Characterisation of open reading frames m29 and m29.1 in murine cytomegalovirus

By

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ABSTRACT

Murine cytomegalovirus (MCMV) in its natural host, the mouse, is an excellent model for studying the biology of cytomegalovirus infection. Mostly this model has been used to study gene homologues of human cytomegalovirus (HCMV). Of the predicted 170 MCMV open reading frames (ORFs) only 78 have significant amino acid identity with genes in HCMV. To better understand the biological mechanisms underlying the differences between the viruses, for example their species specificity and immune evasion genes, MCMV unique ORFs need to be examined. Here the role of m29 and m29.1 ORFs in the MCMV (Smith strain), which have no homology with ORFs of any other cytomegalovirus, have been examined.

The m29 and m29.1 ORFs are overlapping and encoded on opposite strands of the double-stranded DNA genome. Sequence analysis over this region showed a discrepancy to the published sequence. An additional G (guanine) nucleotide was found at nucleotide position 36,198 that alters the predicted ORFs, m29 being 242 amino acids shorter and m29.1 210 amino acids longer than the predicted sequence. This was confirmed by sequencing the MCMV Birmingham K181 strain, the Birmingham Smith strain and MCMV wild type isolates- N1, K17A and G4. Transcripts from the newly identified m29 and m29.1 ORFs were confirmed by reverse transcriptase PCR (RT-PCR). They were produced at early (3h) and immediate-early (2h) times post-infection respectively as determined by cycloheximide and phosphonoacetic acid treatment but were continuously expressed up to at least 24h post-infection. 5' and 3'-RACE (rapid amplification of cDNA ends) analysis from m29.1 ORF confirmed the production of a ~2.4 kb transcript and a low abundance spliced transcript from which a 123bp intron had been removed.

Mutants of ORF m29 and m29.1 have been produced in which ET recombination was used to introduce stop codon mutations within these overlapping ORFs. This was achieved by single base alterations near to the 5` end of each ORF that prevented translation but not transcription of each ORF

individually. Linear dsDNAs containing the mutations were introduced into the Smith MCMV BAC replacing an antibiotic cassette that had been inserted into the gene of interest. Mutant viruses, Rc29 and Rc29.1 respectively, were recovered from these mutant BACs by *in vitro* passage in tissue culture cells. Revertant virus (Rv29.1) was made by a further 2 step process in which the mutant m29.1 ORF was first replaced by the antibiotic cassette and then by the wt ORF.

These mutants were characterized both in tissue culture and in immunocompetent BALB/c and immunodeficient SCID mice. Both mutants produced their expected transcripts but Rc29.1 virus produced no corresponding protein as examined by western blot using an antibody produced in rabbits to bacterially expressed protein. Failure to express the m29 ORF in bacteria and failure of a synthetic peptide to generate rabbit antibodies that bound to denatured m29 protein meant that protein expression of the m29 gene in either mutant could not be determined.

Mutant virus Rc29 replicated similarly to wild type virus both in tissue culture and in BALB/c mice. Mutant virus Rc29.1 replicated poorly with lower yields, a delay of about 2-3 days in reaching peak titres and an earlier decline compared to wt and revertant (Rv29.1) virus in tissue culture. Rc29.1 virus also showed delayed replication in the salivary glands of BALB/c mice compared to wt and Rv29.1 viruses and in SCID mice peak titres occurred later and mice became sick and had to be humanely killed approximately 8 days later that mice infected with wt virus. These results suggest that m29 and m29.1 ORFs are dispensable for viral replication *in vitro* in NIH 3T3 cells and in animal hosts. However, the m29.1 ORF is required for optimal viral growth *in vitro* and *in vivo*.

DEDICATION

This thesis is dedicated to my late loving father, who always cherished that his youngest son would go for higher studies.

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I would like to thank my supervisor, Dr Clive Sweet for his warmth and patience during my time as PhD student in his laboratory. His guidance and support was unstinting and matched only by his enthusiasm for the science. I should acknowledge his untiring efforts to go through my drafts keenly and providing me feedbacks in no time to finish everything in time.

