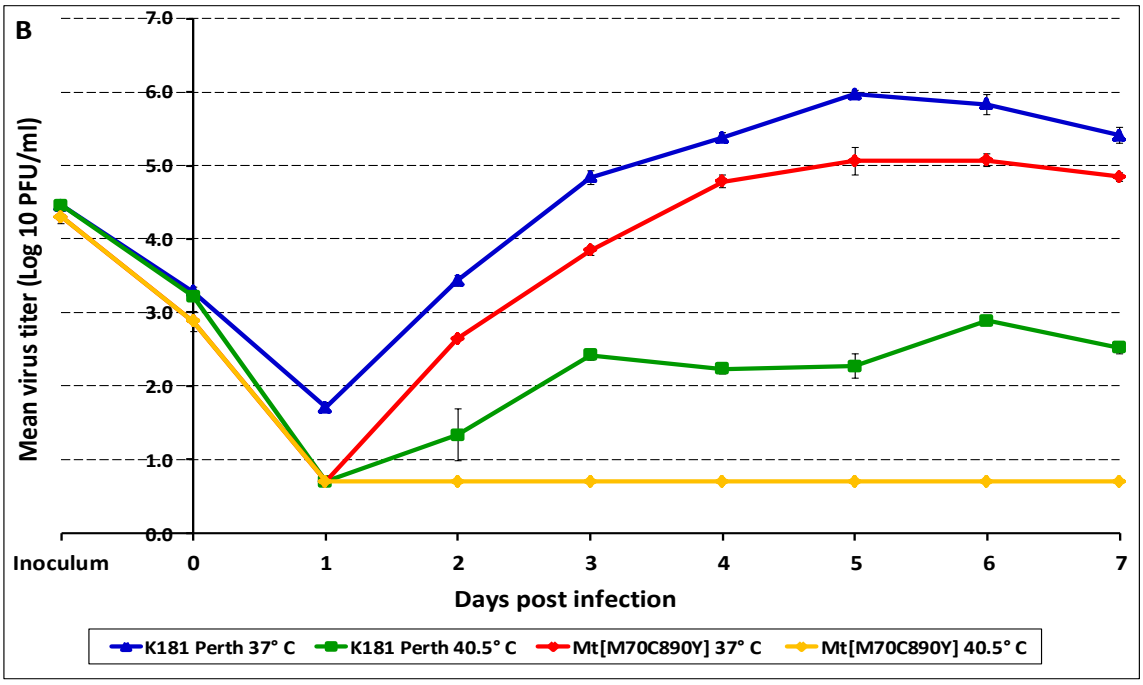


Figure 3.30: Replication of Mt[M70^{C890Y}] virus compared to *wt* K181 (Perth) virus. Viral titres were determined in cell culture supernatants 1-7 days post infection at 37ºC and 40ºC [A] or 37ºC and 40.5ºC [B]. Results are the mean ± SD of three replicates for K181 (Perth) and mutant Mt[M70^{C890Y}] viruses. The limit of detection is 0.7 log10 PFU/ml.

3.4.1.4 Replication of mutant Mt[M27^{A658S}], Mt[M36^{V54I}] and Mt[M27^{A658S}M36^{V54I}] viruses

The replication pattern of the mutant Mt[M27^{A658S}], Mt[M36^{V54I}] and Mt[M27^{A658S}M36^{V54I}] viruses at 37°C were very similar to that of their revertants Rv[M27^{S658A}], Rv[M36^{I54V}] and Rv[M27^{S658A}M36^{I54V}] respectively and to the *wt* virus (Figures 3.31A, 3.32A, and 3.33A). No significant differences were identified between mutant and *wt* virus yields at any day post infection ($p>0.05$).

Also, at 40°C Mt[M27^{A658S}] and Mt[M36^{V54I}] as well as their revertants and the wt viruses replicated similarly and no significant differences were identified between their yields on days 2 to 7 post infection ($p>0.05$) (Figure 3.31B and 3.33B). Whereas the double mutant virus Mt[M27^{A658S}M36^{V54I}] was lower than those of its revertant and *wt* virus (Figure 3.33B), but they were higher than those of the *ts* Mt[M70^{C890Y}] virus. Statistical analysis of mutant Mt[M27^{A658S}M36^{V54I}] virus yields for time points days 2 to 7 showed that virus yields were significantly different from those of its revertant and *wt* virus at each time point ($p<0.05$). Although virus yields of the double mutant Mt[M27^{A658S}M36^{V54I}] on MEF cells at the permissive temperature (37°C) were similar to that of its revertant and *wt* viruses, the plaque size produced by the double mutant was very small compared to *wt* K181 (Perth), the double revertant Mt[M27^{A658S}M36^{V54I}] and the 2 individual single mutants Mt[M27^{A658S}], Mt[M36^{V54I}] and their revertants (Figure 3.34B). Sequence analysis of the double mutant at the end of the growth curve studies revealed that there were no changes to the mutations introduced into both M27 and M36 genes at base position 32,302 and 49,108 respectively of the published K181 (Perth) sequence (data not shown).

At day 7 of the growth curves, infected MEF cells were collected, DNA extracted and sequenced for analysis of each recombinant virus. The presence of mutations in the mutant Mt[M27^{A658S}], Mt[M36^{V54I}] and Mt[M27^{A658S}M36^{V54I}] viruses were confirmed (data not shown).

In conclusion, the presence of either M27 (M27^{A658S}) or M36 (M36^{V54I}) mutations separately do not show a phenotype different from *wt* at either 37°C or 40°C, but presence of both mutations together in one construct Mt[M27^{A658S}M36^{V54I}] resulted in an attenuated phenotype at 40°C and could contribute to the attenuation of *tsm*5 virus *in vitro* at 40°C. Furthermore, these mutations decreased the ability of virus to spread to surrounding cells or the death of infected cells (Figure 3.34).

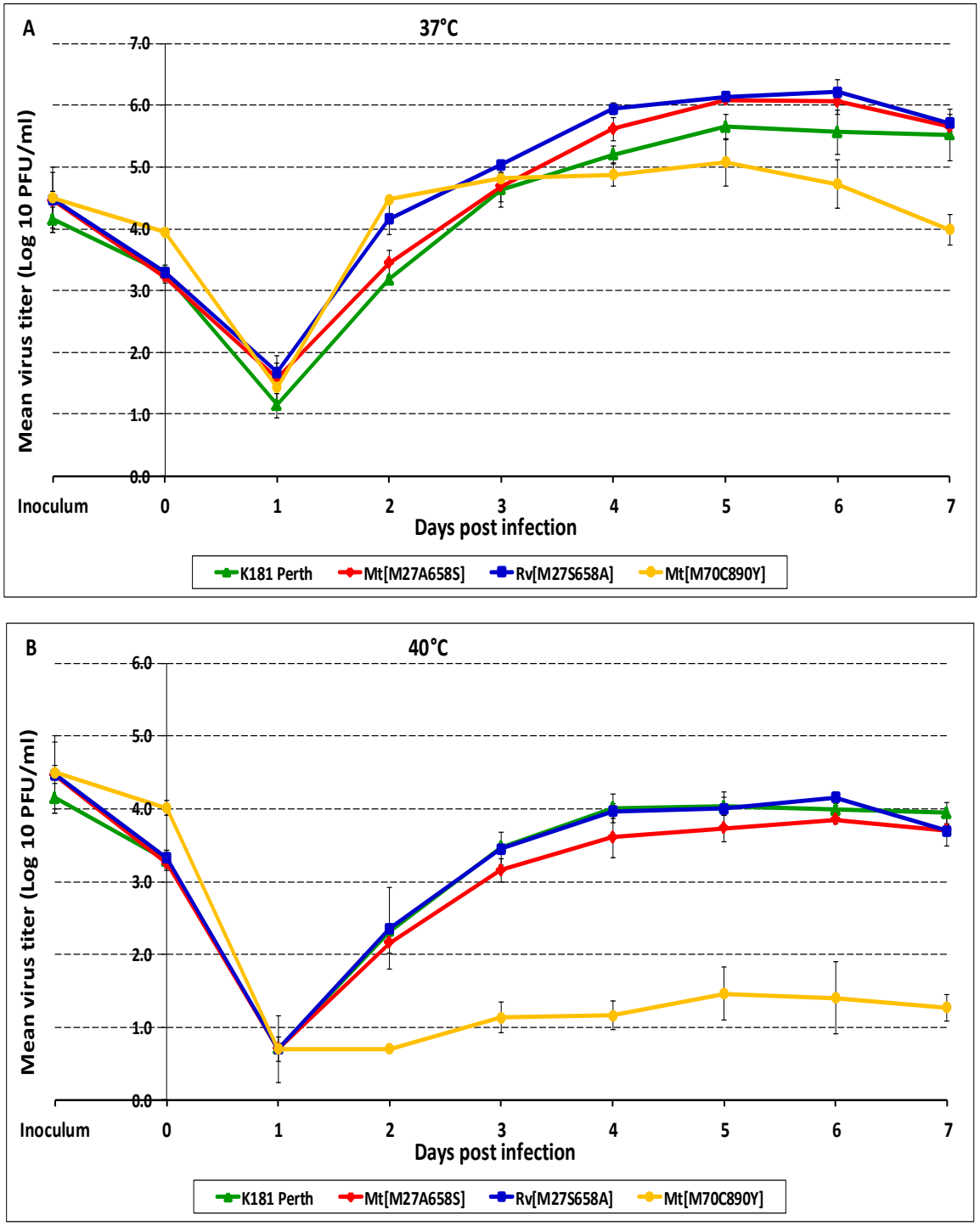


Figure 3.31: Replication of mutant Mt[M27^{A658S}] virus compared to its revertant Rv[M27^{S658A}], *wt* K181 (Perth) and the *ts* Mt[M70^{C890Y}] viruses at the permissive (37°C) and non-permissive (40°C) temperatures. MEF cells were infected with virus at an MOI of 0.05 PFU per cell and incubated at: [A] 37°C or [B] 40°C. Viral titres were determined in cell culture supernatants 1-7 days post infection by plaque assay. Results are the mean ± SD of two experiments, each with three replicates. The limit of detection is 0.7 log10 PFU/ml.

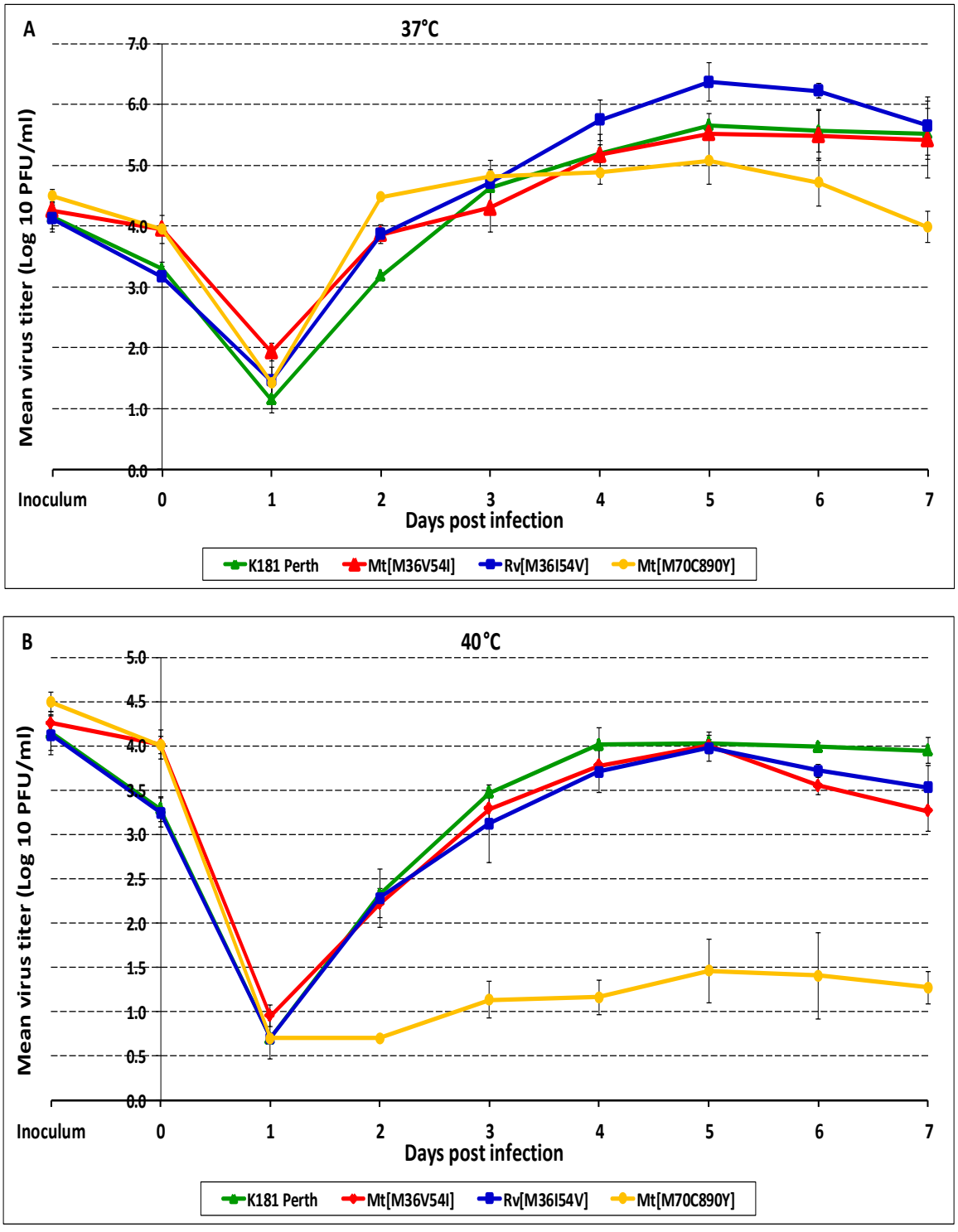


Figure 3.32: Replication of mutant Mt[M36^{V54I}] virus compared to its revertant Rv[M36^{I54V}], *wt* K181 (Perth) and the *ts* Mt[M70^{C890Y}] viruses at the permissive (37°C) and non-permissive (40°C) temperatures. MEF cells were infected with virus at an MOI of 0.05 PFU per cell and incubated at: [A] 37°C or [B] 40°C. Viral titres were determined in cell culture supernatants 1-7 days post infection by plaque assay. Results are the mean ± standard deviation (SD) of two experiments, each with three replicates.

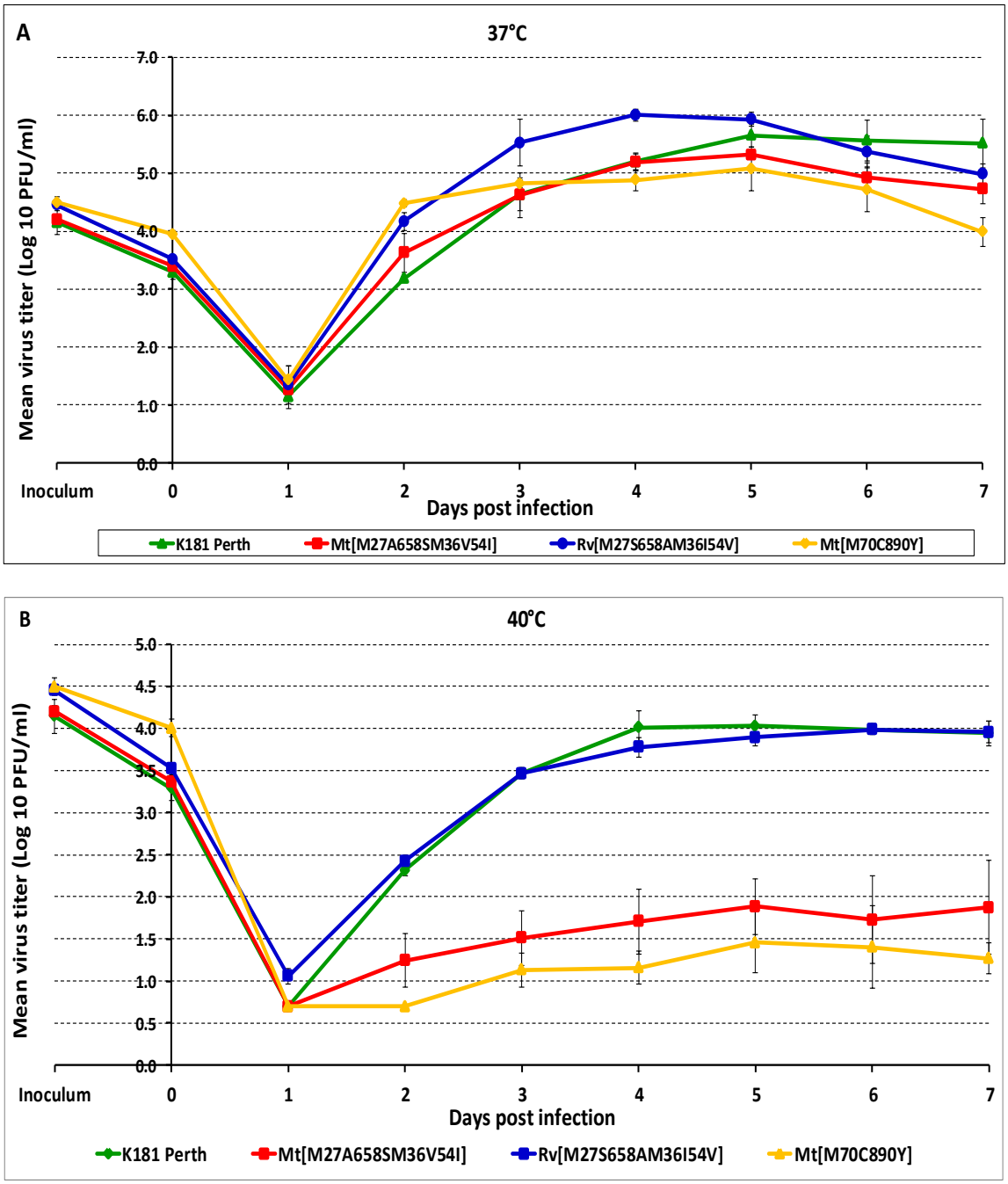


Figure 3.33: Replication of the double mutant Mt[M27^{A658S}M36^{V54I}] virus compared to its revertant Rv[M27^{S658A}M36^{I54V}], *wt* K181 Perth and the *ts* Mt[M70^{C890Y}] viruses at the permissive (37°C) and non-permissive (40°C) temperatures. MEF cells were infected with virus at an MOI of 0.05 PFU per cell and incubated at: [A] 37°C or [B] 40°C. Viral titres were determined in cell culture supernatants 1-7 days post infection by plaque assay. Results are the mean ± standard deviation (SD) of two experiments, each with three replicates.

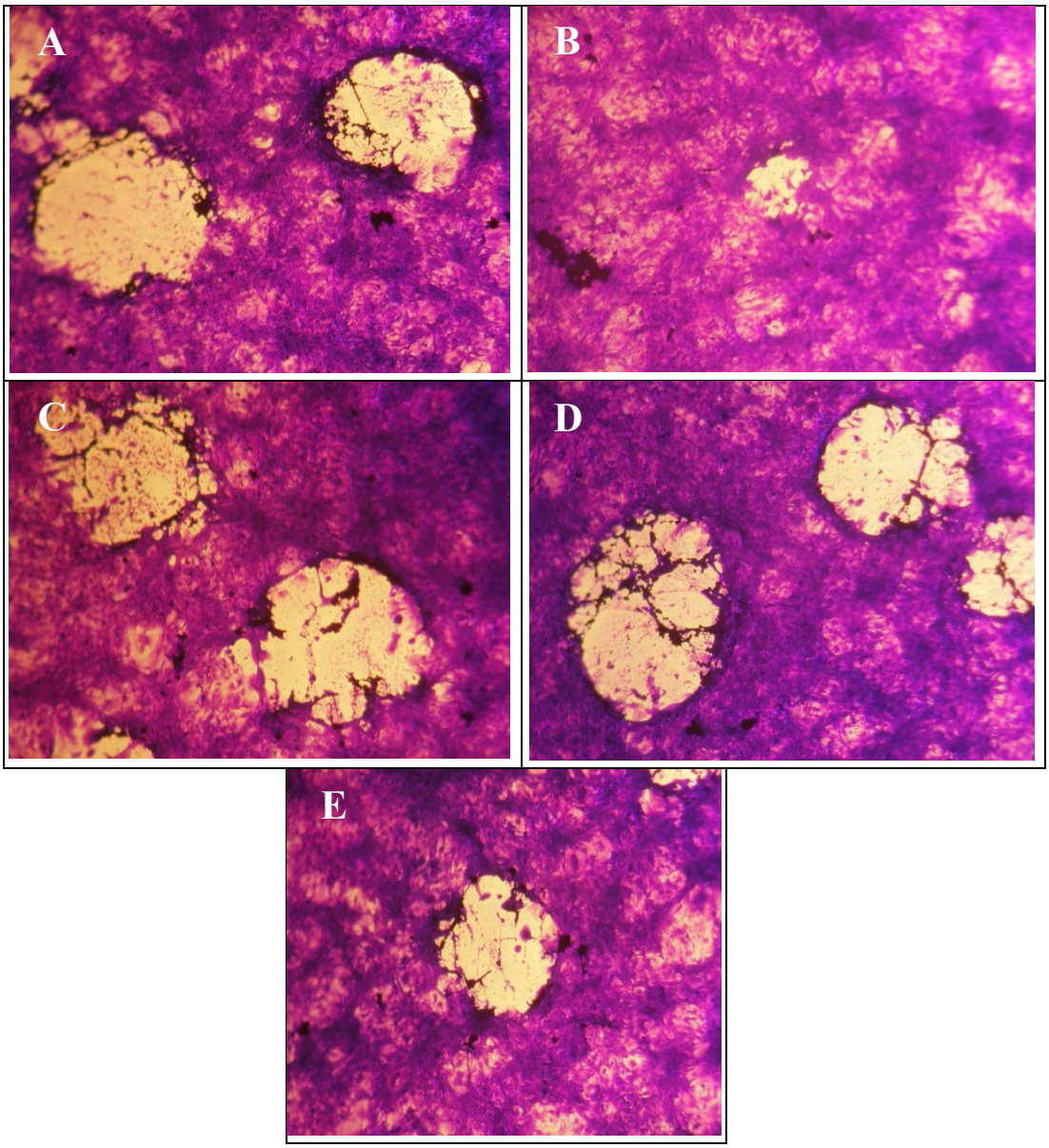


Figure 3.34: Plaque size of *wt* (A), Mt[M27^{A658S}M36^{V54I}] (B), Rv[M27^{S658A}M36^{I54V}] (C), Mt[M27^{A658S}] (D), and Mt[M36^{V54I}] (E) viruses. The viruses were grown and titrated on MEF cells. The images were taken using an inverted microscope attached to a digital camera at 60x magnification.