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ABBREVIATIONS

BAC	Bacterial artificial chromosome
CPE	Cytopathic effect
DMEM	Dulbecco's modified Eagle's medium
E	Early gene
EGFR	Epidermal growth factor receptor
FOS	Foscarnet
GM	Growth medium
GCV	Ganciclovir
GSP	Gene specific primer
HSPGS	Heparan sulphate proteoglycans
HCMV	Human cytomegalovirus
IFN	Interferon
IL	Interleukin
IE	Immediate early gene
L	Late gene
MCMV	Murine cytomegalovirus
MM	Maintenance medium
MIEP	Major immediate-early promoter
MEF	Mouse embryo fibroblasts
MOI	Multiplicity of infection
MHC	Major histocompatability complex
NF-κB	Nuclear Factor kappaB
NK cell	Natural killer cell
NCS	Newborn calf serum
nt	Nucleotide
ORF	Open reading frame
PBS	Phosphate buffered saline
PFU	Plaque forming unit
PCR	Polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SCID	Severely combined immunodeficient
TLRS	Toll-like receptors
wt	Wild type

1. INTRODUCTION

1.1 The Herpesviridae

Cytomegalovirus belongs to the family Herpesviridae comprising more than one hundred viruses. These viruses have some features in common: host restriction, nuclear replication, damage of host cell and latency. They are grouped into three subfamilies- the Alphaherpesvirinae (α), the Betaherpesvirinae (β) and the Gammaherpesvirinae (γ) on the basis of their biological properties and sequence homology, genome sequence arrangements and similarity between the viral proteins (162). The members of the Alphaherpesvirinae have some unique features. They can infect a wide range of hosts, have short reproductive cycles, are capable of spreading rapidly in cultures, can efficiently destroy infected cells and can produce latent infections in sensory ganglia. Viruses belonging to the subfamily Betaherpesvirinae have a restricted host range. Their reproductive cycles are prolonged, replication occurs slowly in tissue culture and the infected cells become enlarged (cytomegalia). Cytomegalovirus belongs to this subfamily. The virus is maintained in a latent state in a variety of tissues including secretory glands and kidneys. Members of the last subfamily, the Gammaherpesvirinae, are also host specific. They replicate in cells of lymphoblastoid origin and sometimes also cause lytic reactions in epithelial and fibroblastic cells. The members of this family have specificity for either T or

B lymphocytes (135). Table 1.1 illustrates the properties of the human herpesvirus family members.

DNA extracted from Herpesviruses is linear and double stranded. An icosahedral nucleocapsid comprising 162 capsomers surrounds the genome of this virus. The genome is released directly into the host cell nucleus where it circularises. The size of the DNA varies between different herpesviruses, being approximately 120-250kbp. The herpesvirus genome contains internal and terminal repeats in varying copy number. Sometimes spontaneous deletions occur giving rise to mutants. The G+C content varies from 31% to 75% (135). The Herpes virus nucleocapsid is surrounded by an envelope composed of a lipid bi-layer embedded with viral glycoproteins. The space (tegument) between the envelope and the nucleocapsid contains virally encoded proteins and enzymes involved in the initiation of replication (162).

The HCMV virion is 150-200 nm in diameter with a 100 nm icosahedral capsid. Three major layers constitute the HCMV virion. The first layer is composed of nucleocapsid, which contains a linear 230-kbp double stranded DNA genome. The adjacent layer is protein rich tegument layer, which is surrounded by an envelope composed of a host derived lipid bi-layer with viral glycoproteins (162) (Figure 1.1).

Table 1.1 Properties of Herpesvirus

Human herpes type	Common name	Sub Family	Target cell type	Site of latency	Mode of transmission
1	Herpes simplex-1 (HSV-1)	*Alphaherpesvirinae*	Mucoepithelia	Neurones	Close contact
2	Herpes simplex-2 (HSV-2)	*Alphaherpesvirinae*	Mucoepithelia	Neurones	Close contact, usually sexual
3	Varicella Zoster virus (VSV)	*Alphaherpesvirinae*	Mucoepithelia	Neurones	Contact or respiratory route
4	Epstein-Barr Virus (EBV)	*Gammaherpesvirinae*	B lymphocyte, epithelia	B lymphocytes	Saliva
5	Cytomegalovirus (CMV)	*Betaherpesvirinae*	Epithelia, monocytes, lymphocytes	Monocytes, lymphocytes and possibly others	Contact, blood transfusions, transplantation, congenital
6	Herpes lymphotropic virus	*Betaherpesvirinae*	T lymphocytes and others	T lymphocytes and others	Contact, respiratory route
7	Human herpes virus-7 (HHV-7)	*Betaherpesvirinae*	T lymphocytes and others	T lymphocytes and others	Unknown
8	Human herpes virus-8 (HHV-8)	*Gammaherpesvirinae*	Endothelial cells	Unknown	Exchange of body fluids?
	Kaposi's sarcoma- associated herpes virus (KSHV)				