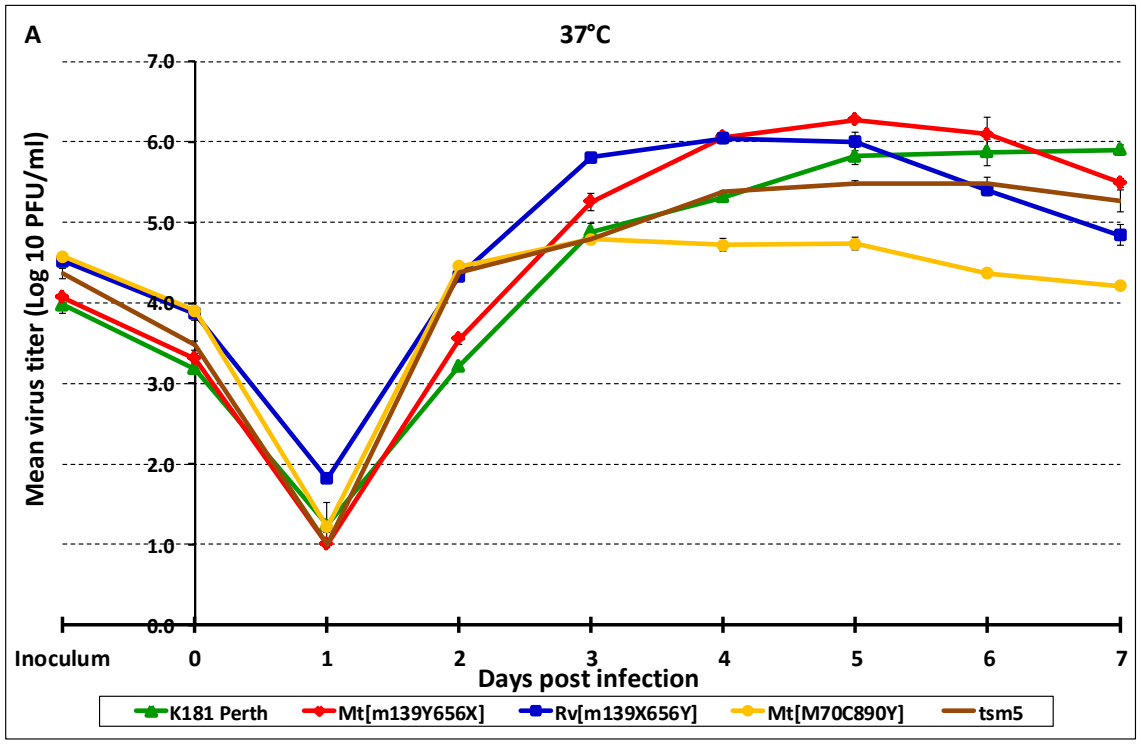
3.4.1.6 Replication of Mt[m139^{Y656X}], Mt[m141^{V195M}] and Mt[m143^{M232I}] mutant viruses

The replication kinetics of the mutant Mt[m139^{Y656X}], Mt[m141^{V195M}] and Mt[m143^{M232I}] viruses at 37°C and were very similar to that of their revertants Rv[m139^{X656Y}], Rv[m141^{M195V}] and Rv[m143^{I232M}] respectively and to the *wt* virus (Figures 3.35A, 3.36A, and 3.37A). No significant differences were identified between mutant and *wt* virus yields at any day post infection ($p>0.05$).

Yields of mutant Mt[m139 ^{Y656X}] virus at 40°C were lower than that of its revertant and *wt* virus, but very similar to that of *tsm5* (Figure 3.35B). Mutant virus was first detected on day 2 post infection; on days 3 and 4 mutant Mt[m139 ^{Y656X}] virus yields were approximately one log lower than those of the revertant and *wt* viruses. Its yields increased gradually and reached the maximum level of $10^{3.5}$ PFU/ml by day 5 post infection. Statistical analysis showed that only the yields of days 3 and 4 were significantly different from those of its revertant and *wt* virus at any time point ($p<0.05$).

In contrast, Mt[m141^{V195M}] and Mt[m143^{M232I}] growth pattern at 40°C (data not shown) and 40.5°C were not significantly different ($p>0.05$) from the revertant and *wt* virus yields (Figure 3.36B and 3.37B).

In conclusion, the mutation Mt[m139 ^{Y656X}] virus has a *ts* phenotype at 40°C and therefore it is likely that this mutation also contributes to the attenuation of *tsm*5 virus *in vitro* at 40°C. Whereas, Mt[m141^{V195M}] and Mt[m143^{M232I}] appear not to pay a role in the *ts* phenotype of *tsm*5.



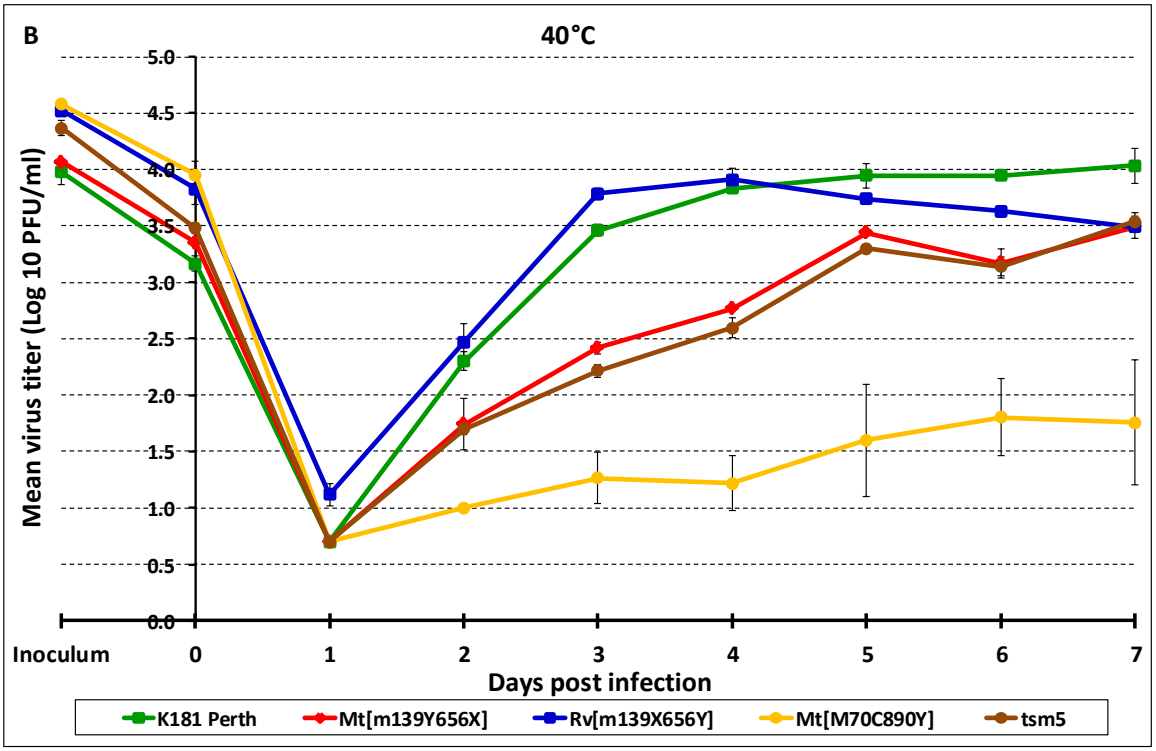


Figure 3.35: Replication of mutant Mt[m139^{Y656X}] compared to its revertant Rv[m139 ^{X656Y}], *wt* K181 (Perth), the *ts* mutant Mt[M70^{C890Y}] and *tsm5* viruses at the permissive (37°C) and non-permissive (40°C) temperatures. MEF cells were infected with virus at an MOI of 0.05 PFU per cell and incubated at: [A] 37°C or [B] 40°C. Viral titres were determined in cell culture supernatants 1-7 days post infection by plaque assay. Results are the mean ± standard deviation (SD) of two experiments, each of three replicates.

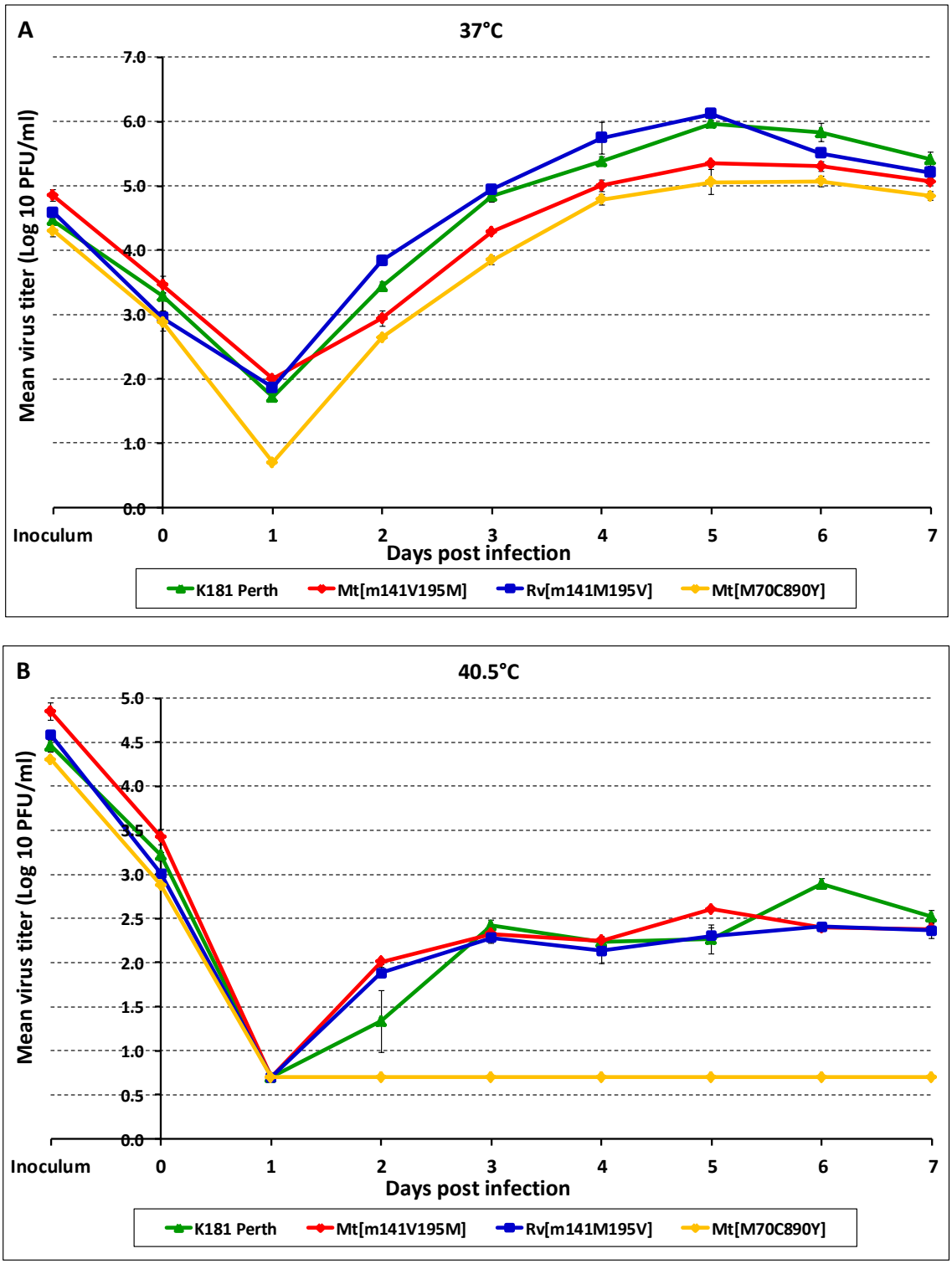
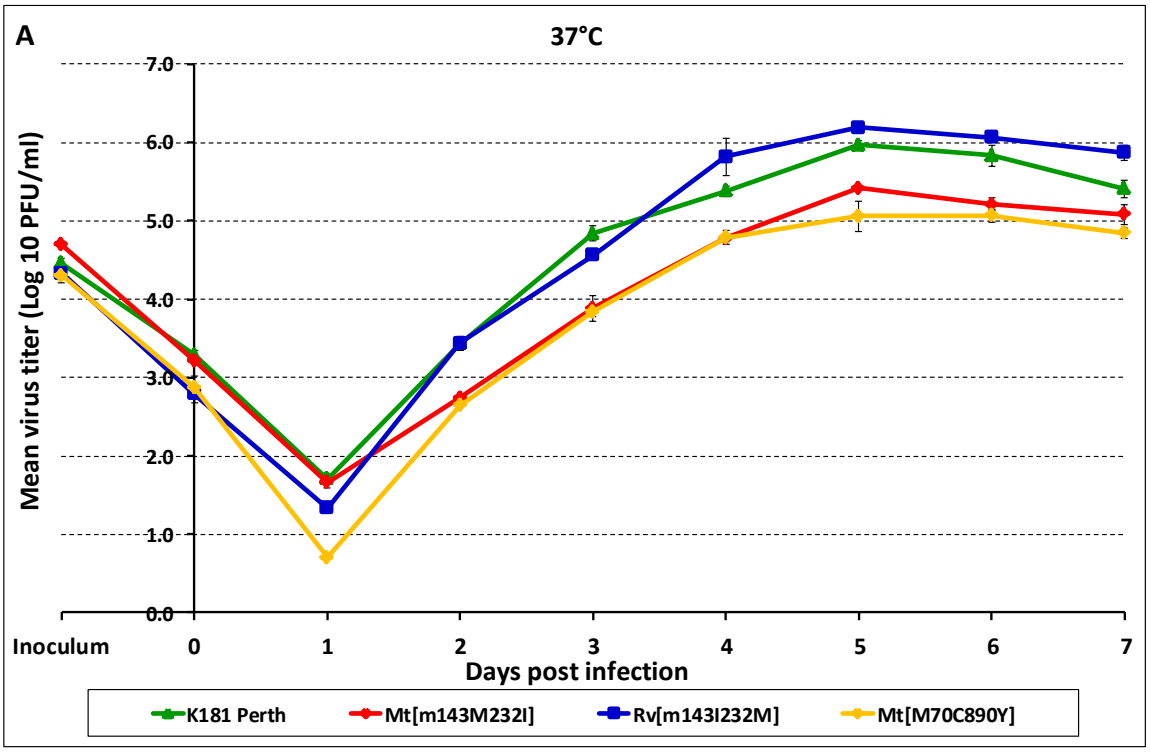


Figure 3.36: Replication of mutant Mt[m141^{V195M}] virus compared to its revertant Rv[m141^{M195V}], *wt* K181 (Perth) and the *ts* Mt[M70^{C890Y}] viruses at the permissive (37°C) and non-permissive (40.5°C) temperatures. MEF cells were infected with virus at an MOI of 0.05 PFU per cell and incubated at: [A] 37°C or [B] 40.5°C. Viral titres were determined in cell culture supernatants 1-7 days post infection by plaque assay. Results are the mean ± standard deviation (SD) of two experiments, each of three replicates.



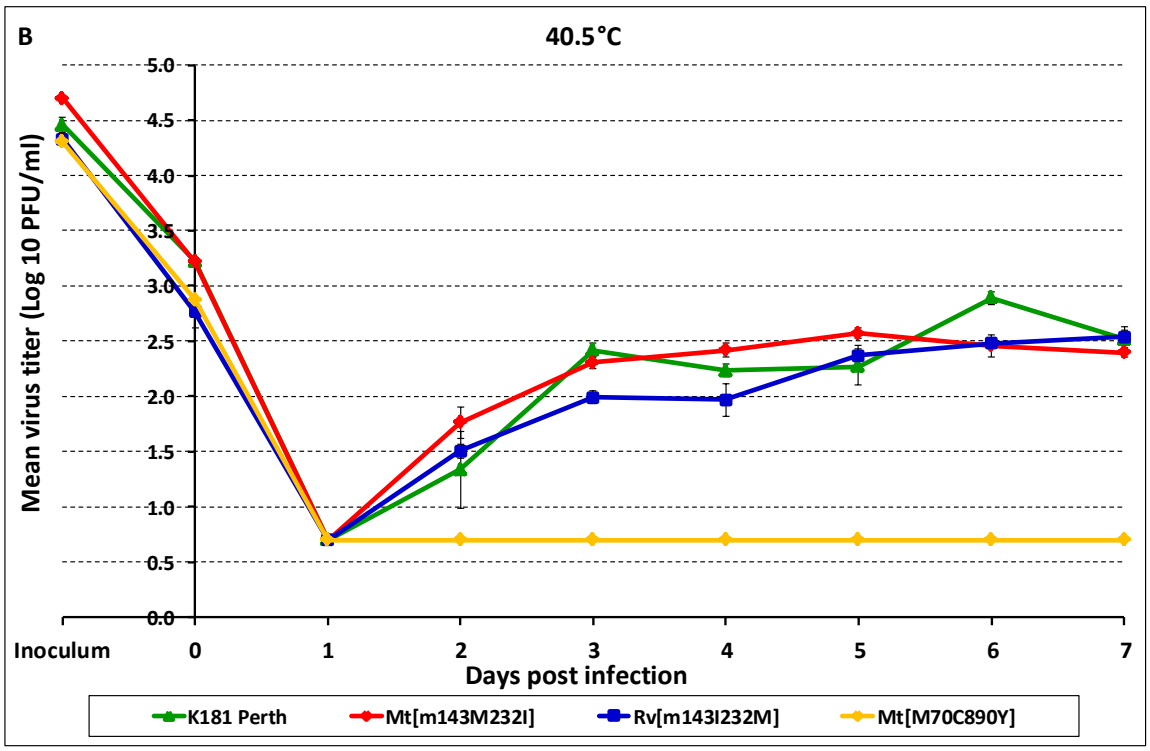


Figure 3.37: Replication of mutant Mt[m143^{M232I}] compared to its revertant Rv[m143^{I232M}], *wt* K181 (Perth) and the *ts* Mt[M70^{C890Y}] viruses at the permissive (37°C) and non-permissive (40.5°C) temperatures. MEF cells were infected with virus at an MOI of 0.05 PFU per cell and incubated at: [A] 37°C or [B] 40.5°C. Viral titres were determined in cell culture supernatants 1-7 days post infection by plaque assay. Results are the mean ± standard deviation (SD) of two experiments, each of three replicates.

3.4.2 Characterisation of virus phenotype on macrophage cell lines:

M27, M36, m139, m141 and m143 are immunoevasion genes and studies showed that are important for virus growth in macrophages (Hanson *et al.*, 1999; Hanson *et al.*, 2001; Menard *et al.*, 2003). Based on that the growth kinetics of the viruses were also studied on the Raw 264.7 macrophage cell line but at 37°C only. Initial experiments showed that, although the macrophages were viable at 40°C, yields were very low or undetectable even for *wt* virus.

3.4.2.1 Replication of *wt* MCMV K181 (Perth)

Raw 264.7 macrophage cell were infected with the *wt* at an MOI= 0.1 to study its growth kinetics. K181 (Perth) virus yields were first detected on day 2 post infection and increased rapidly reaching the maximum yield of ~$10^{7.1}$ PFU/ml on day 6 post infection (Figure 3.38).

3.4.2.2 Replication of mutant Mt[M70^{C890Y}] virus

Unexpectedly mutant Mt[M70^{C890Y}] virus replication in Raw 264.7 macrophages was impaired at 37°C and virus yields were 2-3 logs below those of the *wt* K181 (Perth) virus (Figure 3.38). Mutant Mt[M70^{C890Y}] virus yield were first detected on day 2 post infection (~$10^{2.5}$ PFU/ml) then gradually increased to reach its maximum yield of ~$10^{4.6}$ PFU/ml on day 6 post infection.

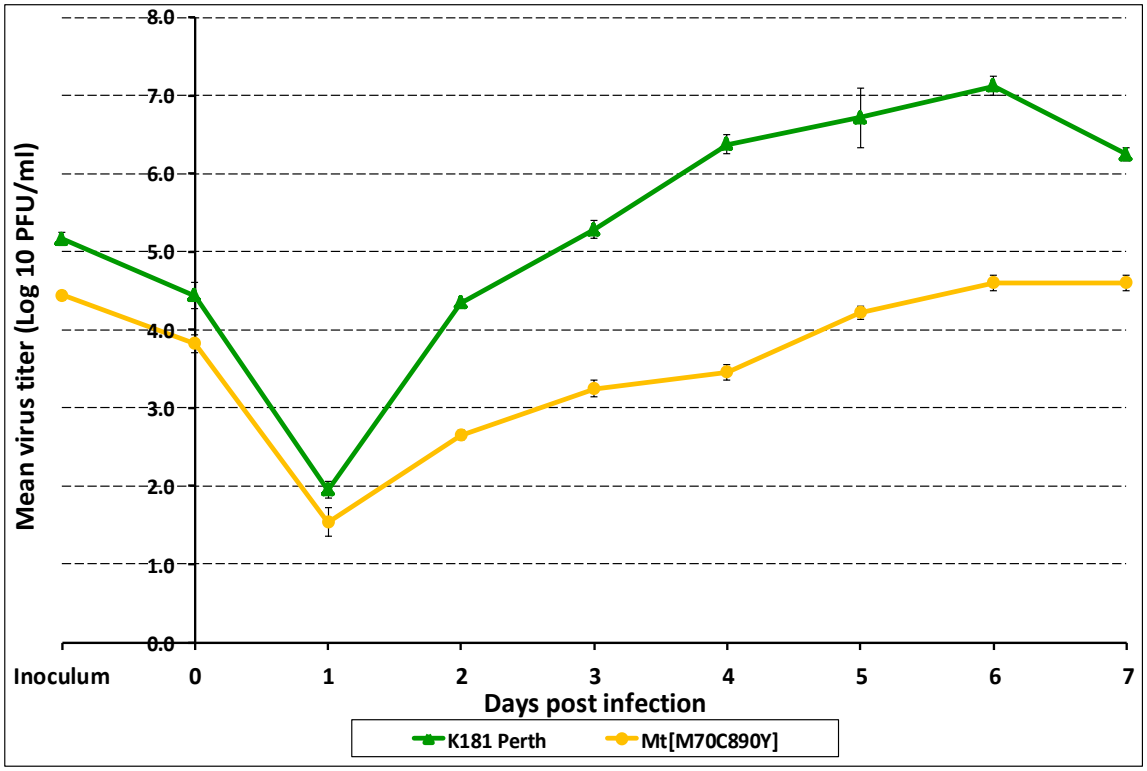


Figure 3.38: Replication of the *wt* K181 (Perth) and mutant Mt[M70^{C890Y}] viruses in Raw 264.7 macrophages. Macrophages were infected with virus at an MOI of 0.1 PFU per cell and incubated at 37°C. Viral titres were determined in cell culture supernatants 1-7 days post infection by plaque assay on MEF cells. Results are the mean ± standard deviation (SD) of two experiments, each of three replicates.

3.4.2.3 Replication of mutant Mt[M27^{A658S}], Mt[M36^{V54I}], Mt[M27^{A658S}M36^{V54I}] Mt[m139^{Y656X}], Mt[m141^{V195M}] and Mt[m143^{M232I}] viruses

As in MEF cells, the replication of mutant Mt[M27^{A658S}], Mt[M36^{V54I}], Mt[m139^{Y656X}], Mt[m141^{V195M}] and Mt[m143^{M232I}] in Raw 264.7 cells were similar to that of their revertants and the K181 (Perth) variant parent viruses (Figure 3.39-3.40 and Figure 3.42-3.44).

Interestingly, replication of the double mutant Mt[M27^{A658S}M36^{V54I}] was attenuated in Raw 264.7 macrophages, compared to its revertant Rv[M27^{S658A}M36 ^{I54V}] virus and the parent *wt* K181 (Perth) variant. Initially, the yield of Mt[M27^{A658S}M36^{V54I}] on day 2 was only slightly lower than that of the double revertant, but at later time points the yields increased at a slower rate reaching a maximum of ~$10^{5.2}$ PFU/ml on day 7 compared to maximums of ~$10^{7.1}$ PFU/ml on day 6 for the *wt* virus and $10^{6.3}$ PFU/ml on day 5 for the revertant virus (Figure 3.41). The yield of mutant Mt[M27^{A658S}M36^{V54I}] was more than 300 fold lower on day 4 than the *wt* and more than 150 fold on day 5. The replication of the double mutant was statistically similar to that of the M70 mutant [MtM70^{C890Y}] and statistically lower than both the *wt* and revertant viruses.

From this growth pattern, we can conclude that apart of the double mutant Mt[M27^{A658S}M36^{V54I}], all other mutant constructs do not have a phenotype in Raw 264.7 cells.