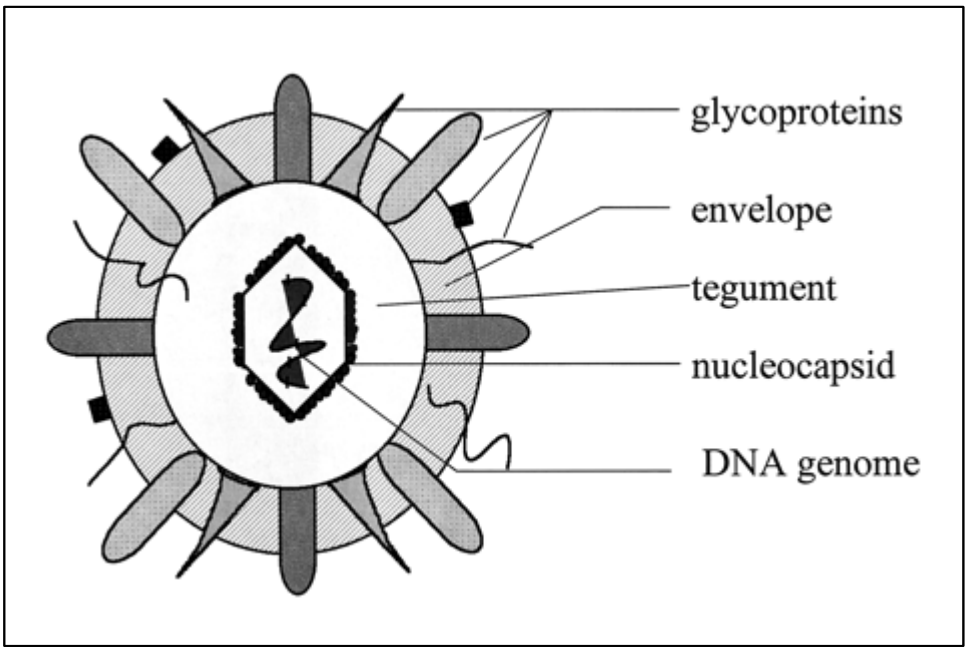


Figure 1.1 Structure of CMV virion. From Brennan, 2001 (18)

1.2 Transmission of Cytomegalovirus

Cytomegalovirus (CMV) infection is prevalent in a significant proportion of the population. Transmission occurs in most cases either perinatally or congenitally. The former route involves virus infection in the birth canal or after birth by virus from the mother while in the latter route, infection detected within the first few months of pregnancy, is transmitted transplacentally or within the uterus. In addition, the virus may be spread through most secretions, particularly saliva, vaginal secretions, semen and also in urine. Surprisingly it is found in highest levels in semen. Therefore, this virus is also transmitted sexually. Again it can be spread to patients who have blood transfusions and transplants. In seropositive mothers, the virus may be transmitted to infants via breast milk (156). Though not highly contagious, it has been found to infect day care workers in day centres. Women who care for children younger than two

years of age have higher levels of serum antibodies than women who care for children older than that age (1).