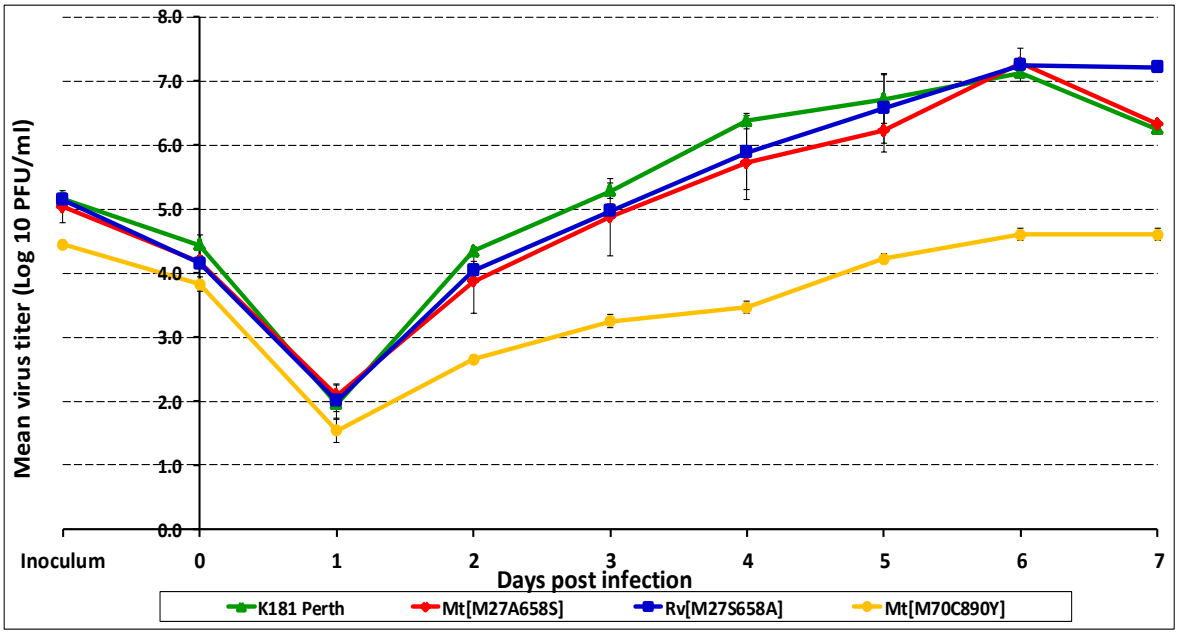


Figure 3.39: Replication of mutant Mt[M27^{A658S}] virus compared to its revertant Rv[M27$^{S658\ A}$], *wt* K181 (Perth) and Mt[M70^{C890Y}] viruses at 37°C. Raw 264.7 macrophage cells were infected with virus at an MOI of 0.1 PFU per cell and incubated at 37°C. Viral titres were determined in cell supernatants 1-7 days post infection by plaque assay on MEF cells. Results are the mean ± standard deviation (SD) of two experiments, each of three replicates.

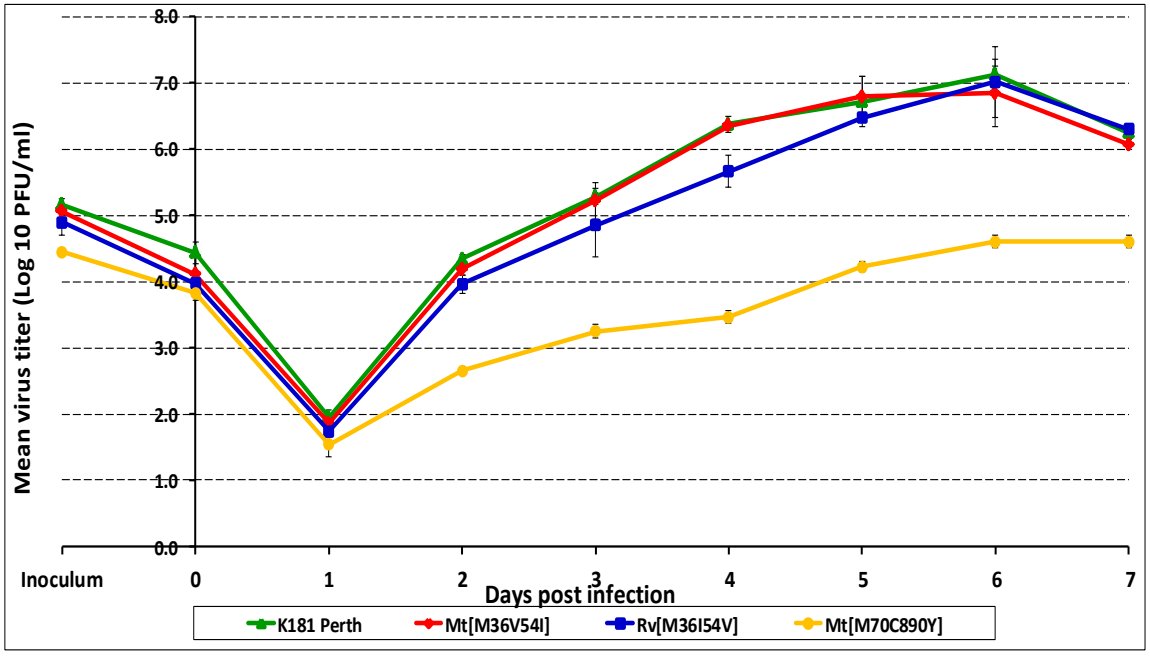


Figure 3.40: Replication of mutant Mt[M36^{V54I}] virus compared to its revertant Rv[M36^{I54V}], *wt* K181 (Perth) and Mt[M70^{C890Y}] viruses at 37°C. Raw 264.7 macrophage cells were infected with virus at an MOI of 0.1 PFU per cell and incubated at 37°C. Viral titres were determined in cell culture supernatants 1-7 days post infection by plaque assay on MEF cells. Results are the mean ± standard deviation (SD) of two experiments, each of three replicates.

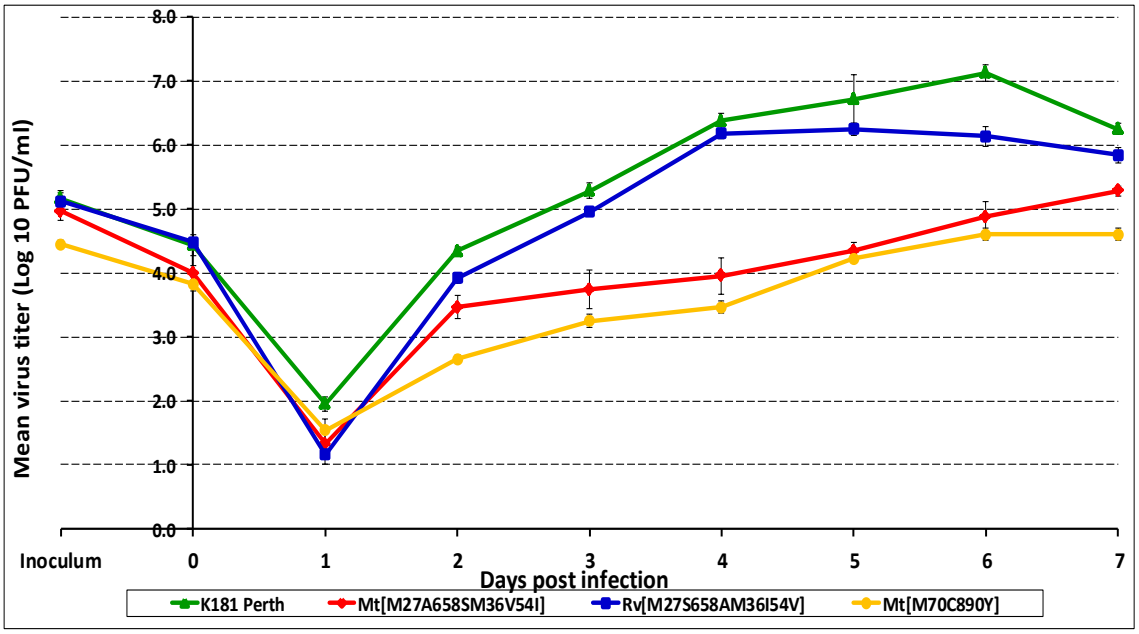


Figure 3.41: Replication of mutant Mt[M27^{A658S}M36^{V54I}] virus compared to its revertant Rv[M27^{S658A}M36 ^{I54V}], *wt* K181 (Perth) and Mt[M70^{C890Y}] viruses at 37°C. Raw 264.7 macrophage cells were infected with virus at an MOI of 0.1 PFU per cell and incubated at 37°C. Viral titres were determined in cell culture supernatants 1-7 days post infection by plaque assay on MEF cells. Results are the mean ± standard deviation (SD) of two experiments, each of three replicates.

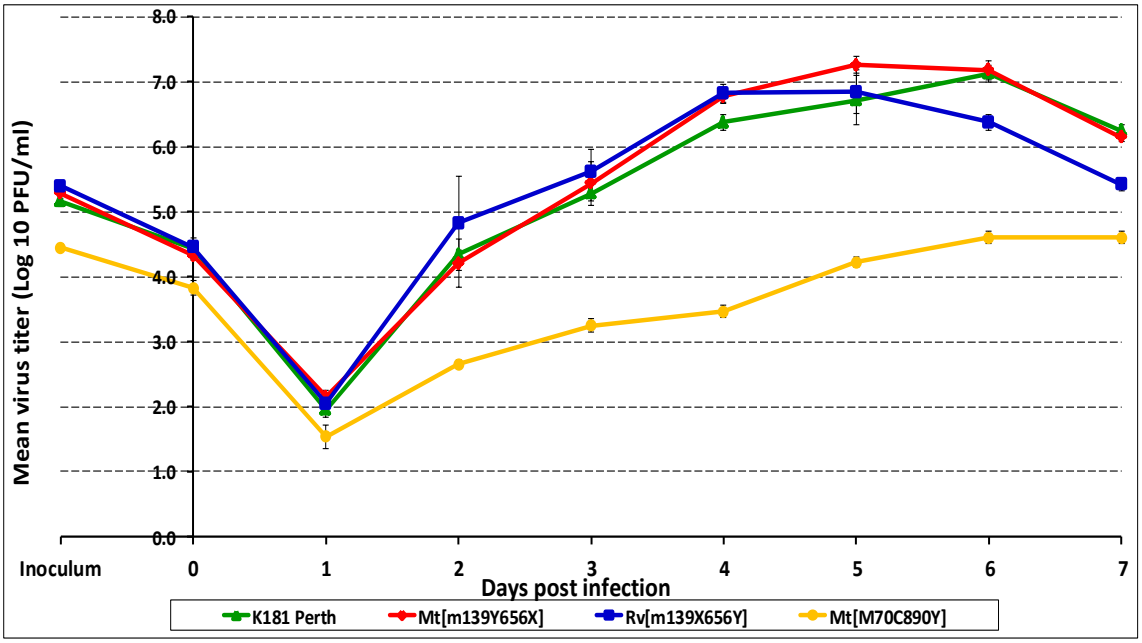


Figure 3.42: Replication of mutant Mt[m139^{Y656X}] virus compared to its revertant Rv[m139^{X656Y}], *wt* K181 (Perth) and Mt[M70^{C890Y}] viruses at 37°C. Raw 264.7 macrophage cells were infected with virus at an MOI of 0.1 PFU per cell and incubated at 37°C. Viral titres were determined in cell culture supernatants 1-7 days post infection by plaque assay on MEF cells. Results are the mean ± standard deviation (SD) of two experiments, each of three replicates.

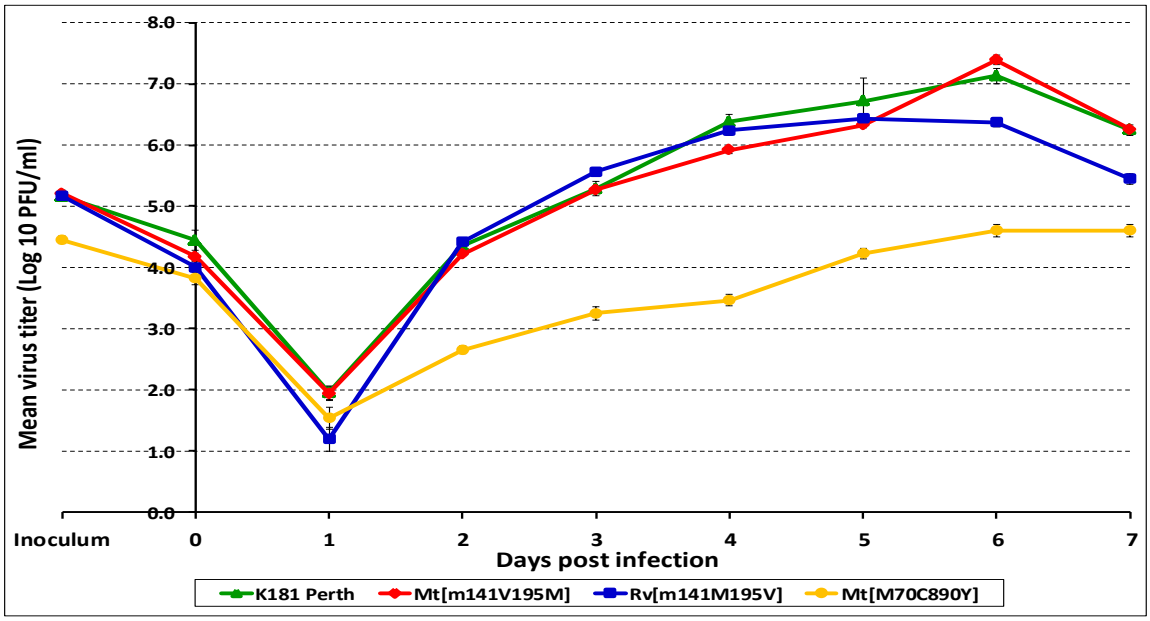


Figure 3.43 Replication of Mt[m141^{V195M}] mutant virus compared to its revertant Rv[m141^{M195V}], *wt* K181 Perth and Mt[M70^{C890Y}] viruses at 37°C. Raw 264.7 macrophage cells were infected with virus at an MOI of 0.1 PFU per cell and incubated at 37°C. Viral titres were determined in cell supernatants 1-7 days post infection by plaque assays (on MEF cells). Results are the mean ± standard deviation (SD) of two experemnets three replicates in each.

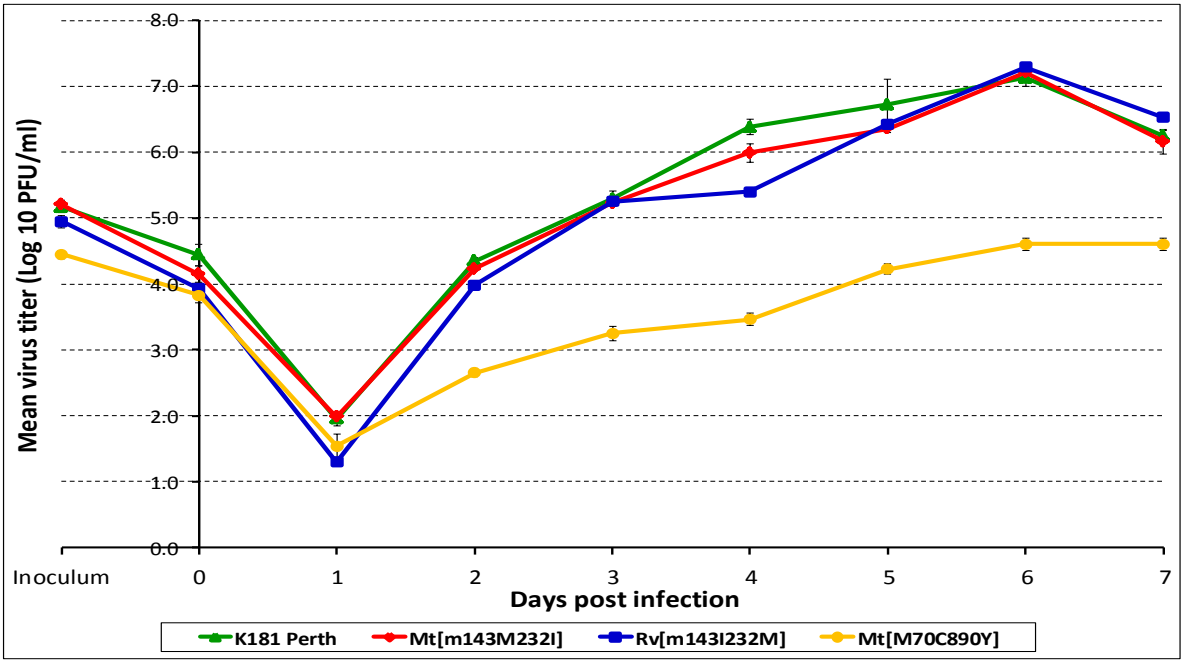


Figure 3.44: Replication of mutant Mt[m143^{M232I}] virus compared to its revertant Rv[m143^{I232M}], *wt* K181 (Perth) and Mt[M70^{C890Y}] viruses at 37°C. Raw 264.7 macrophage cells were infected with virus at an MOI of 0.1 PFU per cell and incubated at 37°C. Viral titres were determined in cell culture supernatants 1-7 days post infection by plaque assay on MEF cells. Results are the mean ± standard deviation (SD) of two experiments, each of three replicates.

3.5 Changing codon preferences in the M70 ORF

It is believed that large DNA viruses, including herpesviruses, have low mutation rates as estimated by methods such as analysis of RFLP or function of individual genes (Sakaoka *et al.*, 1994). The high genome stability of MCMV at the single-nucleotide level in the absence of obvious selection pressure was also supported by the analysis of MCMV using whole genome sequencing that showed that sequence of three sister plaques showed only two single-base-pair substitutions after *in vitro* passage. Furthermore, passage of MCMV in the mouse, revealed that mutation rates were also low (Cheng *et al.*, 2010). However, other studies have shown that mutations many emerge in both HCMV and MCMV as a result of the presence of selective pressure (French *et al.*, 2005; Sanchez *et al.*, 2004). Ganciclovir resistance of HCMV in up to 25% of immunosuppressed patients infected with HCMV resulted from mutations in the UL97 ORF (Sanchez *et al.*, 2004).

On the other hand, MCMV has been shown to acquire *de novo* mutations after *in vivo* passage. Passage of MCMV in mice lacking adaptive immunity but carrying the Cmv1r allele, which encodes the Ly49H activation receptor on NK cells, resulted in emergence of viruses carrying mutations in the m157 ORF (encoding the only known ligand for Ly49H). These mutants were characterized by an increased virulence in naive Cmv1r mice, which was attributed to the presence of single amino-acid substitutions or premature stop codons (French *et al.*, 2004). The adjacent ORFs (m156 and m158) showed no mutations suggesting that mutations emerged under selective pressure (Cheng *et al.*, 2010).

A previous study in our laboratory, in which mutations were introduced into the M56, M70 and M98 genes by Dr. Olga Timoshenko, showed that a C to T mutation in the M70 gene at base position 99,286 made the recombinant Mt[M70^{C890Y}] virus temperature sensitive where virus replication was drastically reduced at 40ºC compared to the parent *wt* K181 (Perth) virus (Timoshenko *et al.*, 2009b). *In vivo*, the virus was not detectable in the salivary glands of BALB/c mice 21 days post infection. From *in vitro* investigation at 40ºC, it was noticed in some experiments that the T mutation at base position 99,286 reverted to the *wt* C. Astonishingly, in some experiments using a triple mutant (Mt98^{324S}70^{890Y}56^{439R}) virus, the codon of the introduced mutation of M70 had mutated from the mutant UAC (ATG on the complementary strand) coding tyrosine to UCC (AGG on the complementary strand) coding for serine as early as one-day post infection, or more likely during passage of the virus to remove the BAC (Timoshenko *et al.*, 2009b). Furthermore, this tyrosine to serine mutation was also observed at the permissive temperature (37ºC) (Timoshenko *et al.*, 2009b).

According to the genetic code, all amino acids (except methionine and tryptophan) in the protein coding sequence can be encoded by more than one synonymous codon (Table 3.5). The synonymous codons for each amino acid are used in unequal frequencies and might have developed with the cell's translation machinery to evade excessive use of suboptimal codons which often correspond to rare or less used tRNAs (Gustafsson *et al.*, 2004). This phenomenon was termed as "synonymous codon bias" which varies greatly between evolutionarily distant species and perhaps even between different tissues in the same species (Plotkin *et al.*, 2004). Analysis of codon usage bias in 43 herpesviruses showed that most of the herpesviruses do not have high codon bias, with the exceptions of only simplex viruses and some varicella viruses (Fu, 2010).

Codon manipulation is widely used in modern molecular microbiology, where codon optimization via changing the gene's synonymous codon into codons used most frequently by the host cell is used to increase the efficiency of cross-species protein expression (Gustafsson *et al.*, 2004). Whereas the opposite approach of reducing expression by intentional introduction of suboptimal synonymous codons has been rarely used. Recently, with advances in synthetic biology, researchers were able to proceed in live attenuated vaccine development as it is now possible to synthesise large segments of synthetic DNA for poliovirus. The synthetic DNA is then used to produce the entire genome of the infectious agents (Cello *et al.*, 2002). In addition, it is possible now to create viable viruses by chemical synthesis in which large segments of the viral genome are modified without altering the amino acid sequence, leading to controlled and deliberate attenuation of the virus (Burns *et al.*, 2006; Mueller *et al.*, 2006). Replacement of natural codons used in the Sabin type 2 (Sabin 2) oral poliovirus vaccine strain for the capsid region with synonymous non-preferred (minor) codons resulted in a significant reduction in the virus yield and virus plaque sizes, which was proportional to the number of non-preferred codons incorporated into the capsid region sequences. Serial passage (for 25 passages in HeLa cells) of the virus with altered codon compositions showed that the relative fitness of the modified viruses remained lower than that of the unmodified virus (Burns *et al.*, 2006). Mice, transgenic for the poliovirus receptor (CD155tg mice), infected with equal amounts of virus particles of the *wt* virus or the virus with altered codons, revealed that the latter was over 100-fold neuro-attenuated compared to the *wt* virus (Mueller *et al.*, 2006). The synthetic viruses with synonymous non-preferred codons were stable and may prove suitable as attenuated substrates for the production of poliovirus vaccines (Mueller *et al.*, 2006).

In MCMV, to reduce the possibility of reversion/mutation of the tyrosine residue 890 to cysteine or serine, a synthetic M70 gene with many synonymous mutations was introduced into the M70 ORF, the hypothesis being that with many mutations present it would be less likely that the tyrosine codon would revert. The aim was to replace M70 codons with the least preferred or sub-optimally codons used by the mouse (*Mus musculus domesticus*) (Table 3.5).

Several strategies were examined for changing the codon sequence of the M70 ORF to those used less frequently by the mouse. Initially, all codons that were not already used least frequently were changed to the least frequently used mouse codons. This resulted in an M70 ORF with a G + C content of 43% compared to the K181 (Perth) ORF of 64% (leop.all line in Figure 3.45). To maintain the high G + C content an alternative strategy was used in which the 9 amino acids that have more than 2 codons were changed to the codon used sub-optimally by the mouse with the highest GC content ($M70^{SO304Cys}$ in Figure 3.45). This produced an M70 ORF with a G + C content of 66% and a CpG of 379 compared to a CpG content of 381 for the K181 (Perth) ORF (Figure 3.46); 403 of the 964 codons (42%) were changed (Figure 3.45). The numbers of the particular codons in each construct are shown in Table 3.6.

Table 3.5: Codon preferences for the mouse (*Mus musculus domesticus*).

Source: http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=10092

Amino Acid	Codon	Number/ 1000	Fraction	Amino Acid	Codon	Number/ 1000	Fraction
Alanine (Ala or A)	GCG	325	6.1	**Leucine (Leu or L)**	CTA	665	12.6
	GCA	913	17.3		CTT	616	11.6
	GCT	705	13.3		CTC	984	18.6
	GCC	1087	20.5	**Lysine (Lys or K)**	AAG	1701	32.1
Arginine (Arg or R)	AGG	739	14.0		AAA	237	44.8
	AGA	1016	19.2	**Methionine (Met or M)**	ATG	1183	22.4
	CGG	397	7.5	**Phenylalanine (Phe or F)**	TTT	793	15.0
	CGA	291	5.5		TTC	1231	23.3
	CGT	178	3.4	**Proline (Pro or P)**	CCG	305	5.8
	CGC	385	7.3		CCA	995	18.8
Asparagine (Asn or N)	AAT	970	18.3		CCT	746	14.1
	AAC	1396	26.4		CCC	872	16.5
Aspartic Acid (Asp or D)	GAT	973	18.4	**Serine (Ser or S)**	AGT	489	9.2
	GAC	1542	29.1		AGC	847	16.0
Cysteine (Cys or C)	TGT	506	9.6		TCG	220	4.2
	TGC	616	11.6		TCA	781	14.8
Glutamic Acid (Glu or E)	GAG	1739	32.9		TCT	587	11.1
	GAA	1673	31.6		TCC	755	14.3
Glutamine (Gln or Q)	CAG	2608	49.3	**Threonine (Thr or T)**	ACG	244	4.6
	CAA	845	16.0		ACA	1206	22.8
Glycine (Gly or G)	GGG	618	11.7		ACT	702	13.3
	GGA	832	15.7		ACC	828	15.6
	GGT	498	9.4	**Tryptophan (Trp or W)**	TGG	800	15.1
	GGC	916	17.3	**Tyrosine (Tyr or Y)**	TAT	655	12.3
Histidine (His or H)	CAT	600	11.3		TAC	933	17.6
	CAC	1135	21.5	**Valine (Val or V)**	GTG	988	18.7
Isoleucine (Ile or I)	ATA	905	17.1		GTA	428	8.1
	ATT	838	15.8		GTT	321	6.1
	ATC	1334	25.2		GTC	540	10.2
Leucine (Leu or L)	TTG	479	9.1	**End (Stop codon or X)**	TGA	30	0.6
	TTA	333	6.3		TAG	50	1.0
	CTG	1639	31.0		TAA	30	0.6

```
atgaccgtcgtgctgttcgccaccgaatacgacaccccaaatatcgtagtcaatatgctg  K181P
atgacggttgttttatttgcgacggaatatgatacgccgaatattgttgttaatatgtta  leop.all
atgacggtcgtcctcttcgccacggaatacgacacgcccaatattgtcgtcaatatgctc  M70so403Cys
atgaccgtcgtgctgttcgccaccgaatacgacaccccaaatatcgtagtcaatatgctg  M70so155Cys
 M  T  V  V  L  F  A  T  E  Y  D  T  P  N  I  V  V  N  M  L

tcggagacaccgacggagcaccacctgttccccctgatgattaaatacaaaccgtcgaac  K181P
tcggaaacgccgacggaacatcatttatttccgttaatgattaagtataagccgtcgaat  leop.all
tcggagacgcccacggagcaccacctcttccccctcatgattaaatacaaacccgaac    M70so403Cys
tcggagacaccgacggagcaccacctgttccccctgatgattaaatacaaaccgtcgaac  M70so155Cys
 S  E  T  P  T  E  H  H  L  F  P  L  M  I  K  Y  K  P  S  N   40

cgtatagaattcgtacttcagacccagaggtgcccggattcgacccgggtccgccccgtt  K181P
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cgtatagaattcgtacttcagacccagaggtgcccggattcgacccgggtccgccccgtt  M70so155Cys
 R  I  E  F  V  L  Q  T  Q  R  C  P  D  S  T  R  V  R  P  V

ttcatatgcgacgcgcgccgcctctccctgtccgaatacgtgtccaccaacacaccgcta  K181P
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ttcatatgcgacgcgcgccgcctctccctgtccgaatacgtgtccaccaacacaccgcta  M70so155Cys
 F  I  C  D  A  R  R  L  S  L  S  E  Y  V  S  T  N  T  P  L   80

cccgcgcgcgttatctgcgccggcatagacgccgacgctactcgcgagttgtacgaacac  K181P
ccggcgcgtgtttatttgtgcgggtattgatgcggatgcgacgcgtgaattatatgaacat  leop.all
cccgcccgggtcatttgcgccgggattgacgccgacgccacgcgggagctctacgaacac  M70so403Cys
cccgcgcgcgttatctgcgccggcatagacgccgacgctactcgcgagttgtacgaacac  M70so155Cys
 P  A  R  V  I  C  A  G  I  D  A  D  A  T  R  E  L  Y  E  H

ctgttcgatcgtaaaaaagacgagacgggtcatgacgaggagaacgggtccgccggcggc  K181P
ttatttgatcgtaagaaggatgaaacgggtcatgatgaagaaaatggttcggcgggtggt  leop.all
ctcttcgatcggaaaaaagacgagacgggcatgacgaggagaacgggtcggccgggggg   M70so403Cys
ctgttcgatcgtaaaaaagacgagacgggtcatgacgaggagaacgggtccgccggcggc  M70so155Cys
 L  F  D  R  K  K  D  E  T  G  H  D  E  E  N  G  S  A  G  G   120

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gacctcttctcggacctcacgtcgacgctcaaatgcctcgtccattacaatcggtcggcc  M70so403Cys
gacctcttctccgacctcaccagcaccctcaaatgcctggtccattacaatcgcagcgcg  M70so155Cys
 D  L  F  S  D  L  T  S  T  L  K  C  L  V  H  Y  N  R  S  A

atcttacgctatctcaacaacaccttcctgtcccccacgtccccctcctggttcctgagc  K181P
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 I  L  R  Y  L  N  N  T  F  L  S  P  T  S  P  S  W  F  L  S   160

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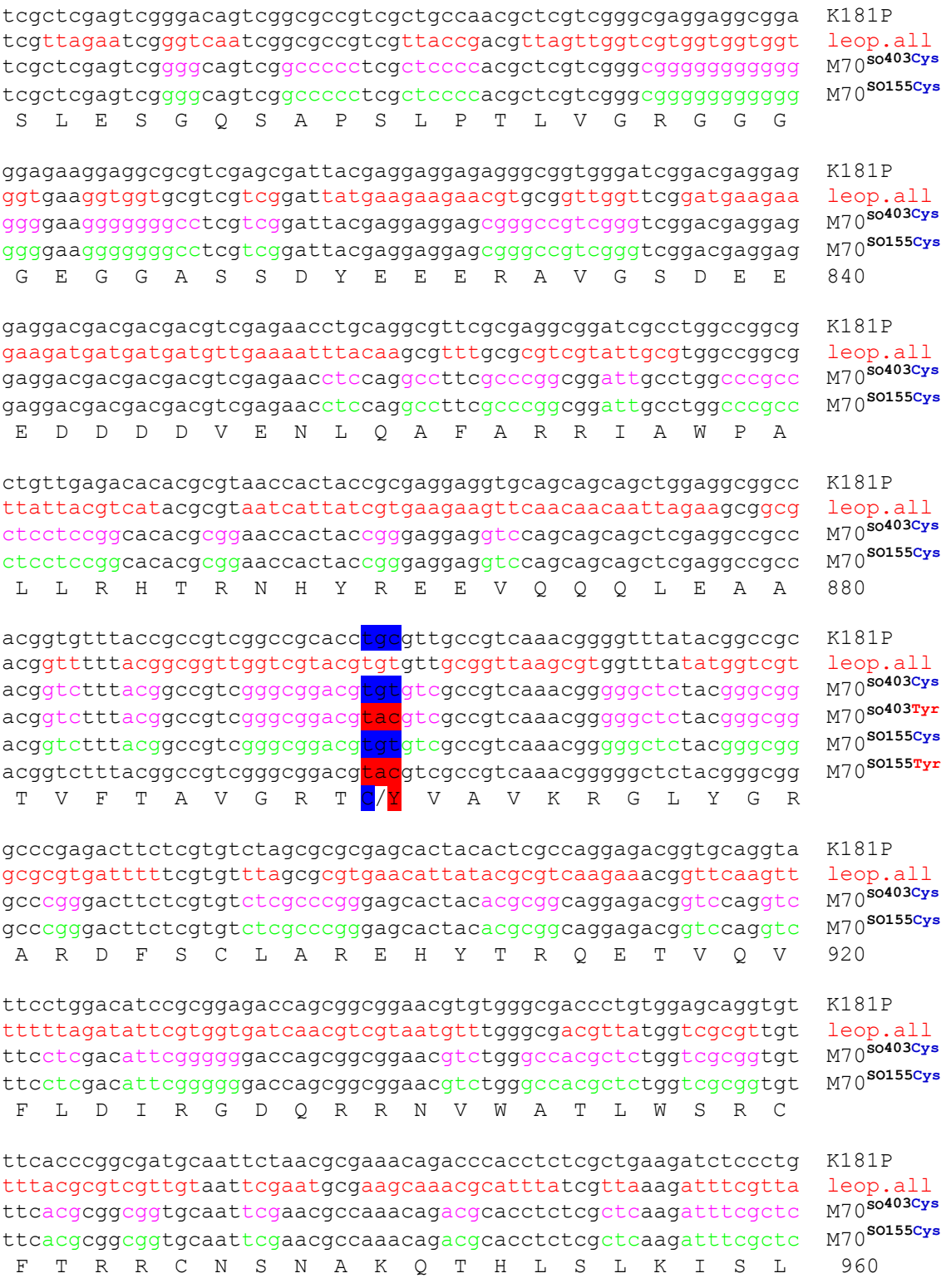
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ccctcgcagtat                                    M70SO155Cys
 P   S   Q   Y
```