1.3 Pathogenesis of Cytomegalovirus

The immune status of the host determines the pathogenesis of CMV disease. Innate and adaptive immune systems control the typical primary infection of this virus but the latent infection is maintained for life. Primary infection, enduring latency, and recurrent spread from reactivation generally occur without any obvious disease consequences. Primary infection classically commences with replication in mucosal epithelium as a consequence of direct contact with contagious secretions from an infected person (106). A general phase of infection spreads virus in the host via a leukocyte-associated viraemia that may last for several months (132). Cell-free infectious virus is not found in blood, even if viral DNA escapes degradation, which is identified in plasma from immunocompromised individuals (154). Peripheral blood (PB) neutrophils, monocytes, and endothelial cells have also been detected to transmit infectious virus in immunocompromised hosts (48). Cell culture studies on spread of clinical strains in the absence of full replication have demonstrated neutrophils might act as carrier to transmit infectious virus and viral particles (47). PB monocytes are vehicles for transmission of CMV (161). The systemic phase of primary infection in adults occurs with persistent viral release in urine, saliva, breast milk, and genital secretions, which may be the key basis for spread between hosts. The delivery of virus during acute infection is best understood from studies in the immunocompromised host, but a range of

endothelial, epithelial, and haematopoietic cells in tissues might be susceptible in immunocompetent individuals (120). Myeloid-lineage haematopoietic cells develop as the primary cell type for viral latency. Viraemia lasts for a long period of time after an adaptive immune reaction can be first detected (132). Although an early persistent infection is evident, all immunocompetent individuals are able to clear the infection and elicit a slow rise in cell-mediated immunity. The lack of immunity in infants allows a relatively long period of persistence and continual virus secretion over this period ensures efficient transmission to uninfected individuals (106).

Transplantation of solid organs or bone marrow and transfusion of whole blood also aid transmission of CMV. PB mononuclear cells of the myeloid lineage can also maintain latent infection. In healthy seropositive individuals, latent infection arises from bone marrow (106).

Therefore, pathogenesis of CMV infection is directly related to the condition of the immune system of the host. Both primary infection and reactivation from latency play important roles in pathogenesis. Innate and adaptive immune systems manage acute viral infection, maintain latency, and alter virus during reactivation. Immune deficiencies such as in the case of bone marrow transplantation, stem cell allografting, and PB stem cell autografting (106) render the host vulnerable to CMV infection and consequent disease.

1.4 Clinical manifestations

The incidence of HCMV infection in the population varies between 30 - 70% (118). CMV is more prevalent and contracted earlier in populations of developing countries and within lower socio-economic groups (60). The presence of specific antibodies to CMV correlates with increasing age and with sexual promiscuity (134). Therefore, elderly people are more likely to be CMV seropositve than younger individuals, while sex workers and those who are in immediate contact with small children also have high risk of infection with HCMV (117, 155). HCMV infection of immunocompetent hosts is asymptomatic; very few infected individuals exhibit symptoms and then of a non-specific nature. Mononucleosis, malaise, headache, fatigue, lymphadenopathy, pharyngitis, splenomegaly, fever and hepatomegaly are the clinical signs and symptoms seen in symptomatic individuals (19, 117).

1.4.1 Infection in pregnant women

During a primary infection of the pregnant woman, HCMV can spread via the placenta to the foetus resulting in congenital abnormalities, which includes microcephaly, rash, jaundice, brain calcification and hepatosplenomegaly (19). This may lead to unilateral or bilateral hearing loss and mental retardation. In latently infected pregnant females, reactivation of the virus in the cervix produces less severe symptoms in the infected foetus and congenital abnormalities are generally absent. Another mode of HCMV transmission happens perinatally. CMV remains latent in the cervical epithelium and

reactivates in the process of immunosuppression associated with pregnancy and virus may infect the baby as it is passing through the birth canal. The child may also be infected after birth through the process of lactation as the breast epithelium harbours virus. But in both cases, the infant remains asymptomatic (117).

1.4.2 Infection in immunosuppressed/immunocompromised patients

The care of immunosuppressed patients may be complicated as HCMV is one of the most common and difficult of opportunistic pathogens. Latent virus may be reactivated, reinfection may occur in these patients who have previously had this infection, or a primary infection may occur; all these facilitate the infection to be common. Again, transfusions or transplants in the patients can simultaneously aid the transmission of CMV. Because, at these times, the patients become maximally immunosuppressed and diagnosis and causes of disease make the case critical (117). Again in case of immunocompromised patients, HCMV has the risk of reactivating if they are seropositive or can be infected from an exogenous source if they are seropositive or seronegative. Specifically, immunosuppression is required in solid organ or bone marrow transplantation for the survival of the graft, and here viral infection can cause death (117). In immunocompromised AIDS patients or immunosuppressed transplant patients, reactivation leads to uncontrolled HCMV replication and often results in high levels of mortality and morbidity. Thus, reactivation of latent HCMV constitutes a very severe clinical problem (146). It has also been found in organ transplant patients that reactivation occurs from the transplant