Figure 3.45: Nucleotide sequence of K181 M70 ORF. The K181P is published M70 sequence for K181(Perth) variant (EMBL Acc. No. AM886412), Leop.all = all codons changed to those used least optimally, i.e. the least frequent codon for that amino acid, M70^{SO403Cys} and M70^{SOTyr} are the cysteine and tyrosine versions respectively where only the 9 amino acids with more than 2 codons were changed to codons used suboptimally with the highest CG content, M70^{SO155Cys} and M70^{SO155Tyr} are the cysteine and tyrosine versions respectively where the 9 amino acids were changed in the carboxy terminus only (Table 3.6).

```
K181P      MTVVLFATEYDTPNIVVNMLSETPTEHHLFPLMIKYKPSNRIEFVLQTQRCPDSTRVRPV 60
leop.all   MTVVLFATEYDTPNIVVNMLSETPTEHHLFPLMIKYKPSNRIEFVLQTQRCPDSTRVRPV 60
M70SO403Cys MTVVLFATEYDTPNIVVNMLSETPTEHHLFPLMIKYKPSNRIEFVLQTQRCPDSTRVRPV 60
M70SO155Cys MTVVLFATEYDTPNIVVNMLSETPTEHHLFPLMIKYKPSNRIEFVLQTQRCPDSTRVRPV 60
           ************************************************************

K181P      FICDARRLSLSEYVSTNTPLPARVICAGIDADATRELYEHLFDRKKDETGHDEENGSAGG 120
Leop.all   FICDARRLSLSEYVSTNTPLPARVICAGIDADATRELYEHLFDRKKDETGHDEENGSAGG 120
M70SO403Cys FICDARRLSLSEYVSTNTPLPARVICAGIDADATRELYEHLFDRKKDETGHDEENGSAGG 120
M70SO155Cys FICDARRLSLSEYVSTNTPLPARVICAGIDADATRELYEHLFDRKKDETGHDEENGSAGG 120
           ************************************************************

K181P      DLFSDLTSTLKCLVHYNRSAILRYLNNTFLSPTSPSWFLSTYGTHEGTLILTMSYYLFER 180
Leop.all   DLFSDLTSTLKCLVHYNRSAILRYLNNTFLSPTSPSWFLSTYGTHEGTLILTMSYYLFER 180
M70SO403Cys DLFSDLTSTLKCLVHYNRSAILRYLNNTFLSPTSPSWFLSTYGTHEGTLILTMSYYLFER 180
M70SO155Cys DLFSDLTSTLKCLVHYNRSAILRYLNNTFLSPTSPSWFLSTYGTHEGTLILTMSYYLFER 180
           ************************************************************

K181P      QYSTIQTTRDYTKCFTADPGRNLFTYINMRDFMATMNGSRFRKQTARFAAFAKARNARDR 240
Leop.all   QYSTIQTTRDYTKCFTADPGRNLFTYINMRDFMATMNGSRFRKQTARFAAFAKARNARDR 240
M70SO403Cys QYSTIQTTRDYTKCFTADPGRNLFTYINMRDFMATMNGSRFRKQTARFAAFAKARNARDR 240
M70SO155Cys QYSTIQTTRDYTKCFTADPGRNLFTYINMRDFMATMNGSRFRKQTARFAAFAKARNARDR 240
           ************************************************************

K181P      RELEYVDAKINAFREESRLAADSCVYYVYLAYRTALCREKFLQYCEHTAYDKNLPDDQQC 300
Leop.all   RELEYVDAKINAFREESRLAADSCVYYVYLAYRTALCREKFLQYCEHTAYDKNLPDDQQC 300
M70SO403Cys RELEYVDAKINAFREESRLAADSCVYYVYLAYRTALCREKFLQYCEHTAYDKNLPDDQQC 300
M70SO155Cys RELEYVDAKINAFREESRLAADSCVYYVYLAYRTALCREKFLQYCEHTAYDKNLPDDQQC 300
           ************************************************************

K181P      AAEENYLGRSLDAELISIMNTYFSVEGYFGSYIHVDRAKLSPPHSYRGYDWNTEADTMVG 360
Leop.all   AAEENYLGRSLDAELISIMNTYFSVEGYFGSYIHVDRAKLSPPHSYRGYDWNTEADTMVG 360
M70SO403Cys AAEENYLGRSLDAELISIMNTYFSVEGYFGSYIHVDRAKLSPPHSYRGYDWNTEADTMVG 360
M70SO155Cys AAEENYLGRSLDAELISIMNTYFSVEGYFGSYIHVDRAKLSPPHSYRGYDWNTEADTMVG 360
           ************************************************************

K181P      YSSTATNLAISLRKLNSTCESLFSPLPPTLMGLLKLCASDRYVPRAEKSRKRTSGGREKE 420
Leop.all   YSSTATNLAISLRKLNSTCESLFSPLPPTLMGLLKLCASDRYVPRAEKSRKRTSGGREKE 420
M70SO403Cys YSSTATNLAISLRKLNSTCESLFSPLPPTLMGLLKLCASDRYVPRAEKSRKRTSGGREKE 420
M70SO155Cys YSSTATNLAISLRKLNSTCESLFSPLPPTLMGLLKLCASDRYVPRAEKSRKRTSGGREKE 420
           ************************************************************

K181P      DETRVCRRNYLLNDTSRPIGPMPVFRVEMPEKRHVFCAVSAENWTRRLLPKDLMKNLPSE 480
Leop.all   DETRVCRRNYLLNDTSRPIGPMPVFRVEMPEKRHVFCAVSAENWTRRLLPKDLMKNLPSE 480
M70SO403Cys DETRVCRRNYLLNDTSRPIGPMPVFRVEMPEKRHVFCAVSAENWTRRLLPKDLMKNLPSE 480
M70SO155Cys DETRVCRRNYLLNDTSRPIGPMPVFRVEMPEKRHVFCAVSAENWTRRLLPKDLMKNLPSE 480
           ************************************************************

K181P      YVSDECLTDAVWLREDIAASCEVGEQLYRTRHEMFNENLPVFNFVGDVDLKLREDLQGLS 540
Leop.all   YVSDECLTDAVWLREDIAASCEVGEQLYRTRHEMFNENLPVFNFVGDVDLKLREDLQGLS 540
M70SO403Cys YVSDECLTDAVWLREDIAASCEVGEQLYRTRHEMFNENLPVFNFVGDVDLKLREDLQGLS 540
M70SO155Cys YVSDECLTDAVWLREDIAASCEVGEQLYRTRHEMFNENLPVFNFVGDVDLKLREDLQGLS 540
           ************************************************************

K181P      RQEVFDLCRALRRTLIGAWRHLFPEVDPDSHPVFFFKSACPQNAAGAADEAMLYGGGGYD 600
Leop.all   RQEVFDLCRALRRTLIGAWRHLFPEVDPDSHPVFFFKSACPQNAAGAADEAMLYGGGGYD 600
M70SO403Cys RQEVFDLCRALRRTLIGAWRHLFPEVDPDSHPVFFFKSACPQNAAGAADEAMLYGGGGYD 600
M70SO155Cys RQEVFDLCRALRRTLIGAWRHLFPEVDPDSHPVFFFKSACPQNAAGAADEAMLYGGGGYD 600
           ************************************************************


K181P      EDDDPRPEHAAAMVDYGDAVRRPPFCVCRRKLGLRVIIPFPPRTAAIGAQTLKRLAGILD 660
Leop.all   EDDDPRPEHAAAMVDYGDAVRRPPFCVCRRKLGLRVIIPFPPRTAAIGAQTLKRLAGILD 660
M70SO403Cys EDDDPRPEHAAAMVDYGDAVRRPPFCVCRRKLGLRVIIPFPPRTAAIGAQTLKRLAGILD 660
M70SO155Cys EDDDPRPEHAAAMVDYGDAVRRPPFCVCRRKLGLRVIIPFPPRTAAIGAQTLKRLAGILD 660
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K181P      HTLCLDRDLVCKLNAISHPGECFDTGIYSHGRSIRMPLMYKLDEASGLILHSRLNPIFIV 720
Leop.all   HTLCLDRDLVCKLNAISHPGECFDTGIYSHGRSIRMPLMYKLDEASGLILHSRLNPIFIV 720
M70SO403Cys HTLCLDRDLVCKLNAISHPGECFDTGIYSHGRSIRMPLMYKLDEASGLILHSRLNPIFIV 720
M70SO155Cys HTLCLDRDLVCKLNAISHPGECFDTGIYSHGRSIRMPLMYKLDEASGLILHSRLNPIFIV 720
           ************************************************************

K181P      PAGYRDRPAEFVLQQLCPQNLTHHGRPPRRDGSADQLTEVVLHITDRACADSDGNFLQSR 780
Leop.all   PAGYRDRPAEFVLQQLCPQNLTHHGRPPRRDGSADQLTEVVLHITDRACADSDGNFLQSR 780
M70SO403Cys PAGYRDRPAEFVLQQLCPQNLTHHGRPPRRDGSADQLTEVVLHITDRACADSDGNFLQSR 780
M70SO155Cys PAGYRDRPAEFVLQQLCPQNLTHHGRPPRRDGSADQLTEVVLHITDRACADSDGNFLQSR 780
           ************************************************************

K181P      ARRAMSRRRLPLGPLLRAHLSLESGQSAPSLPTLVGRGGGGEGGASSDYEEERAVGSDEE 840
Leop.all   ARRAMSRRRLPLGPLLRAHLSLESGQSAPSLPTLVGRGGGGEGGASSDYEEERAVGSDEE 840
M70SO403Cys ARRAMSRRRLPLGPLLRAHLSLESGQSAPSLPTLVGRGGGGEGGASSDYEEERAVGSDEE 840
M70SO155Cys ARRAMSRRRLPLGPLLRAHLSLESGQSAPSLPTLVGRGGGGEGGASSDYEEERAVGSDEE 840
           ************************************************************

K181P      EDDDDVENLQAFARRIAWPALLRHTRNHYREEVQQQLEAATVFTAVGRTCVAVKRGLYGR 900
Leop.all   EDDDDVENLQAFARRIAWPALLRHTRNHYREEVQQQLEAATVFTAVGRTCVAVKRGLYGR 900
M70SO403Cys EDDDDVENLQAFARRIAWPALLRHTRNHYREEVQQQLEAATVFTAVGRTCVAVKRGLYGR 900
M70SO403Tyr EDDDDVENLQAFARRIAWPALLRHTRNHYREEVQQQLEAATVFTAVGRTYVAVKRGLYGR 900
M70SO155Cys EDDDDVENLQAFARRIAWPALLRHTRNHYREEVQQQLEAATVFTAVGRTCVAVKRGLYGR 900
M70SO155Tyr EDDDDVENLQAFARRIAWPALLRHTRNHYREEVQQQLEAATVFTAVGRTYVAVKRGLYGR 900
           ************************************************ **********

K181P      ARDFSCLAREHYTRQETVQVFLDIRGDQRRNVWATLWSRCFTRRCNSNAKQTHLSLKISL 960
Leop.all   ARDFSCLAREHYTRQETVQVFLDIRGDQRRNVWATLWSRCFTRRCNSNAKQTHLSLKISL 960
M70SO403Cys ARDFSCLAREHYTRQETVQVFLDIRGDQRRNVWATLWSRCFTRRCNSNAKQTHLSLKISL 960
M70SO155Cys ARDFSCLAREHYTRQETVQVFLDIRGDQRRNVWATLWSRCFTRRCNSNAKQTHLSLKISL 960
           ************************************************************

K181P      PSQY 964    G+C = 64%     CpG = 381
Leop.all   PSQY 964    G+C = 43%
M70SO403Cys PSQY 964    G+C = 66%     CpG = 378
M70SO155Cys PSQY 964    G+C = 65%     CpG = 367
           ****
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Figure 3.46: Translated amino acids sequences of, Leop.all, M70^{SO403Cys}, M70^{SO403Tyr}, M70^{SO155Cys} and M70^{SO155Tyr} compared to the *wt* K181 (Perth) sequence.

Table 3.6: Number of codons changed in the constructed mutants, M70^{SO403Cys}, M70^{SO403Tyr}, M70^{SO155Cys} and M70^{SO155Tyr}, where only the 9 amino acids (indicated with *) with more than 2 codons were changed to codons used suboptimally with the highest GC content.

Amino Acid	Codon	Mouse frequency (per 1,000)	K181	BAC^{403Cys}	BAC^{403Tyr}	BAC^{155Cys}	BAC^{155Tyr}
Ala*	GCT	13.3	1	0	0	1	1
	GCC	20.5	30	78	78	50	50
	GCA	17.3	1	0	0	0	0
	GCG	6.1	46	0	0	27	27
Arg*	CGT	3.4	9	0	0	5	5
	CGC	7.3	42	0	0	27	27
	CGA	5.5	8	0	0	1	1
	CGG	7.5	26	96	96	59	59
	AGA	19.2	3	0	0	2	2
	AGG	14	8	0	0	2	2
Asn	AAT	18.3	5	5	5	5	5
	AAC	26.4	31	31	31	31	31
Asp	GAT	18.4	9	9	9	9	9
	GAC	29.1	54	54	54	54	54
Cys	TGT	9.6	8	9	8	9	8
	TGC	11.6	20	19	19	19	19
Gln	CAA	16	1	1	1	1	1
	CAG	49.3	27	27	27	27	27
Glu	GAA	31.6	15	15	15	15	15
	GAG	32.9	47	47	47	47	47
Gly*	GGT	9.4	4	0	0	3	3
	GGC	17.3	27	0	0	17	17
	GGA	15.7	7	0	0	0	0
	GGG	11.7	15	53	53	33	33
His	CAT	11.3	2	2	2	2	2
	CAC	21.4	24	24	24	24	24
Ile*	ATT	15.8	3	32	32	16	16
	ATC	25.2	26	0	0	13	13
	ATA	17.1	3	0	0	3	3
Leu*	TTA	6.3	3	0	0	1	1
	TTG	9.1	3	0	0	1	1
	CTT	11.6	1	0	0	1	1
	CTC	18.6	13	99	99	45	45
	CTA	12.6	2	0	0	1	1
	CTG	31	77	0	0	50	50
Lys	AAA	44.8	13	13	13	13	13
	AAG	32.1	16	16	16	16	16

Amino Acid	Codon	Mouse frequency (per 1,000)	K181	BAC^{403Cys}	BAC^{403Tyr}	BAC^{155Cys}	BAC^{155Tyr}
Met	ATG	22.4	19	19	19	19	19
Pro*	CCT	14.1	2	0	0	0	0
	CCC	16.5	20	50	50	32	32
	CCA	18.8	2	0	0	1	1
	CCG	5.8	26	0	0	17	17
Phe	TTT	15	5	5	5	5	5
	TTC	23.3	36	36	36	36	36
Ser*	TCT	11.1	2	0	0	1	1
	TCC	14.3	15	0	0	14	14
	TCA	14.8	0	0	0	0	0
	TCG	4.2	26	64	64	35	35
	AGT	9.2	1	0	0	1	1
	AGC	16	20	0	0	13	13
Thr*	ACT	13.3	3	0	0	1	1
	ACC	15.6	28	0	0	22	22
	ACA	22.8	2	0	0	2	2
	ACG	4.6	27	60	60	35	35
Trp	TGG	15.1	8	8	8	8	8
Tyr	TAT	12.4	6	6	6	6	6
	TAC	17.6	35	35	36	35	36
Val*	GTT	6.1	4	0	0	2	2
	GTC	10.2	15	51	51	28	28
	GTA	8.1	4	0	0	3	3
	GTG	18.7	28	0	0	18	18

Both the cysteine and tyrosine versions of the suboptimal M70 ORF sequence (plus 50 base homology arms at both terminals) were synthesised by ShineGene Molecular Biotech, Inc., Shangai, China and cloned into the multiple cloning site of the pPCR-Script Amp SK(+) plasmid. These modified ORFs were then used to replace the *wt* M70 ORF in the K181 (Perth) BAC using two-step homologous recombination mutagenesis (Section 2.7). In the first step, the *wt* M70 gene was replaced with the RpsL-neo cassette and in the second step the cassette was replaced with either the cysteine or the tyrosine version of the synthetic M70 gene [obtained by *BamH*I/*Hin*dIII digestion of the pPCR-Script Amp SK(+) plasmid].

Isolated recombinant BACs were PCR screened and analysed using RpsL-neo cassette primers and primers for the synthetic M70 genes (Table 2.3). Revertants were generated using a similar approach (Section 2.7). PCR products of the inserted M70 gene (cysteine and tyrosine versions) were sequenced to verify that no changes had occurred in the inserted sequence and BAC RFLPs were analysed in comparison to the *wt* and revertant BAC (data not shown).

Several attempts to transfect NIH3T3 or MEF cells (Section 2.3.1) with the recombinant BACs containing the synthetic M70 genes were made, but neither the cysteine nor the tyrosine version of the modified BACs produced virus despite successful transfection (Figure 3.50). Fluorescent cells were detected and continued to fluoresce for more than 12 days post infection but the infection did not spread from the initially infected cells and no infectious virus was produced although the cells were monitored for up to 30 days until the cells started to detach from the plastic surface. Attempts to infect fresh NIH 3T3 cells with culture supernatant from the transfected cells produced neither florescence nor cytopathic effect.

Based on this outcome, another strategy was investigated in which only the amino acids at the carboxy terminal end of the M70 ORF were changed suboptimally using two step homologous recombination (Section 2.7). The RpsL-neo cassette was amplified using M70HA FOR + M70HA-RpsL-neo Rev1 primers (Table 2.8), whereas the M70 fragments for both M70$^{\mathbf{SO155Cys}}$ and M70$^{\mathbf{SO155Tyr}}$ (Figure 3.45 and Figure 3.46) were amplified from the pPCR-Script Amp SK(+) plasmid containing the synthetic M70 gene using M70HA FOR + M70HA*wt*-Rev1 primers (Table 2.8). Insertion of the amplified regions (M70$^{\mathbf{SO155Cys}}$ and M70$^{\mathbf{SO155Tyr}}$) from the modified M70 ORFs resulted in M70 ORFs with a G + C content of 66% (CpG 367) for the cysteine ORF and 66% (368) for the tyrosine ORF; 155 codons were

changed (16% of the ORF). DNA from mutant BACs Mt[M70^{SO155Cys}] and Mt[M70$^{SO155\ Tyr}$] as well as their revertant BACs were amplified by PCR and sequenced (data not shown) and an RFLP analysis carried out on the mutants (Figures 3.47 – 3.49).

Again, NIH 3T3 cells were successfully transfected with both constructs as detected by the green florescence of the infected cells (Figure 3.50), but only the cysteine construct Mt[M70^{SO155Cys}] produced infectious virus. Replication of this virus was examined in the next section.

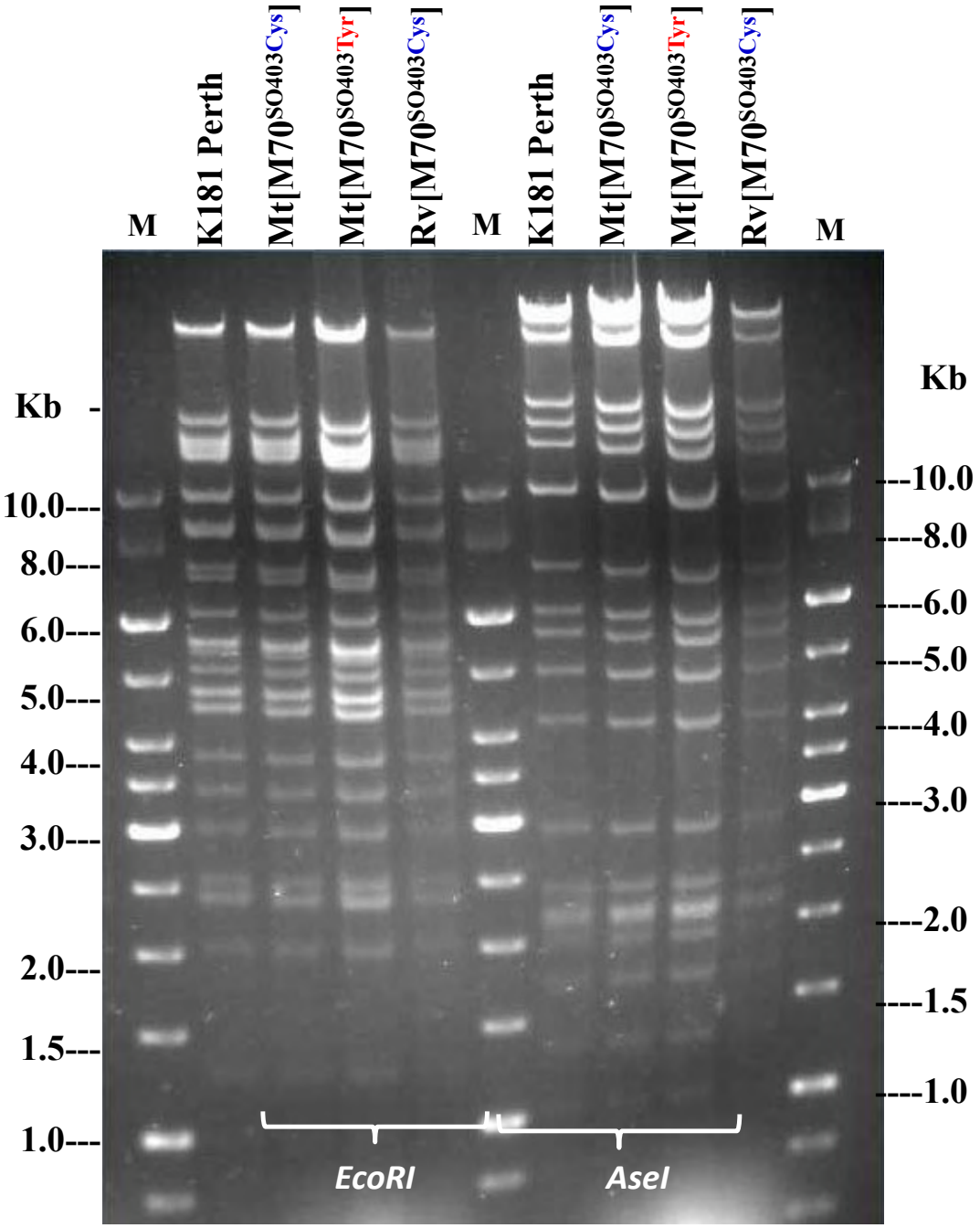


Figure 3.47: Recombinant MCMV BAC genomes RFLP analysis using *Eco*RI and *Ase*I restriction enzymes digestion. Recombinant and *wt* K181 Perth BAC DNA (~2 µg) was digested with 1 µl of *Eco*RI or *Ase*I restriction enzyme and resulting fragments were separated on a 0.4% agarose gel for ~19 hours at 2 V/cm. No genome rearrangements were detected in either of the recombinant BACs compared to the *wt* K181 BAC. Molecular size markers (lanes M), in kilobases, are indicated.

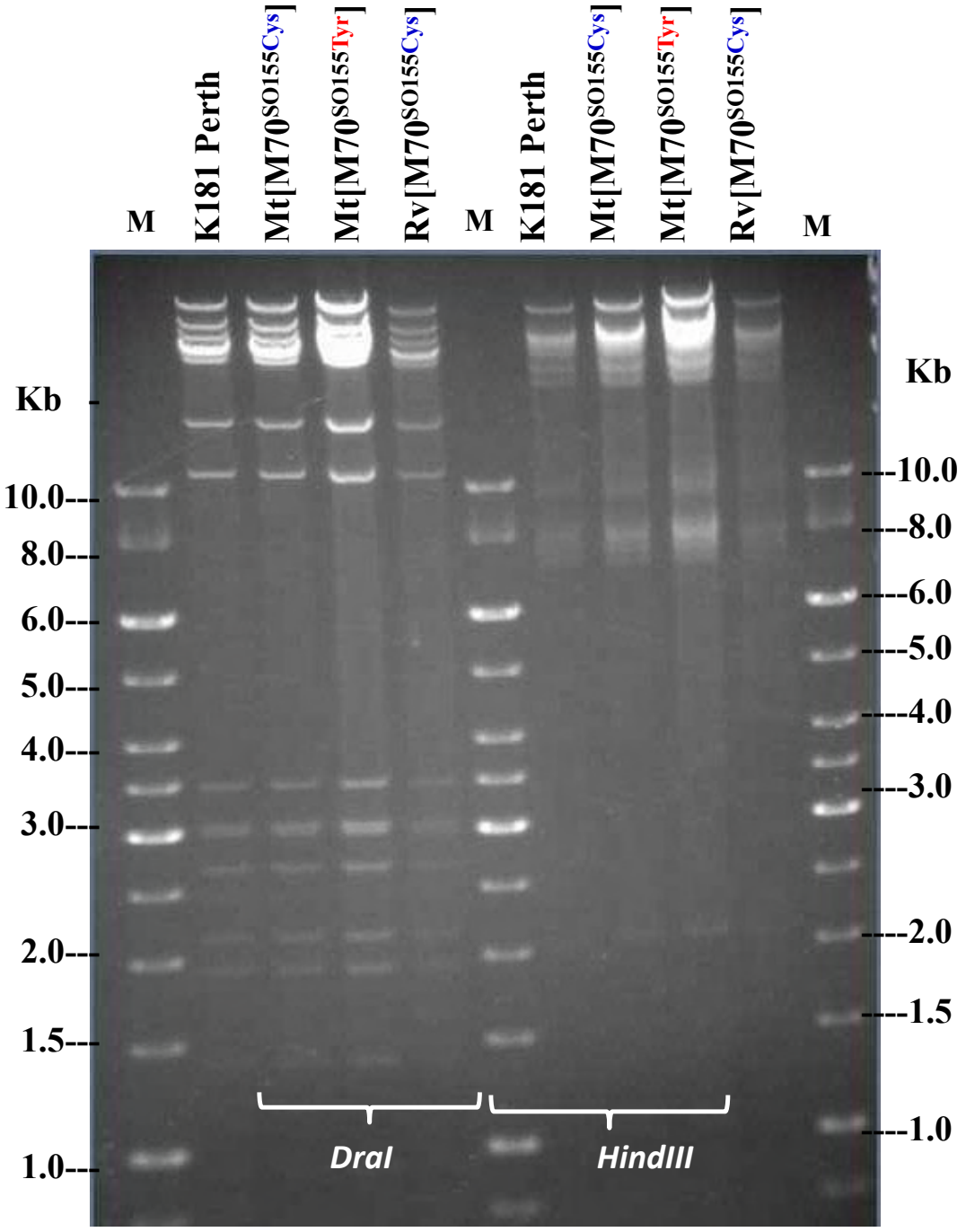


Figure 3.48: Recombinant MCMV BAC genomes RFLP analysis using *Dra*I and *Hind*III restriction enzymes digestion. Recombinant and *wt* K181 (Perth) BAC DNA (~2 µg) was digested with 1 µl of *Dra*I or *Hind*III restriction enzyme and resulting fragments were separated on a 0.4% agarose gel for ~19 hours at 2 V/cm. No genome rearrangements were detected in either of the recombinant BACs compared to the *wt* K181 BAC. Molecular size markers (lanes M), in kilobases, are indicated.

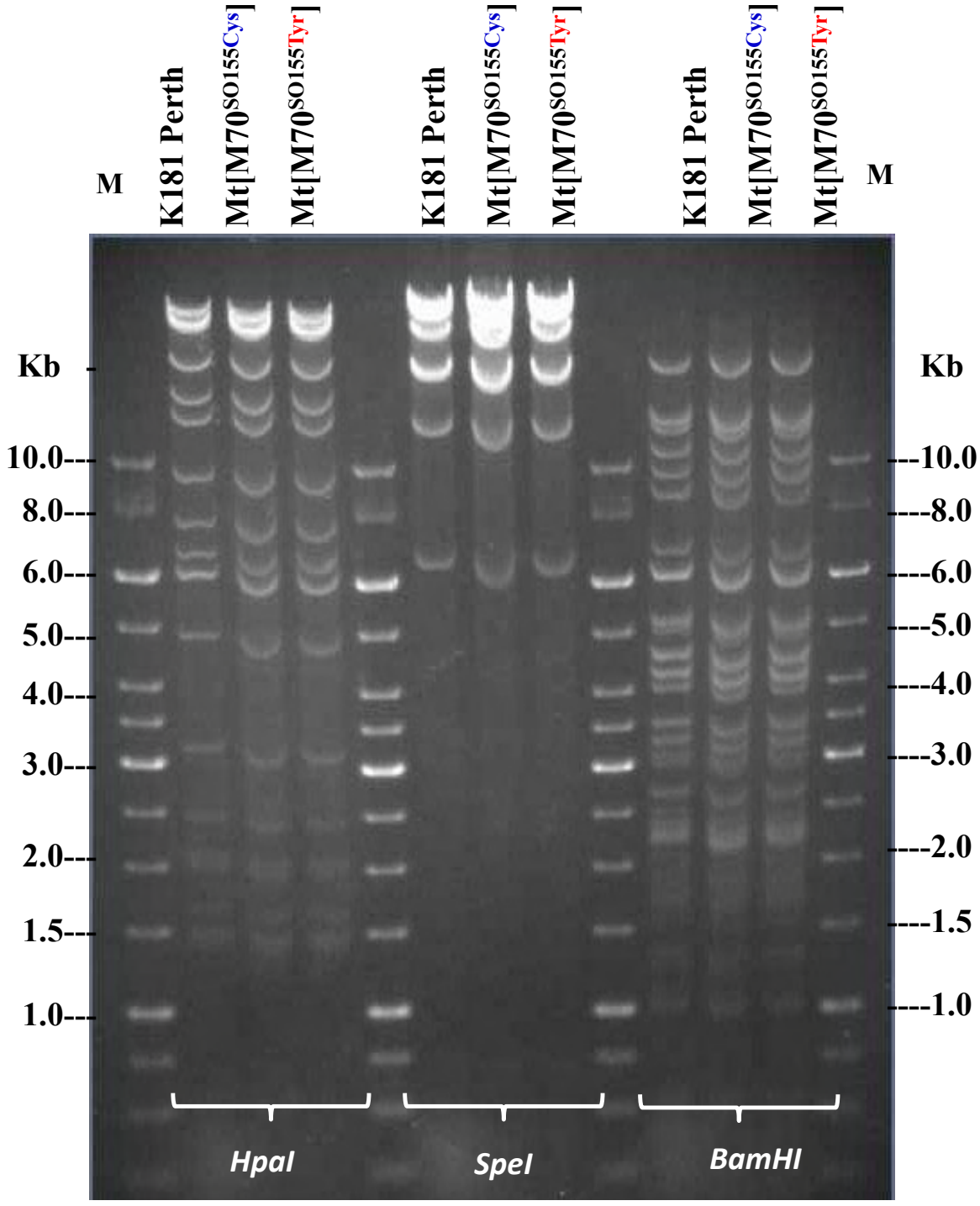


Figure 3.49: RFLP analysis of recombinant MCMV BAC genomes using *Hpa*I, *Spe*I and *Bam*HI restriction enzymes digestion. Recombinant and *wt* K181 (Perth) BAC DNA (~2 µg) was digested with 1 µl of *Hpa*I, *Spe*I or *Bam*HI restriction enzyme and resulting fragments were separated on a 0.4% agarose gel for ~19 hours at 2 V/cm. No genome rearrangements were detected in either of the recombinant BACs compared to the *wt* K181 BAC. Molecular size markers (lanes M), in kilobases, are indicated.

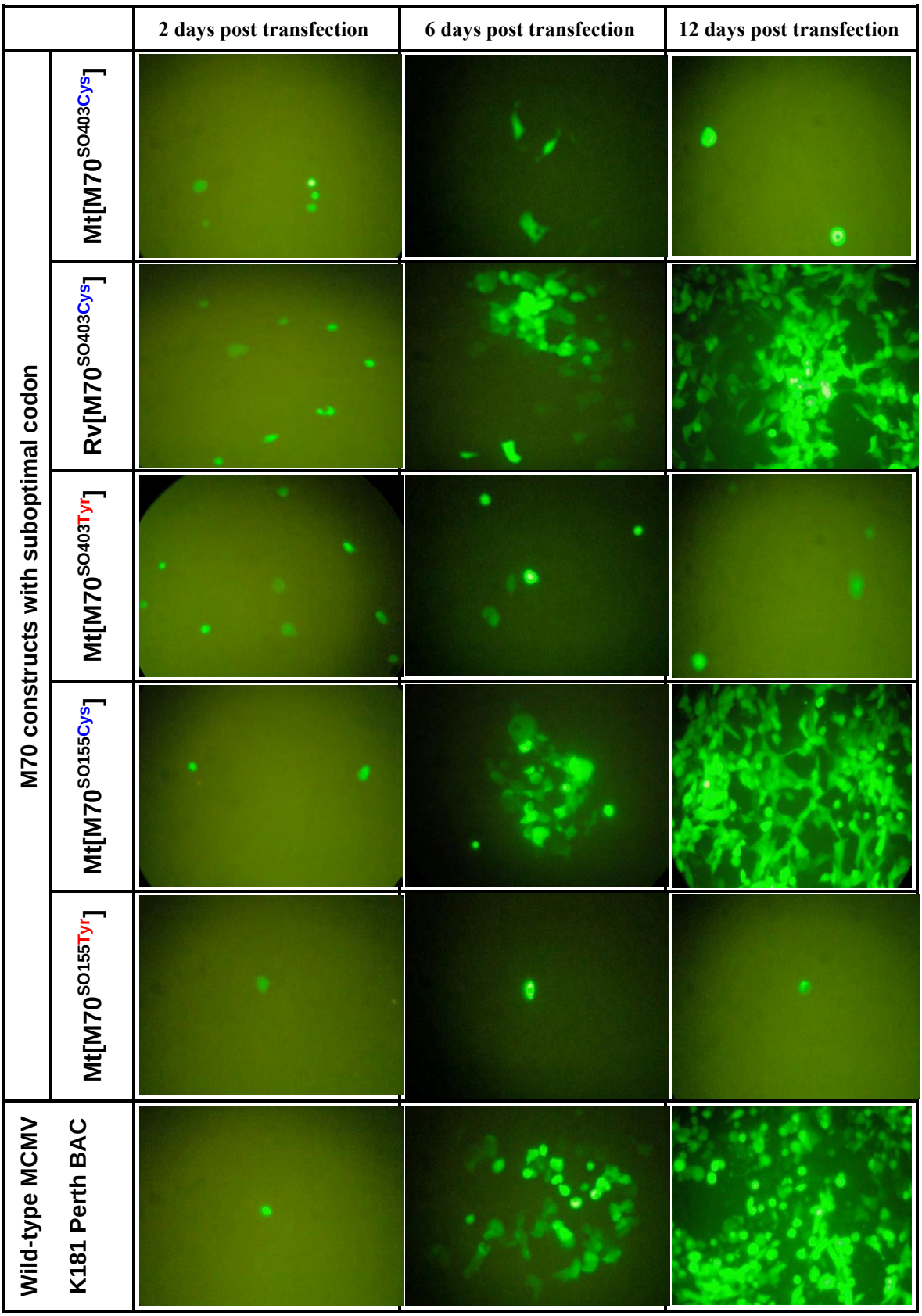


Figure 3.50 : Transfection of NIH 3T3 cells with the *wt* K181 (Perth) BAC and modified BACs containing the re-constructed M70 ORFs.

3.5.1 Replication of Mt[M70^{SO155Cys}] mutant virus

The replication pattern of the mutant Mt[M70^{SO155Cys}] virus at 37°C was very similar to that of that of *wt* virus (Figure 3.51). The maximum yield was $10^{5.54}$ PFU/ml on day 5 compared to $10^{5.83}$ PFU/ml for the K181 (Perth) virus. No significant differences were identified between mutant and *wt* virus yields at any day post infection ($p>0.05$).

At 40°C the mutant virus replicated with the same efficiency as the *wt* K181(Perth) virus (Figure 3.51). No significant differences were identified between mutant and *wt* virus yields on days 2 to 7 post infection ($p>0.05$).

In conclusion, mutant Mt[M70^{SO155Cys}] does not show a phenotype different from *wt* at either 37°C or 40°C and therefore changing the 155 codons at the distal terminal of the M70 gene in the presence of cysteine does not affect replication of the virus *in vitro* in MEF cells at37°C or 40°C.

Furthermore, and to study the tendency for reversion of the changed codons in recombinant Mt[M70^{155C}], the virus was passaged 10 times in tissue culture. Sequence analysis revealed that none of the altered codons had mutated (data not shown).

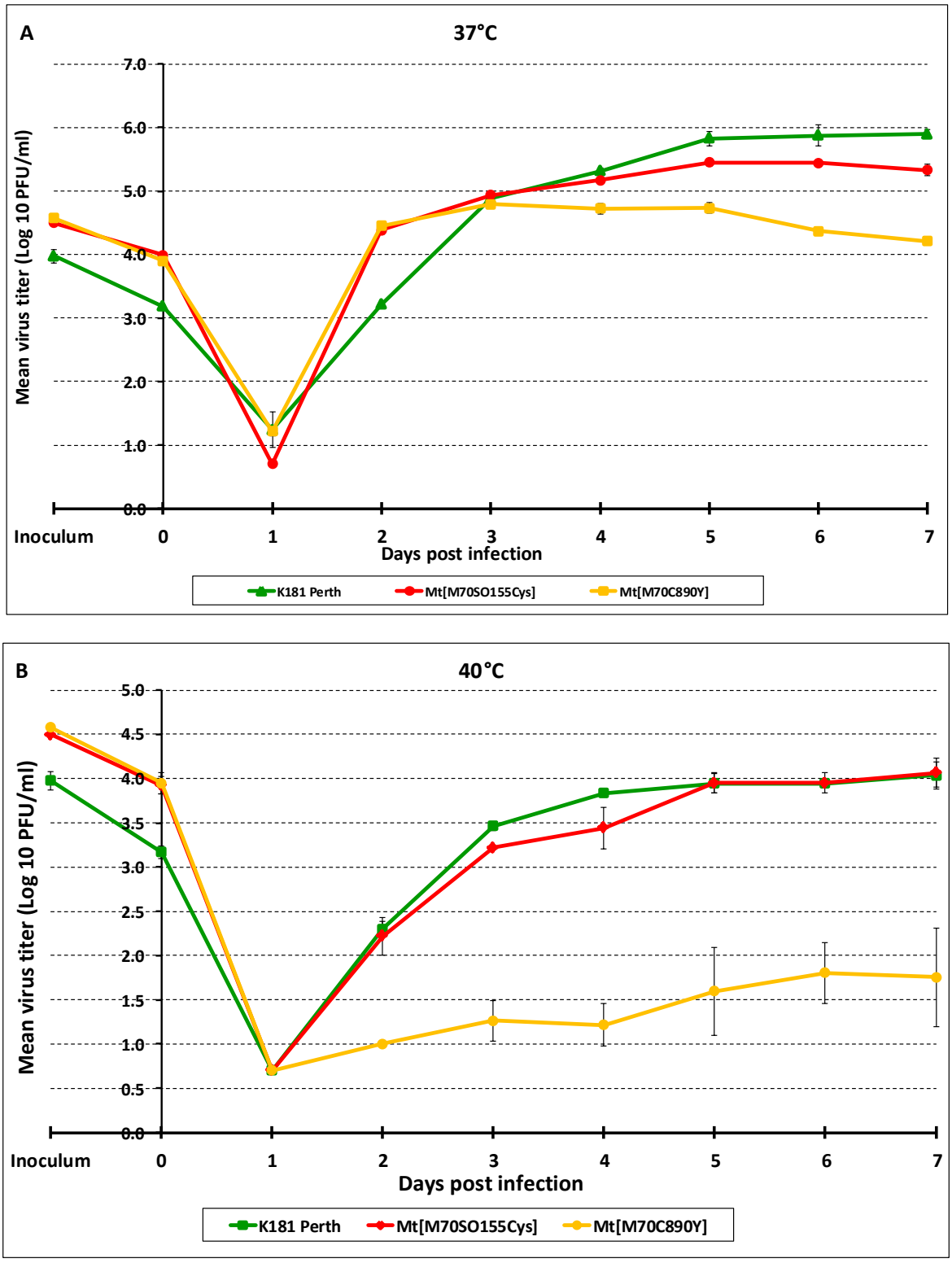
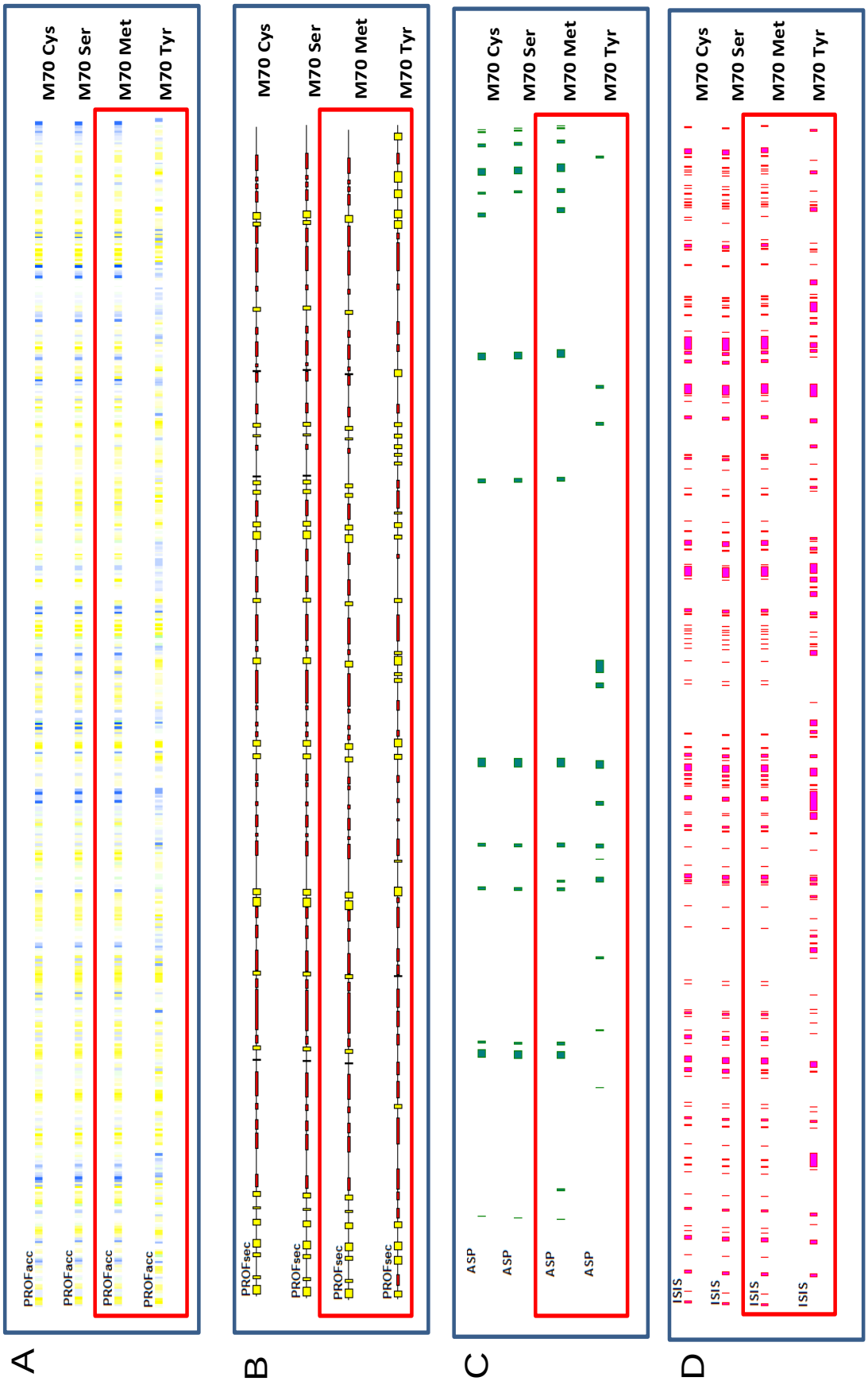


Figure 3.51: Replication of mutant Mt[M70^{SO155Cys}] compared to K181 (Perth) and Mt[M70^{C890Y}] viruses at the permissive (37°C) and non-permissive (40°C) temperatures. MEF cells were infected with virus at an MOI of 0.05 PFU per cell and incubated at: [A] 37°C or [B] 40°C. Viral titres were determined in cell culture supernatants 1-7 days post infection by plaque assay. Results are the mean ± standard deviation (SD) of three replicates.

3.5.2 Metagenomic analysis of M70 structure

The lack of viability of Mt[M70^{SO155Tyr}] mutant was unexpected as it was previously suggested that the ability of amino acid residue 890 to interact chemically with other amino acids in the structure was not important (Timoshenko *et al.*, 2009b). Although no structure for any herpes virus primase is available, we have used PredictProtein to examine the possible effect of the cysteine to tyrosine mutation on the secondary structure of the M70 protein (Figure 3.52).

While it is unknown what effect this change will actually have it appears that the change from cysteine (M70 Cys) to tyrosine (M70 Tyr) has a marked effect on predicted solvent accessibility (PROFacc), secondary structure (PROFsec), amino acid subsequences that are most likely to switch between different types of secondary structure (ASP), DNA interaction sites (ISIS), protein disorder prediction-unstructured loops (NORSnet) prediction of residue mobility (PROFbval), and prediction of meta-disorder (MD) but shows no obvious changes in the prediction of natively unstructured regions (Ucon). It is interesting that when the cysteine residue is replaced by serine (M70Ser) or methionine (M70Met), which produced mutant viruses with a wild-type phenotype (Timoshenko *et al.*, 2009b), the predicted structure was similar to the wild-type structure (Figure 3.52).


A
M70 Cys
M70 Ser
M70 Met
M70 Tyr
PROFacc
PROFacc
PROFacc
PROFacc
B
M70 Cys
M70 Ser
M70 Met
M70 Tyr
PROFsec
PROFsec
PROFsec
PROFsec
C
M70 Cys
M70 Ser
M70 Met
M70 Tyr
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ASP
ASP
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D
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ISIS
ISIS
ISIS
ISIS

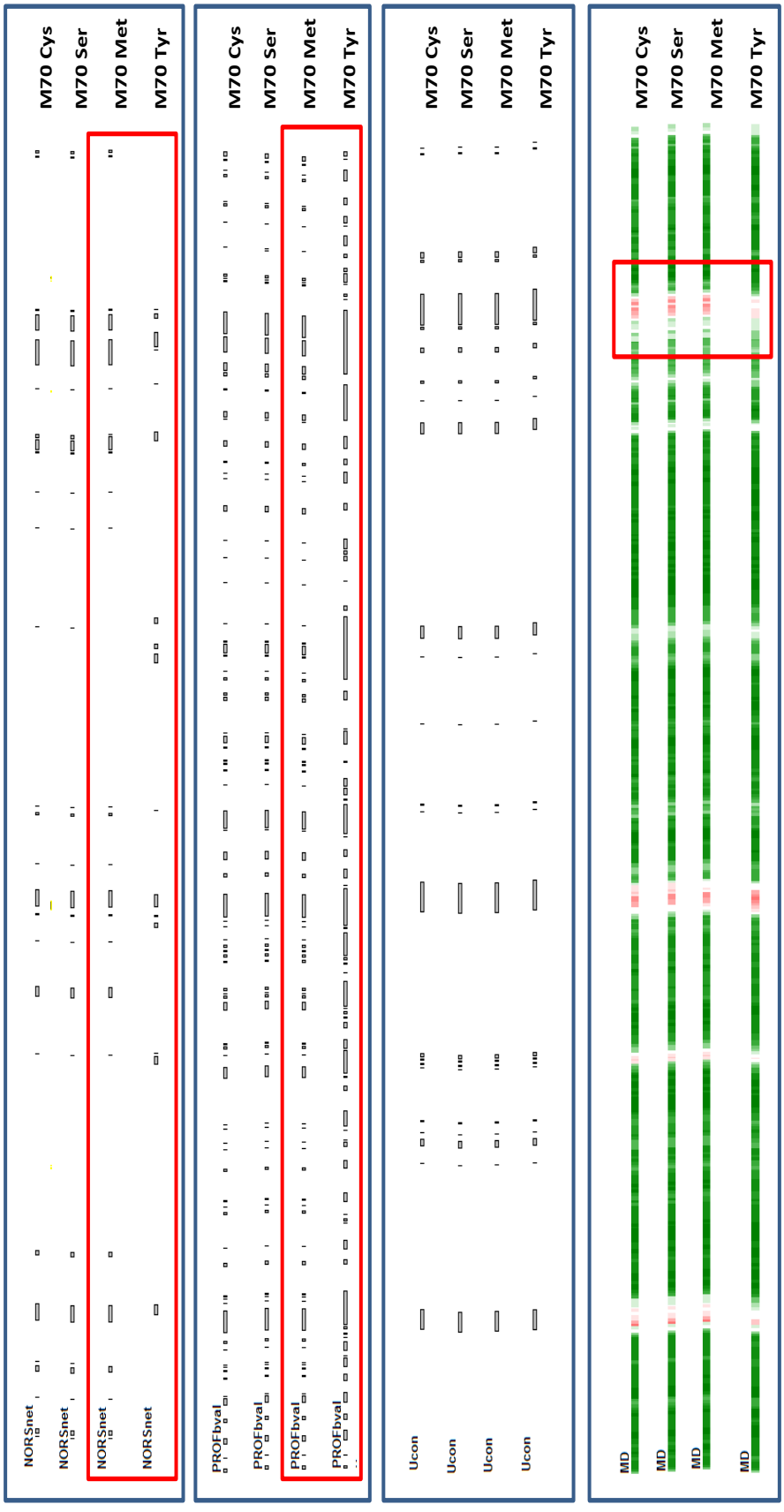

Figure 3.52: Metagenomic analysis of M70 structure of the *wt* (M70 Cys), $M70^{C850Y}$ (M70 Tyr), $M70^{C850S}$ (M70 Tyr) and $M70^{C850M}$ (M70 Tyr) using PredictProtein (http://www.predictprotein.org), showing the effect of the residue change at position 850 compared to the wild type. Analysis showed that change from cysteine (M70 Cys) to tyrosine (M70 Tyr) has a marked effect on predicted solvent accessibility (A), secondary structure (B), amino acid subsequences that are most likely to switch between different types of secondary structure (C), DNA interaction sites (D), protein disorder prediction-unstructured loops (E) prediction of residue mobility (F), and prediction of meta-disorder (H) but no obvious changes in prediction of natively unstructured regions (G). When the cysteine residue was replaced by serine (M70Ser) or methionine (M70Met), the predictions showed no effect.

3.6 HCMV Merlin BAC

A C to T nucleotide substitution introduced into the M70 ORF of the K181 (Perth) variant of MCMV at base position 99,286 leads to tyrosine substitution for cysteine at residue 890 and results in attenuation of virus growth *in vitro* (at 40ºC) and *in vivo* (Timoshenko *et al.*, 2009b). In MEF cells, the mutant was not detectable until day 5 post infection compared to the parent and revertant viruses that were readily detectable from day 1 post infection. Furthermore, DNA synthesis of the mutant virus at the non-permissive temperature was 100-1000 fold lower than that of the *wt* and revertant (Timoshenko *et al.*, 2009b).

Studies in BALB/c mice infected with the mutant virus revealed that no infectious virus was detectable in the salivary glands for up to three weeks post inoculation (Timoshenko *et al.*, 2009b). These findings suggested that the M70 protein is vital for MCMV growth and pathogenicity and the mutant could be an excellent candidate for a live attenuated virus model within mice. Introduction of the same mutation into the UL70 of HCMV might reveal whether this mutation also produces an attenuated human cytomegalovirus that may hold the potential for the production of a successful HCMV vaccine.

The UL70 homologue, together with UL102 and UL105, are predicted to encode proteins important for the formation of the replicative helicase-primase complex (McMahon & Anders, 2002). UL70 is the human cytomegalovirus equivalent of the M70 protein within the helicase-primase complex. UL70 encodes a 974 amino acid protein approximately 107 kDa in size.

This part of the study was carried out as a summer student project in which I prepared most of the constructs and did the planning with some help from the student. We planned to introduce a single point mutation (C to T) at base position 102,094 within the UL70 gene of HCMV Merlin BAC. This mutation will result in a cytosine to tyrosine substitution at position 865, the homologous region to position 99,286 of C890Y within MCMV. This in turn will affect the interactions of the complex, potentially reducing DNA synthesis. The mutation was produced using site directed mutagenesis (Section 2.6.2) and introduced into the *wt* HCMV Merlin BAC using two-step homologous recombination (Section 2.7).

Linear DNA of UL70 was prepared using UL70 primers (Table 2.3), cloned into pGEM®-T Easy Vector (Section 2.6.1) and the mutation introduced into the cloned segment using the site directed mutagenesis technique (Section 2.6.2). PAL1111 (SW102 containing the HCMV Merlin BAC) was used for construction of the Mt[UL70^{C890Y}] BAC. The RpsL-neo cassette flanked with 50 bases of homology arms at its 5'and 3'termini was prepared using UL70 RpsL-Fwd and UL70 RpsL-Rev primers (Table 2.10). The RpsL-neo cassette was inserted successfully into the UL70 gene of the HCMV Merlin BAC and isolates were selected on LBA plates containing kanamycin that were then screened and analysed by PCR (Figure 3.53 and Figure 3.55). Mutated linear DNA segments were then used to replace the cassette. Isolates were selected on streptomycin, PCR screened and analysed using pairs of virus specific and RpsL-neo specific primers (Figure 3.54 and Figure 3.55). Furthermore, PCR amplified segments of the UL70 gene of the promising isolates were sequenced and the mutation was confirmed (Figure 3.55). RFLP analysis was performed to ensure that no rearrangements had occurred elsewhere in the BAC (Figure 3.56 and Figure 3.57).

Revertants of the HCMV mutants were also constructed to ensure that the phenotypic properties of the constructed mutants were attributed to the mutations not to other factors or mistakes during their construction. In a similar way, the revertants were constructed using the two-step homologous recombination technique. They were then PCR analysed and sequenced as described for the mutant. Mutant Mt[UL70^{C865Y}], revertant Rv[UL70^{Y865C}] and *wt* Merlin BACs were extracted as described in section 2.5.3.2. Viruses have been rescued from these BAC construct and the study of their properties will be investigated by Dr Brian Martin in the College of Medical and Dental Sciences at The University of Birmingham.

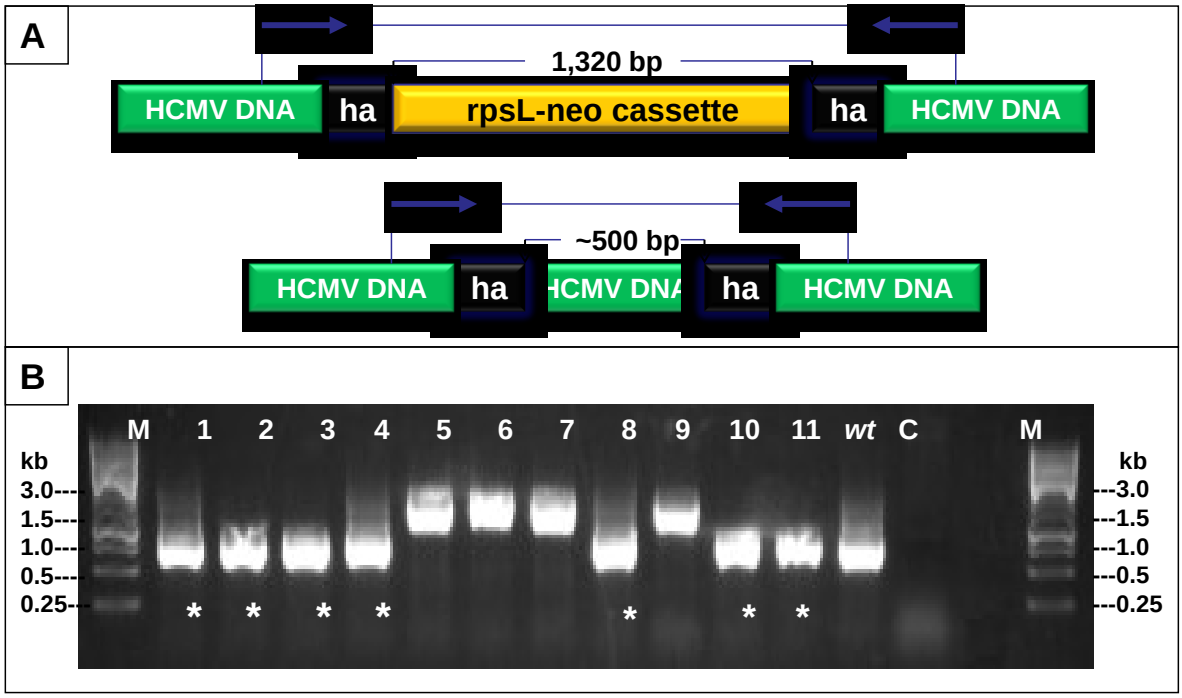

Figure 3.53: PCR screening of the rpsL-neo cassette insertion into the HCMV BAC using primers outside the recombination region. A. schematic diagram showing the difference in the expected product size before and after insertion or replacement of RpsL-neo cassette using primers outside the recombination region. B. PCR screening of 11 different isolates for replacement of the *wild-type* region of the UL70 gene segment with the RpsL-neo cassette in the UL70 gene. This gives PCR products that are about 800 bp larger than the PCR products produced from the *wt* HCMV Merlin BAC. Isolates with non-successful RpsL-neo cassette insertion are indicated by the stars. (*wt*) is *wt* HCMV UL70 gene segment negative control for RpsL-neo cassette insertion and (C) is a PCR negative control (PCR with no DNA). M is a 1kb molecular size marker (Ladder), in kilobases.

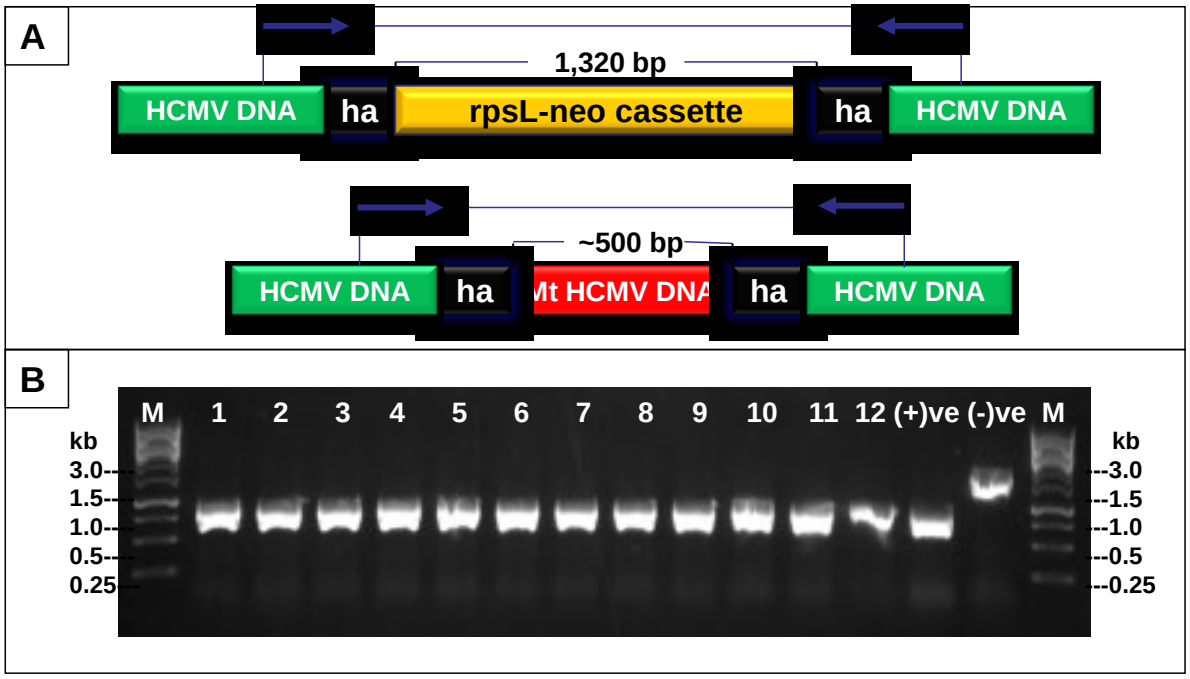

Figure 3.54: PCR screening for the RpsL-neo cassette replacement with the mutated linear DNA segment in the HCMV Merlin BAC using primers outside the recombination region. A. Schematic representation of the primer binding sites. ha – homology arms. B. PCR screening of 12 different isolates for replacement of RpsL-neo cassette in the UL70 gene segment with the HCMV sequence containing the desired mutation which gives PCR products that are about 800 bp smaller than the PCR products of the RpsL-neo cassette [(-)ve]. [(+)ve] is HCMV segment positive control. M is a 1kb molecular size marker (Ladder), in kilobases.

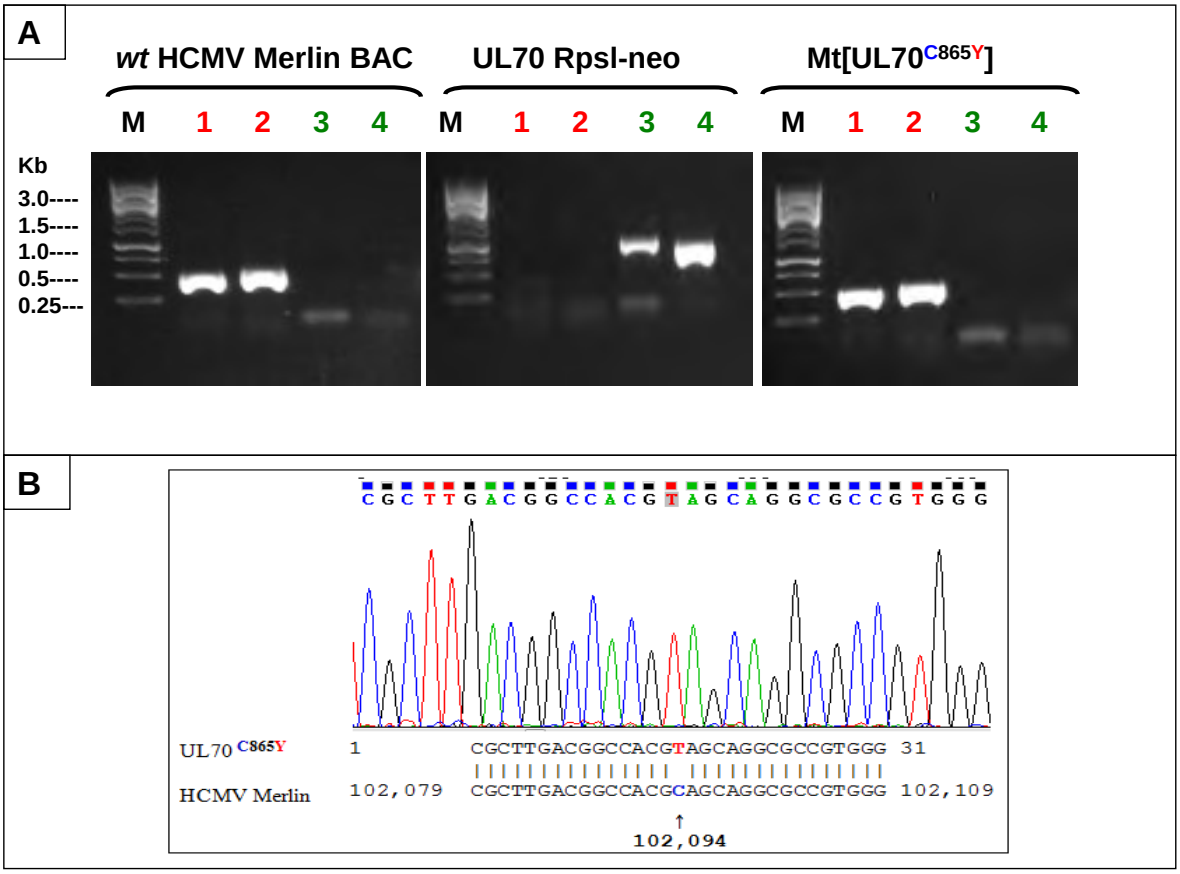

Figure 3.55: Mt[UL70^{C865Y}] BAC construction. [A] shows the detailed PCR analysis (see Figure 3.10 for further explanation) and the steps of UL70 mutant construction starting with *wt* HCMV Merlin BAC, the UL70 RpsL-neo cassette (insertion of the RpsL-neo cassette), Mt[UL70^{C865Y}] BAC (replacement of the RpsL-neo cassette with the mutated UL70 ORF) using UL70 specific primers in the 1st and 2nd PCR reactions, and RpsL-neo specific primers in 3rd and 4th PCR reactions. Lanes M show 1 kb ladder. B. The sequence profile of region of interest in the UL70 gene in the Mt[UL70^{C865Y}] BAC showing that the mutation of interest (C to T) has been inserted at base position 102,094 of the HCMV Merlin BAC.

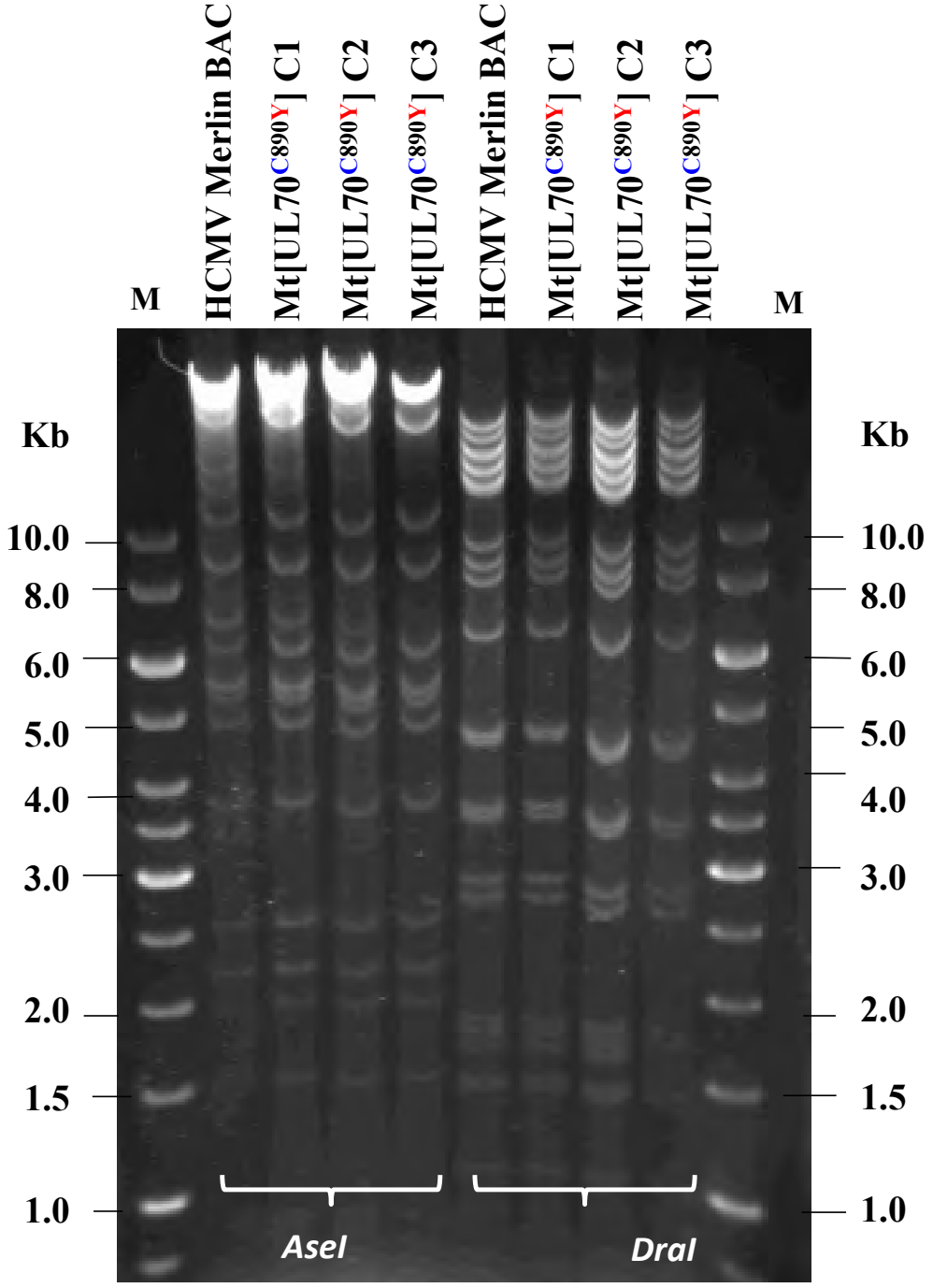

Figure 3.56: RFLP analysis of the recombinant HCMV Merlin BAC using *Ase*I *and Dra*I restriction enzymes digestion. Three recombinant mutant isolates (C1-C3) and the *wt* HCMV Merlin BAC DNA (~2 µg) was digested with 1 µl of *Ase*I or *Dra*I restriction enzyme and the resulting fragments were separated on a 0.4% agarose gel for ~19 hours at 2 V/cm. No genome rearrangements were detected in either of the recombinant BACs compared to the *wt* HCMV Merlin BAC. Molecular size markers (lanes M), in kilobases, are indicated.

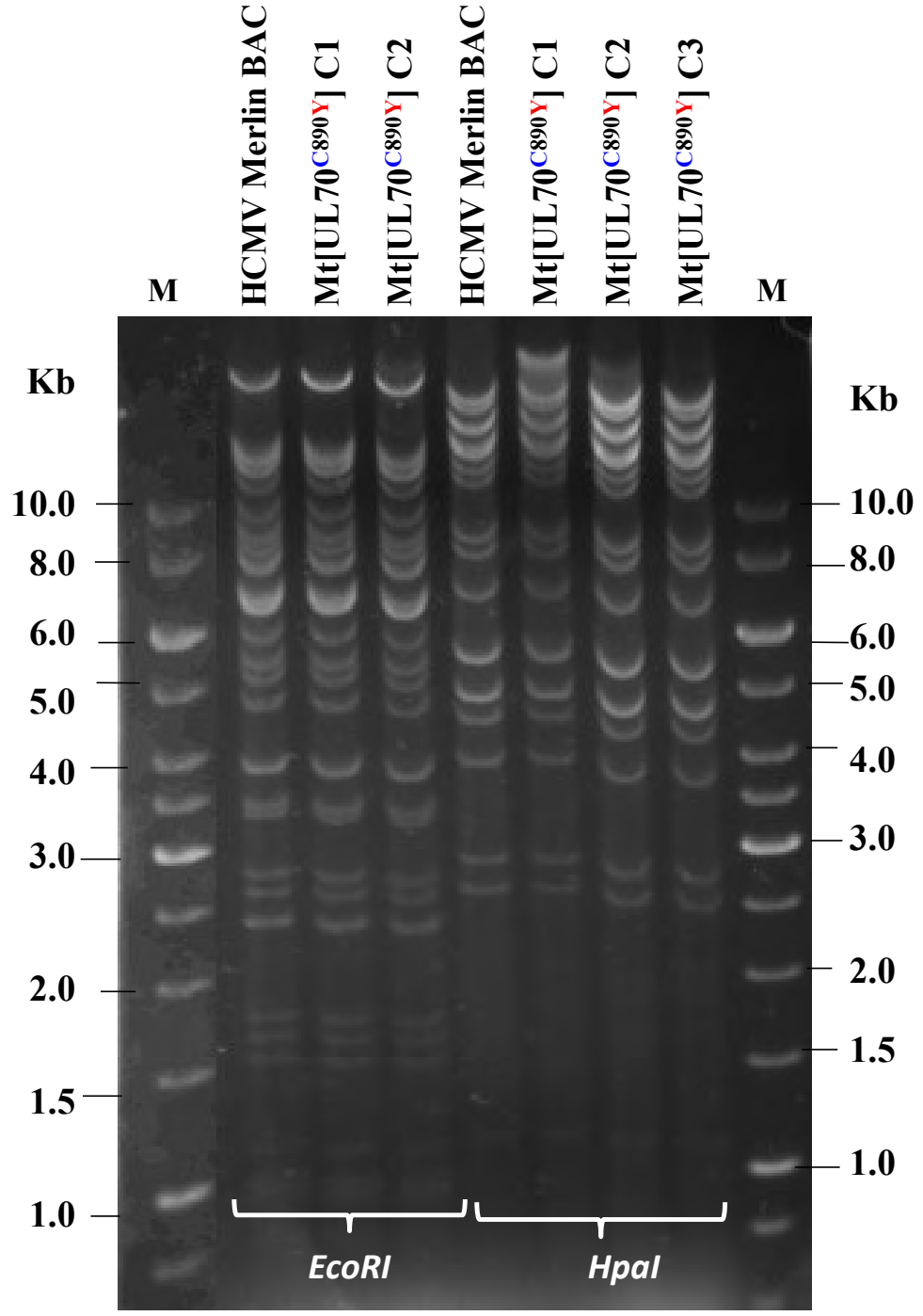

Figure 3.57: RFLP analysis of the recombinant HCMV Merlin BAC using *Eco*RI *and Hpa*I restriction enzyme digestion. Three recombinant mutant isolates (C1-C3) and the *wt* HCMV Merlin BAC DNA (~2 µg) was digested with 1 µl of *Eco*RI or *Hpa*I restriction enzyme and the resulting fragments were separated on a 0.4% agarose gel for ~19 hours at 2 V/cm. No genome rearrangements were detected in either of the recombinant BACs compared to the *wt* HCMV Merlin BAC. Molecular size markers (lanes M), in kilobases, are indicated.

Chapter 4: **General Discussion**

4.1 The sequence polymorphism of *tsm5* mutant virus

Chemical mutagenesis of the *wt* K181 (Birmingham) variant of MCMV resulted in the isolation of many temperature-sensitive mutant viruses. One of them, *tsm5,* was defective in replication in immunocompetent BALB/c mice but able to induce an immune response and to protect animals from a subsequent challenge with a sublethal dose of *wt* K181 (Birmingham) virus (Bevan *et al.*, 1996; Furrarah & Sweet, 1994; Morley *et al.*, 2002; Sammons & Sweet, 1989). The identification of mutations responsible for this phenotype was a necessary requirement to understand the basis of *tsm5* attenuation and to apply this knowledge to the development of an HCMV vaccine. DNA synthesis in *tsm5* virus-infected cells was reduced by approximately 90% at 40°C compared to that at 37°C and to that of the K181 (Birmingham) variant at either temperature. An electron microscopic study revealed that there were no C capsids in the *tsm5*-infected cells (Sweet *et al.*, 2007).

Previously, five non-synonymous mutations were identified in *tsm5* compared to its parent K181 (Birmingham) virus (Sweet *et al.*, 2007). Two mutations were identified in immune evasion genes: a C to A mutation leading to an A658S residue change in the inhibitor of IFN-γ signalling (M27) and a C to T mutation (V54I) in the inhibitor of apoptosis (M36). Three mutations were found in DNA replication, processing and packaging genes: a C to T mutation (G439R) in the large terminase subunit (M56), a C to T mutation (C890Y) in the primase component (M70) of the helicase-primase complex and a C to T mutation (P324S) in the alkaline nuclease (M98) (Sweet *et al.*, 2007). In addition,

many other mutations have been identified in this study (Table 3.2 and Table 3.3) using CGS.

In a previous study, it was shown that the mutation in the M70 gene made the virus temperature-sensitive, as its growth was drastically reduced at 40°C (Figure 3.30). In addition, the chimaeric Smith/*tsm5* DGIK virus (contains the central region of the *tsm5* genome and its 3'and 5'ends from the MCMV Smith genome), is temperature-sensitive, although less so than *tsm5*. It does not have a mutation in its M70 gene, but it still does not replicate similar to *wt* virus (Sweet *et al.*, 2007; Timoshenko *et al.*, 2009b). This suggested that there may be mutations other than the M70 mutation that contribute to the *ts* phenotype of *tsm5*. Based on these results, the present study was to introduce the mutations identified in M27, M36, m139, m141 and m143 of *tsm5* into the *wt* K181 (Perth) virus to identify their role in the *tsm5* temperature-sensitive phenotype.

From sequence analysis of mutations present in all available *tsm5* stocks, it was found that *tsm5* was polymorphic, as both mutant and *wt* nucleotides were present as a double peak at the positions of the mutations identified previously. In total, six *tsm5* stocks, the earliest produced in 1990 and the latest in 2004, were available for this study. Sequencing revealed that all *tsm5* stocks were polymorphic in at least two out of the five loci and the proportion of the *wt* and mutant variants varied between the stocks (Table 3.1). In the 1990 stock, the M36 and M70 mutations were confirmed by sequencing, but following one passage, for the preparation of a seed stock, both mutations showed double peaks. The *tsm5* stock has been passaged *in vitro* (Thesis; by Dr. Olga Timoshenko) 11 times at 37°C and 4 times at 40°C, but this did not lead to the loss of either of the variants, *wt* or mutant, at any of the five loci, and a mixed population was obtained. Likewise, Varicella zoster virus vaccine strains Oka Biken and V-Oka-GSK contain mixtures of

strains represented in variable proportion from lot to lot, where sequence analysis has revealed that there are SNPs in twelve genes and eight nucleotide substitutions in ORF 62 (Sauerbrei *et al.*, 2004).

The presence of both mutations as a single peak in the first passage excludes the possibility that the *tsm5* polymorphism is due to contamination with *wt* virus. However, it is difficult to differentiate between the *tsm5* polymorphism being due to the presence of different *tsm5* viruses, in the original plaque when the virus was created, or due to reversion of these mutations to wild type during subsequent passages. The presence of the M27 and M36 gene mutations with clear single peaks in cosmid H (one of 11 cosmids generated from *tsm5*) (Sweet *et al.*, 2007) might suggest that these double peaks are due to reversion of these mutations to the *wt*. Similarly, reversion of the T nucleotide at base position 99,286 to the *wt* C nucleotide (double peaks) in the BAC derived mutant Mt[M98^{P324S}70^{C890Y}] *in vitro* at 40ºC supports this view. Again, the T to G mutation of the M70 triple mutant Mt[M98^{324S}70^{890Y}56^{439R}] virus, which resulted in a codon change that led to substitution of tyrosine in the mutant by serine instead of the *wt* cysteine, which occurred one-day post infection of mouse fibroblasts at 37ºC (Timoshenko *et al.*, 2009b), also argues in support of the view that these double peaks resulted from reversion rather than due to a mixed population in *tsm5*.

On the other hand, passage of the *tsm5* stock created on 27/07/2004 for 11 times did not result in complete reversion of any of the identified mutations. Furthermore, the M36 and M56 mutations were still mutants as single peaks in this stock. Absence of the *wt* nucleotide excludes the possibility that double peaks are due to contamination with *wt* virus and supports the view that the *tsm5* stocks are polymorphic and contain several mutant viruses. Similarly, sequence of VZV vaccine virus (GlaxoSmithKline Oka vaccine) isolated

from patients with zoster disease revealed that it had six *wt* SNP and 62 mutations. However, none of the six revertant SNPs was able to dominate or discriminate the Oka vaccine virus from the *wt* (Sauerbrei *et al.*, 2004).

Tsm5 was produced using N-methyl-N'-nitro-N-nitrosoguanidine, which results in random mutagenesis, and thus progeny viruses need to be purified by plaque purification to eliminate *wt* contaminants and mutants (Tonari & Minamishima, 1983). In addition, it is difficult to isolate pure MCMV because of its ability to form multicapsid virions in tissue culture (Weiland *et al.*, 1986) and *in vivo*, for example, in the lungs (Reddehase *et al.*, 1985). These multicapsid virions can hold up to fifteen capsids surrounded by a single membrane (Chong & Mims, 1981; Reddehase *et al.*, 1985) and might constitute up to 40% of multicapsid virions in the purified virion preparation (Kurz *et al.*, 1997). In the original study (Sammons and Sweet, 1989) precautions were taken to try and eliminate multicapsid virions by sonication and filtration, but it appears this was not successful.