recipient's own latent HCMV rather than virus transmitted from donor (153). Factors that govern the infection include whether the graft donor or recipient are seropositive or seronegative, blood products, HLA matching and the level of immunosuppression (117, 149). Use of immunosuppressive and cytotoxic drugs may aid the development of HCMV disease (45). Pneumonitis, hepatitis, adenopathy, splenomegaly, fever, fatigue, gastrointestinal ulceration and fungal infections are clinical signs found in bone marrow and solid organ transplant recipients (19). Hence, CMV-pneumonitis is the leading cause of death in such recipients (52). In the case of AIDS patients, HCMV can be a major problem. It has been found that retinitis occurs in up to 15% of all AIDS patients. Besides this, interstitial pneumonia, colitis, oesophagitis, hepatitis and encephalitis are also observed in these patients (19). In HIV positive individuals, highly active anti-retroviral therapy (HAART) can lower the prevalence of CMV viraemia and is correlated with increasing frequency of CD4+ lymphocytes (39). Anti-CMV therapy can be replaced by HAART in patients responding well to the latter (9). Some reports indicated HAART may give rise to immune reconstitution uveitis (IRU) due to an immunologic response to CMV in the retina (69).

1.5 Genome organisation of human and mouse Cytomegalovirus

Human cytomegalovirus is isolated routinely in different laboratories and several strains such as AD169, Towne and Toledo have been propagated in tissue culture for several generations (106). These HCMV strains have 90-95% similarity in their genome organization. Chee and colleagues (30) described

AD169 as the largest viral genome sequence when it was completely sequenced. The genome size was approximately 230 Kbp and consisted of a total of 208 ORFs, some of which may produce spliced products. At the same time, they also suggested that some ORFs might not actually encode proteins. Davison and colleagues (37) refined this analysis and recognised 164-167 ORFs and G+C content 57.2% in the genome of HCMV.

HCMV has a linear, double stranded DNA. It has two covalently linked unique segments, one long (UL) and the other short (US). Each of the unique regions is flanked by inverted repeats (TRL and IRL, TRS and IRS) (106) (Figure 1.2). The UL region is 166,972bp in size and contains 132 ORFs while, in the US region, there are 36 ORFs of 35,418bp in size. The terminal repeat long (TRL) resides at the 5'end of the UL region and at its 3'end, the inverted repeat long (IRL), collectively known as repeat long (RL) of 11,247bp containing 14 ORFs. The inverted repeat short (IRS) region and the terminal report short (TRS) are together designated as repeat short (RS) of 2,524bp with one ORF in the orientation opposite to the terminal long in the ends. "a" sequence of 578bp is found at both termini of the genome which is the part of RL and RS (30).

Murphy and colleagues (107) sequenced two HCMV laboratory strains (AD169 and Towne) and four HCMV clinical isolates (Toledo, FIX, PH and TR) and identified a total of 252 ORFs with potential to encode proteins that are conserved in all four clinical isolates. Dolan and colleagues (41) sequenced the 235,645 bp genome of a low passage strain (Merlin). Comparative analyses

with the published genome sequence of a high passage strain (AD169) showed that Merlin accurately reflects the wild-type complement of 165 genes.

The murine cytomegalovirus (MCMV) genome consists of a single unique sequence with short direct repeats (31bp) at either end (Figure 1.2). The G+C content of the genome is 59%, which is relatively high but similar to HCMV. The genome is 230,278bp in length and it encodes 170 ORFs; 78 of these have significant homology with HCMV (128). The homologues are found in the central conserved part of the MCMV genome. The designation M, e.g., M28, denotes murine genes with homology to those of HCMV; non homologous genes are labelled m, e.g. m29.1 (128).

The original MCMV genome sequence (128) has recently been reannotated and an additional 126 ORFs predicted (20). Tang and colleagues (170) reanalysed the MCMV genome sequence (128) and predicted 14 additional ORFs. The expression of seven of these ORFs was confirmed by either DNA microarray or RT-PCR analysis. Thus, the final number of ORFs expressed by MCMV has yet to be determined.