Furthermore, quantifying genome copies present in cells infected with equal PFU numbers of *wt* and mutated HCMV, using real-time PCR prior to onset of virus replication, revealed that one PFU of mutant virus corresponded to ~$2.8x10^4$ viral genomes and one PFU of *wt* virus corresponded to 80 viral genomes. Apparently, due to mutant virus defectiveness, 300 times more mutant virions than *wt* virions are required to enter the cell to produce lytic virus infection and plaque formation (Heider *et al.*, 2002).

As plaque picking is not a precise method for isolation of clonal *tsm5* virus containing single mutations to study the possibility of reversion, it could be addressed by cloning *tsm5* DNA into a BAC. Progeny viruses from a single *tsm5* genome could then be passaged in different cells and at permissive and non-permissive temperatures to study

precisely the possibility of reversion of the *tsm5* mutations. However, this would not be a rapid procedure as BACs take some time to produce.

4.2 BAC mutagenesis by Red-mediated homologous recombination:

Manipulation of herpesvirus genomes was made easier after cloning of herpesvirus genomes as BACs in *E. coli* to allow the use of powerful techniques of bacterial genetics for mutagenesis of viral genes to study gene function and phenotype separately.

In the current study, mutagenesis performed in the MCMV K181 (Perth) variant BAC using homologous recombination (through pKD46 plasmid products) in *E. coli*, has been shown to be very effective and we were able to produce all the required mutant and revertant virus constructs. However, some difficulties were experienced during this project and knowledge of these might help the inexperienced researcher in the future.

One difficulty was the presence of the *wt* BAC together with the recombinant BAC, during the process of successful RpsL-neo cassette insertion, in the same bacterial clone thus giving PCR products with both viral as well as RpsL-neo primers (data not shown). This suggests the presence of two copies of the BAC in the same bacterial cell during the transformation. Therefore, introduction of RpsL-neo into one of the two BAC copies while the other BAC remains as *wt* enables the bacterial cell to survive the antibiotic selection. If such a clone is used for the second round (mutant construction), it will result in contamination with *wt* BAC.

The second step of screening for BAC recombinants, where the RpsL-neo cassette is replaced with the viral gene fragment carrying the mutation or *wt* sequence, often reveals that the majority of recombinants do not yield any PCR product with screening primers. The reason for this is that in the second step, the antibiotic selection is aimed at identifying BACs that have lost the RpsL-neo cassette and any unwanted rearrangements resulting in the loss of the RpsL-neo cassette would produce streptomycin resistant isolates and would thus be selected. In addition, unwanted nucleotide substitutions can be found in the re-introduced gene fragment when the RpsL-neo cassette is replaced with the PCR amplified product. The mismatches are most likely introduced during the PCR amplification step. In some cases, rearrangement happens where most of the viral genome in the BAC is lost although the BAC cassette is still present (data not shown). Here, the isolates can be selected on chloramphenicol containing plates, but PCR of the manipulated ORF and other ORFs on either side gives no product at all. So occasionally, more than 50 clones need to be characterized to obtain a BAC with an intact genome and the desired mutation and without unwanted mismatches.

4.3 Recombinant virus growth phenotypes

4.3.1 The M27 mutant Mt[M27^{A658S}]

The M27 gene encodes a 79-kD protein (length 682 aa) shown to bind selectively to and down-regulate, the signal transducer and activator of transcription (STAT)-2, but it has no effect on STAT1 activation or signalling. STAT2 inhibition by the M27 protein confers resistance to IFN-γ for viral replication in tissue culture and in mice. An M27 deletion mutant (ΔM27) was completely inhibited by IFN-γ (Khan *et al.*, 2004b). This inhibition was assumed to be caused by the inability of the mutant virus to neutralize the effect of IFN-γ- mediated gene expression and hence inhibition of viral replication caused by downregulation of STAT2. This provides good evidence that viral interference with IFN-γ signalling is accountable for preventing immunopreatosome expression (Khan *et al.*, 2004b). In addition to the role of M27 in supporting viral growth in the presence of IFN-γ, it was shown that there is an immunomodulatory proviral effect for the M27 gene where IFN-γ stimulates a large number of genes involved in antigen presentation, cell adhesion, chemotaxis and inflammation (Boehm *et al.*, 1997). So, *in vivo*, a dramatic replication deficiency of MCMV resulted from deletion of the M27 ORF (Abenes *et al.*, 2001).

The growth curve of the Mt[M27^{A658S}] mutant showed that this single mutation has no effect on virus growth *in vitro* in MEF cells at either permissive or non-permissive temperatures compared to both the wild-type and M27 revertant viruses (Figure 3.31). Thus, this mutation does not show a *ts* phenotype. Furthermore, mutant Mt[M27^{A658S}] showed similar growth kinetics in macrophages to the revertant and the *wt* K181 (Perth) variant at 37ºC (Figure 3.39).

The mutation introduced into the M27 gene was at nucleotide position 32,302 in which an adenosine substitution for cytosine results in replacement of an alanine with a serine at residue 658 (A658S) at the carboxyl-terminal end of the protein. Analysis of the M27 peptide sequence suggested this substitution is not located in any conserved or consensus regions. This result is consistent with studies suggesting that the M27 carboxyl-terminal sequence is dispensable for viral replication in fibroblasts *in vitro* (Abenes *et al.*, 2001). However, the M27 deleted mutant was attenuated for replication in both BALB/c and SCID mice that were infected intraperitoneally with virus. Titres of the M27 deleted mutant virus in the salivary glands, lungs, spleens, livers, and kidneys of infected SCID mice at 21 days post infection were 50- to 500-fold lower than those of the wild-type virus and the rescued virus (Abenes *et al.*, 2001). In addition, the virulence of the mutant virus was lower as deaths in infected SCID mice occurred for up to 37 days post infection compared to wild type and rescued virus-infected mice that died within 27 days. These results suggest that a disruption of M27 expression results in reduced viral growth and attenuated viral virulence in infected animals (Abenes *et al.*, 2001). The *tsm*5 mutant is also attenuated for mice and the M27 mutation could contribute to this. However, its ability to replicate like *wt* virus in macrophages might suggest otherwise, but studies in mice are necessary to confirm this.

4.3.2 Murine cytomegalovirus US22 genes:

One of the characteristic features of betaherpesviruses, in which they differ from alpha- and gamma-herpesviruses, is the presence of additional gene families such as the US22 gene family. These families are usually found at the ends of the genome (Neipel *et al.*, 1991; Nicholas, 1996). The US22 gene family was initially described in HCMV. It consists of 12 members in both HCMV and MCMV and 11 in rat CMV (Chee *et al.*, 1990).

These members are characterized by stretches of hydrophobic and charged residues and up to four conserved sequence motifs that are specific for betaherpesviruses. In HCMV, motif I varies between US and UL family members (Nicholas, 1996). MCMV genes M23, M24, m25.1, m25.2, M 36 and M43 have UL-like motif I whereas m128, and m139 to m143 genes have the HCMV US-like motif I. Motifs I and II sequences contain short stretches of hydrophobic and charged residues, whereas, motifs III and IV are less well-defined and have stretches of non-polar residues (Hanson *et al.*, 1999; Kouzarides *et al.*, 1988).

Many US22 gene products are viral tegument components and the functions of most US22 genes are unknown (Adair *et al.*, 2002). However, five of the twelve members of the US22 gene family (M36, M43, m139, m140 and m141) products of MCMV are important for virus replication in macrophages (Menard *et al.*, 2003). The M36 gene has an anti-apoptotic function where it encodes an anti-apoptotic protein that inhibits death receptor-mediated induction of apoptosis by binding to procaspase 8 (Menard *et al.*, 2003). UL36 in HCMV, the positional and sequence homologue of M36, was initially anticipated to function as a transcriptional transactivator (Colberg-Poley *et al.*, 1992). A later study showed that the function of UL36 is similar to that of M36, where it encodes a viral inhibitor of caspase 8 activation (vICA) (Menard *et al.*, 2003; Skaletskaya *et al.*, 2001).

Genes m139-m141 have all four motifs, while m142 and m143 (and IRS1/TRS1 of HCMV) do not have motif II. It is also shown that m139, m141, m142 and m143 have an acidic domain which is common to herpesvirus transcriptional activators and specifically to MCMV immediate-early proteins 1 and 2 (Cardin *et al.*, 1995; Munch *et al.*, 1992). Among these genes, UL36 and TRS1/IRS1 in HCMV, and m128, m142, and m143 in MCMV, and positional homologues of HCMV UL36 to UL38, and UL43 of human herpesvirus 6

(Cardin *et al.*, 1995; Munch *et al.*, 1992; Nicholas & Martin, 1994), are found to be transcribed with immediate-early kinetics.

4.3.2.1 The M36 mutant Mt[M36^{V54I}]

Studies have suggested that the M36-encoded protein has an antiapoptotic function where it interacts with procaspase-8. Deletion of the M36 gene resulted in apoptosis of infected macrophages (Menard *et al.*, 2003). Also, deletion of M36 resulted in enhanced apoptosis after pro-apoptotic stimulation of infected fibroblasts. Study of the anti-apoptotic function of wild-type and M36 deleted MCMV infected NIH 3T3 fibroblasts and IC-21 macrophages and mock-infected cells after pro-apoptotic anti-Fas treatment revealed that there was a 4.5-fold difference in the number of surviving cells after infection with wild-type and ΔM36 MCMV, whereas the difference was 65-fold in IC-21 macrophages (Menard *et al.*, 2003).

The growth kinetics of mutant Mt[M36^{V54I}] showed no *ts* effect on virus growth *in vitro* in MEF cells at either permissive or non-permissive temperatures compared to both the *wt* K181 (Perth) parent virus and M36 mutant revertant (Figure 3.32). Thus, this mutation does not show a *ts* phenotype as it replicated to similar titre to its revertant and the *wt* virus. Moreover, mutant Mt[M36^{V54I}] showed similar growth kinetics in Raw macrophages to the revertant and the *wt* K181 (Perth) variant at 37ºC (Figure 3.40).

Our results for replication of mutant Mt[M36^{V54I}] are consistent with other studies which showed that the M36 ORF is dispensable for growth in MEF and NIH3T3 cells (Menard *et al.*, 2003). However, the M36 ORF protein plays a role in viral growth in macrophages, as M36 transposon mutants with transposon insertions 98 bp downstream of the ATG and 363 bp downstream of the start of exon2 resulted in impairment of virus

growth in M36 infected IC-21 macrophages and titres were three logs lower in semi-permissive J774-A1 macrophages compared to wild-type virus (Menard *et al.*, 2003). The mutation introduced into the M36 gene in the present study was into exon1. Little is known of the functional domains of M36 but it appears that the mutation identified in the *tsm5* M36 plays little role in the function of M36.

4.3.2.2 The double mutant Mt[M27^{A658S} M36^{V54I}] virus

As discussed above, the M27 protein plays a role in binding selectively to and then downregulation of STAT2, which explains why *wt* virus replication is resistant to IFN-γ in tissue culture and in mice. Thus, a mutant in which the M27 gene was deleted (ΔM27) was completely inhibited by IFN-γ (Khan *et al.*, 2004b).

On the other hand, the anti-apoptotic function of the M36 gene product and its interaction with procaspase-8 supports the replication of the virus in cells and allows the production of virus progeny. Deletion of the M36 gene resulted in apoptosis of infected macrophages (Menard *et al.*, 2003). Induction of apoptosis resulted in abortive viral infection and shut-down of virus replication. Deletion of the M36 ORF also resulted in an enhanced apoptosis after pro-apoptotic stimulation of infected fibroblasts.

Although the presence of individual M27 and M36 mutations in the constructs of Mt[M27^{A658S}] and Mt[M36^{V54I}] respectively did not affect virus replication in MEFs or macrophages (Section 3.4.1 and 3.4.2), the presence of both mutations in one virus, the Mt[M27^{A658S} M36^{V54I}] mutant, attenuated virus growth in MEF cells at 40ºC and in macrophages at 37ºC. These results suggest that the two mutations might have a synergistic effect whereby previously undetected inhibition of viral replication as a result of IFN-

mediated gene expression in the M27 mutant and the undetected increased apoptosis in the M36 mutant now combine to suppress virus replication in the double mutant. This might explain the low titre of the Mt[M27^{A658S} M36^{V54I}] mutant during production of seed and working stocks and the small plaque size (Figure 3.34) produced by the double mutant compared to its revertant as well as the M27, M36 and *wt* viruses.

Activation of the IFN signalling pathways results in transcription of target genes whose products have an antiviral activity. The eIF2α Protein kinase R (PKR) and the 2'-5' oligoadenylate synthetases are two well-known IFN-inducible components that exert the antiviral response. Each is an essential factor that independently acts to inhibit virus replication and thus results in the establishment of an antiviral state. In interferon-stimulated primary human cells infected with HSV-1, 2'-5' oligoadenylate synthetase production is blocked late in the virus' productive life cycle by the US11 gene product (Sanchez & Mohr, 2007). Other factors, such as IFN-inducible enzymes and molecules involved in the cell cycle or cell death might limit viral replication. These IFN-inducible enzymes are present in cells in an inactive form that can be activated when the cells are virally infected. It is thought that viral co-factors such as dsRNA can play a role in the IFN-inducible enzyme activation (Jacobs & Langland, 1996). It might be that apoptosis enzymes are among the IFN-inducible molecules involved in cell death and thus M27 mutation potentiates the effect of the M36 mutation.

The M27 and M36 proteins might also have activities other than inhibition of IFN-mediated gene expression and apoptosis or they might interact together in either IFN signalling or apoptosis pathways that promote virus growth. For example, recent evidence suggests that reduced expression of STAT2 in cancer cells leads to resistance to type I IFN-induced apoptosis. STAT2 deficient cells were defective in activating the mitochondrial-

dependent death pathway (Romero-Weaver *et al.*, 2010; Scarzello *et al.*, 2007). Similarly, in multiple myeloma, members of the Bcl-2 family such as Bax and Bak have been implicated in mediating type I IFN-induced apoptosis (Panaretakis *et al.*, 2003; Thyrell *et al.*, 2004). A small set of IFN-inducible, STAT2-dependent apoptotic genes have recently been identified on human chromosome 22 (Hartman *et al.*, 2005). It is likely that expression of these genes requires transcriptionally competent STAT2 for mediating type I IFN-induced apoptosis. Thus, a minor increase in STAT2 expression by the M27 mutation coupled with increased IFN induction resulting from the M36 mutation may produce the synergistic effect observed.

It will be interesting to study the effect of presence of both mutations in the mouse where it was shown that deletion of the M27 gene had a dramatic effect on virus replication *in vivo* (Abenes *et al.*, 2001). This effect was attributed to the immunomodulatory proviral effect for M27 gene where IFN-γ stimulates a large number of genes involved in antigen presentation, cell adhesion, chemotaxis, and inflammation (Boehm *et al.*, 1997).

4.3.3 m139, 141 and 143 mutants

In infected fibroblasts and macrophages, the protein products of the m139 (72 and 61 kDa), m140 (56 kDa) and m141 (52 kDa) ORFs are expressed abundantly at early and late times in the nucleus and cytoplasm of infected fibroblasts and macrophages (Hanson *et al.*, 1999; 2001). The products of m139, m140 and m141 function to regulate, directly or indirectly, the replication of MCMV in macrophages, that play a role in CMV pathogenesis and in supporting productive and latent infections (Henry *et al.*, 2000). Deletion or mutation in these genes results in attenuation of virus replication in activated macrophages

in vitro and in severe combined immunodeficient (SCID) mice (Hanson *et al.*, 1999; 2001; Menard *et al.*, 2003).

Studies described a transcriptional transactivation function for all US22 genes except m142 and m143 (Cardin *et al.*, 1995; Colberg-Poley, 1996). Studies with MCMV showed that the IE2 product of m128 is dispensable for growth *in vitro* and *in vivo* (Cardin *et al.*, 1995). A mutant with a deletion that included genes m137 through to m143 was not able to grow like *wt* MCMV, which might indicate that one or more of these genes are important for virus replication (Cavanaugh *et al.*, 1996).

MCMV mutants RV7 (m137 through to m141 genes deleted) and RV10 (m139 through to m141 genes deleted) failed to grow in macrophages *in vitro* and demonstrated altered tissue type distribution *in vivo* (Cavanaugh *et al.*, 1996; Hanson *et al.*, 1999). Hanson and colleagues showed that this phenotype of growth in macrophages was not a property of the product of the m139 where deletion of m139 alone had no influence on viral replication in IC-21 macrophages. However, deletion of the m141 ORF resulted in a 2 $\log_{10}$ reduction in virus growth in IC-21 macrophages. Furthermore, they showed that m140 and m141 influenced viral pathogenesis by functioning both cooperatively and independently to regulate MCMV replication in a cell type-specific manner (Hanson *et al.*, 2001). The product of m139 interacts with the complex formed of m140 and m141 gene products to form a complex that co-localized predominantly at the perinuclear region of the cell (Karabekian *et al.*, 2005).

The m139 ORF encodes two proteins, one of 72 kDa (p72m139) and one of 61 kDa (p61m139), which can be detected by 6 h.p.i. in either fibroblasts or macrophages, and remain at steady-state levels increased throughout the course of infection. However,

p61m139 was consistently less abundant at all times post infection. The 72-kDa m139 protein closely matched the predicted size of 71.8 kDa, suggesting that this protein is a full-length product. The smaller protein may be a processed product derived from p72m139 or may arise from the second transcript (Hanson *et al.*, 2001).

In a comprehensive mutational analysis of all 12 US22 gene family members of MCMV, only m142 and m143 were essential for virus replication *in vitro* (Menard *et al.*, 2003). Mutants with a m142 or m143 deletion were both unable to replicate in non-complementing cells at low and high MOI. It was shown that in cells infected with these mutants, proteins of viral IE and E were expressed, whereas viral DNA replication and synthesis of the late-gene product glycoprotein B were inhibited although late gene mRNAs were present (Valchanova *et al.*, 2006).

Furthermore, synthesis of global protein was diminished in these cells, which is linked with phosphorylation of the double-stranded RNA-dependent protein kinase R (PKR) and its target protein, the eukaryotic translation initiation factor 2α. These findings propose that m142 and m143 are essential for blocking the PKR-mediated shutdown of protein synthesis. Expression of the human cytomegalovirus TRS1 gene, the double-stranded-RNA-binding protein that inhibits PKR activation, partially restored replication of these mutants. This suggests that m142 and m143 were both important for inhibition of the PKR-mediated host antiviral response (Valchanova *et al.*, 2006).

4.3.3.1 The m139 mutant Mt[m139^{Y565X}]

In infected fibroblasts and macrophages, the protein products of m139, m140 and m141 are abundantly expressed in co-ordinate fashion from 3'co-terminal transcripts at early and late times in the nucleus and cytoplasm (Hanson *et al.*, 1999; 2001).

Compared to the *wt* K181 (Perth) parent virus and the m139 revertant, the replication of mutant Mt[M139^{Y656X}] showed no deficiency in virus growth *in vitro* in MEF cells at 37ºC. However, at 40ºC the virus yields were lower and similar to those of *tsm5* (stock 2004) (Figure 3.35). Surprisingly, mutant Mt[M139^{Y656X}] showed similar growth kinetics in Raw macrophages to the revertant and the *wt* K181 (Perth) variant virus at 37ºC (Figure 3.42). This suggests that the M139^{Y656X} mutation contributes to the *ts* phenotype of *tsm*5.

In our study, the mutation introduced into the Mt[M139^{Y656X}] mutant resulted in a stop codon and then truncation of the m139 protein; this resulted in a slight temperature sensitivity where its replication at 40ºC in MEFs was reduced compared to its revertant and the *wt*. However, its replication in macrophages at 37ºC was similar to that of the *wt*, which might indicate that truncation of m139 at residue 656 affects folding of its product at an elevated temperature.

4.3.3.2 The m141 mutant Mt[m141^{V195M}]]

Introduction of the T to C mutation at position 198,731 of the m141 gene did not affect the growth kinetics of the Mt[m141^{V195M}] mutant and showed no *ts* effect on virus growth *in vitro* in MEF cells compared to *wt* K181 (Perth) (Figure 3.36). Moreover, Mt[m141^{V195M}] showed similar growth kinetics in Raw macrophages to the revertant and the *wt* K181 (Perth) variant at 37ºC (Figure 3.43).

Our observation that the m141 mutation has no *ts* phenotype as the mutant replicated to similar titres as its revertant and the *wt* indicates that the introduced mutation has no effect on virus growth in MEFs and macrophages *in vitro* and possibly does not affect the

interaction between m139 and m140 products. An *in vivo* study of m141 mutant may reveal an effect of the introduced mutation on the pathogenicity of the virus.

4.3.3.3 The m143 mutant Mt[m143^{M232I}]

As for the m139 and m141 mutants, the growth kinetics of mutant Mt[m143^{M232I}] were similar to its revertant virus and the *wt* K181 (Perth) virus in MEF cells at both the permissive and non-permissive temperature (Figure 3.37). Thus, this mutation does not show a *ts* phenotype. Moreover, mutant Mt[m143^{M232I}] showed similar growth kinetics in Raw macrophages to the revertant and the *wt* K181 (Perth) variant at 37ºC (Figure 3.44)

The m143 mutation (T to C at position 201, 944) has no effect on virus growth *in vitro* in MEF cells at either 37ºC or 40ºC compared to both the *wt* K181 (Perth) parent virus and its revertant (Figure 3.37). In addition, Mt[m143^{M232I}] showed similar growth kinetics in Raw macrophages to the revertant and the *wt* K181 (Perth) variant at 37ºC (Figure 3.44).

Thus, the T to C at position 201, 944 mutation in m143 gene of MCMV possibly has no effect on virus replication *in vitro* in MEFs and macrophages.

4.4 Changing codon preferences of the M70 ORF

Our working hypothesis in this project was that the replacement of preferred codons with non-preferred codons might lower replicative fitness primarily by reducing the rate of translation (at the level of polypeptide chain elongation) of the MCMV M70 primase potentially disrupting viral DNA synthesis in infected cells. To test this hypothesis, two viruses were constructed with a synthetic M70 gene (cysteine and tyrosine versions) in which 403 of the *wt* codons were changed to the non-preferred codons. Both BACs were not able to produce viable viruses upon transfection into MEF cells although the transfections were successful as they were monitored for 12 day post infection (Figure 3.50). These results suggest that changing 403 of the 964 codons (42%) resulted in shut-off of primase protein synthesis and thus no virus production or CPE was detected although the revertant BACs produced viruses like the *wt* parent virus.

Based on this, another two MCMV BACs (cysteine and tyrosine versions) were produced with 155 out of 964 codons (16%) of the distal region of M70 ORF (at nt 99,061-100081) or 155 out of 359 codons (43.2%) in that segment changed to the non-preferred codons. Upon reduction of the number of codons changed to non-preferred codons the M70 gene primase was produced in sufficient amount to allow the virus to replicate like the wild type virus. However, the presence of tyrosine at residue 890 aggravated the situation for the virus and shut-off primase protein synthesis and thus no virus production or CPE were detected.

Studies with the poliovirus vaccine strain Sabin type 2 where codons in the capsid region were changed to suboptimal codons also showed that attenuation of the virus was proportional to the number of codons changed to the non-preferred or least used ones

(Burns *et al.*, 2006). Thus, reduction of the number of the suboptimal codons in the M70 gene from 403 to 155 allowed mutant Mt[M70^{SO155Cys}] to yield infectious virus and to produce a detectable CPE on MEFs. However, its ability to replicate in macrophage cell culture and *in vivo* still needs to be examined.

The presence of tyrosine at residue 890 in the Mt[M70^{C890Y}] attenuated replication of the virus in MEFs at 40ºC and in macrophages at 37^{o}C (Section 3.4.1.3 and 3.4.2.2), but it replicated similarly to *wt* virus at 37ºC in MEFs (Timoshenko *et al.*, 2009b). However, in Mt[M70^{SO155Tyr}], changing 155 out of 964 codons (16%) into suboptimal codons resulted in combination of two factors that exerted a stress in the expression and function of the M70 primase protein that resulted in its inability to replicate.

Interestingly, serial passage of mutant Mt[M70^{SO155Cys}] 10 times in MEF tissue culture cells revealed that no mutations occurred in the synthetic segment of the M70 gene. Similar results were observed for poliovirus where serial passage (for 25 passages in HeLa cells) of the virus with altered codon composition showed that the relative fitness of the modified virus remained lower than that of the unmodified *wt* virus (Burns *et al.*, 2006). Furthermore, infection of mice, transgenic for the poliovirus receptor (CD155tg mice), with equal amounts of virus particles of *wt* virus or virus with altered codons, revealed that the mutated virus was over 100-fold neuro-attenuated compared to the *wt* virus (Mueller *et al.*, 2006).

The synthetic poliovirus with synonymous non-preferred codons was stable and may prove suitable as an attenuated virus for the production of a poliovirus vaccine (Mueller *et al.*, 2006). Thus, virus containing the modified M70 gene with less than 20-40% of its *wt*

codons might produce a virus suitable for a model vaccine study, especially if the correct combination of non-preferred codons and tyrosine 890 can be found.

4.4.1 M70 gene growth at 37^{o}C

It is generally accepted that the difference in physical properties resulting from a base substitution leading to an amino acid change in the protein product of a mutated gene, which results in a temperature-sensitive mutant, is due to a temperature sensitive folding event that does not influence the properties of the native form of the protein (Smith *et al.*, 1980). At the permissive temperature, the temperature sensitive polypeptide chains reach their final native state, while at higher temperature they fail to reach this state (Smith & King, 1981). Furthermore, following maturation of the mutant protein into its native state at the permissive temperature, it will have a similar thermal stability and biological activity to the wild-type protein (Sturtevant *et al.*, 1989). Based on this it was assumed that the residue substitution in a polypeptide chain resulting in a temperature sensitive mutation is not essential for the maintenance of the native form of the protein but plays a significant role in the stability and/or kinetic fates of the folding intermediates (Mitraki & King, 1992).

The zinc finger motif (cys–his–cys–cys) in the M70 protein is conserved in all herpesviruses and is involved in DNA binding and helicase activity as well as primase activity, where mutations within this motif of the HSV-1 homologue of M70/UL70 affects these functions (Chen *et al.*, 2005). The C890 residue is located 16 amino acids upstream of the zinc finger and is conserved in all cytomegaloviruses and hence it might affect the activities of the zinc finger. Cytosine substitution with tyrosine at residue 890 in the M70 gene in mutant Mt[M70^{C890Y}], did not affect replication of the virus in MEF cells at 37ºC but attenuated its replication in MEFs at 40ºC and in vivo (Timoshenko *et al.*, 2009b).

Surprisingly, replication of mutant Mt[M70^{C890Y}] was also attenuated in macrophages at 37ºC (Section 3.4.2.2). Based on these findings, attenuation of the virus in macrophages and at the non-permissive temperature in MEFs was not necessarily due to improper folding of the M70 protein, but may be due to a defective protein which manifests itself during stress situations, such as the state of the host cells at the non-permissive temperature or between the different cell culture types. As in the cytomegalovirus infection in humans, circulating leukocytes, particularly monocytes, play an important role in the dissemination of MCMV in the mouse in many stages during the infection (Collins *et al.*, 1994), and thus attenuation of mutant Mt[M70^{C890Y}] replication in macrophages probably plays a role in the attenuation of the virus infection *in vivo*.

It was suggested previously that substitution of the cysteine with a tyrosine may have introduced a tyrosine kinase phosphorylation site and the phosphorylated tyrosine could markedly affect primase conformation and stability, but prediction analysis using the ScanProsite software from the Expert Protein Analysis System (ExPASy) proteomics server did not identify Y890 as a predicted tyrosine kinase phosphorylation site (Timoshenko *et al.*, 2009b). It is possible that the large side chain of tyrosine could affect and destabilise the primase protein structure although upon substitution of cysteine with a much smaller amino acid with a chemically active side chain, serine, the virus grew similarly to the *wt* virus. Furthermore, replacement of cysteine with a relatively inactive amino acid, methionine, also re-established *wt* activity (Timoshenko *et al.*, 2009b).

However, prediction of the structure of the M70 primase using PredictProtein (http://www.predictprotein.org), showed that replacement of cysteine with tyrosine has a marked effect on predicted solvent accessibility (PROFacc), secondary structure (PROFsec), amino acid subsequences that are most likely to switch between different types

of secondary structure (ASP), DNA interaction sites (ISIS), protein disorder prediction-unstructured loops (NORSnet) prediction of residue mobility (PROFbval), and prediction of meta-disorder (MD) but shows no obvious changes in the prediction of natively unstructured regions (Ucon). It is interesting that when the cysteine residue is replaced by serine (M70Ser) or methionine (M70Met), which produced mutant viruses with a *wt* phenotype (Timoshenko *et al.*, 2009b), the predicted structure was similar to the wild-type structure (Figure 3.52). According to these results, understanding the factors resulting in the M70 mutated virus attenuation will be possible only when the primase protein structure is available.

Chapter 5: **References**

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Chapter 6: **Appendices**

A. Publication

1- **Timoshenko, O., Al-Ali, A., Martin, B.A. & Sweet, C. (2009).** Identification of mutations in a temperature-sensitive mutant (*tsm*5) of murine cytomegalovirus using complementary genome sequencing. *Journal of Medical Virology* **81,** 511-518.

2- **Timoshenko, O., Al-Ali, A., Martin, B.A. & Sweet, C. (2009).** Role of mutations identified in ORFs M56 (terminase), M70 (primase) and M98 (endonuclease) in the temperature-sensitive phenotype of murine cytomegalovirus mutant tsm5. *Virology* **392,** 114-122.

B. Poster presentation

Alali, A., Martin, B.A. & Sweet, C. (2008). Study of the Role of M27 and M36 Genes in Replication and Pathogenicity of Murine Cytomegalovirus (MCMV). Bioscience Graduate Research School Symposium, The University of Birmingham, March 2008, Birmingham, UK.

C. Oral presentation

Alali, A., Martin, B.A. & Sweet, C. (2009). Study of the Role of Some Members of the US22 Gene Family in Murine Cytomegalovirus Replication and Pathogenicity. Bioscience Graduate Research School Symposium, The University of Birmingham, March 2009, Birmingham, UK.