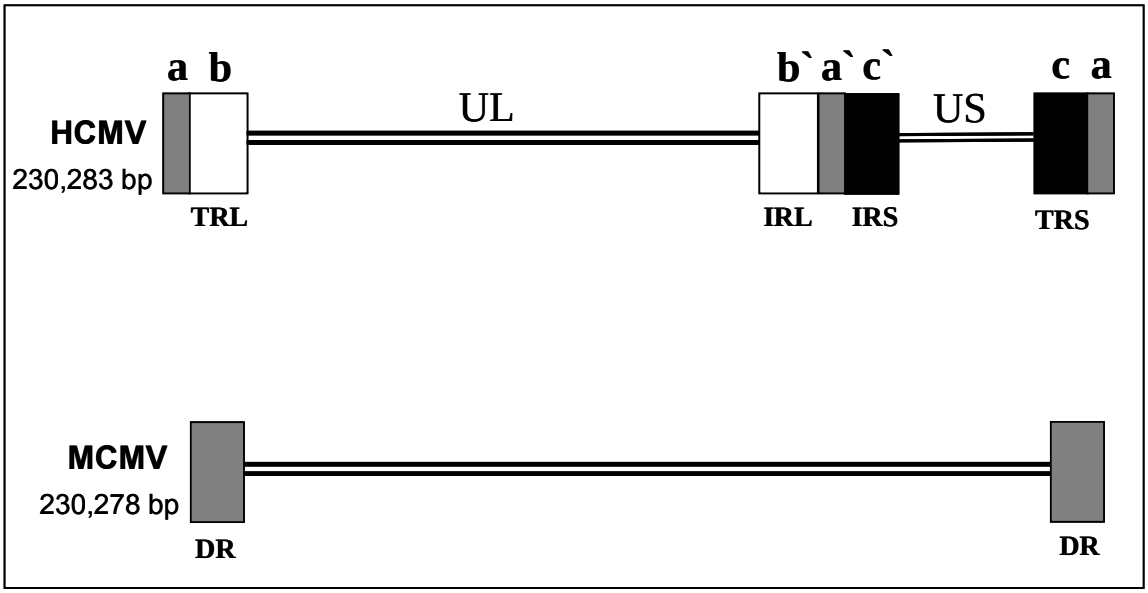


Figure 1.2 Schematic representation of the HCMV and MCMV genomes. Abbreviations: a and DR, direct repeat; a`, inverted repeat; TRL, terminal repeat long; TRS, terminal repeat short; IRL, inverted repeat long; IRS, inverted repeat short; UL, unique sequence long; US, unique sequence short; HCMV, human cytomegalovirus and MCMV, murine cytomegalovirus. Modified from Roizman and Pellet (135).

1.6 CMV replication

1.6.1 Entry

Major cell types including monocyte/macrophages, endothelial cells, epithelial cells, smooth muscle cells, fibroblasts, stromal cells, neuronal cells, neutrophils and hepatocytes are candidates for HCMV infection and capable of maintaining life-long infection within the host (65). Cell lines representative of these cell types are also susceptible to HCMV infection and membrane proteins of these cells bind HCMV. Nonetheless, other host cell factors govern

the viral penetration into these cells (114). Multiple receptors and ubiquitous molecules on the cell surface allow recognition and entrance of HCMV into the host. It utilizes several cellular and viral proteins for entry into such a wide range of cells. A model for HCMV entry (Figure 1.3) illustrates that HCMV infection begins with low affinity tethering to heparan sulphate proteoglycans (HSPGs) (35). Two viral glycoprotein complexes, the heterodimer gM/gN and the gB homodimer of the virion have heparan binding ability. This then leads to firm docking to epidermal growth factor receptors via gB (178). However, susceptible hematopoietic cells lack EGFRs, which argues for the existence of other HCMV receptors. Cellular integrins also interact with gB (33). Eventually the virus envelope fuses with the plasma membrane thus enabling the deposition of viral components in the cytoplasm. Membrane fusion requires the heterotrimeric envelope glycoprotein complex, gH/gL/gO.

1.6.2 CMV gene expression and regulation

Nucleocapsids released into the cytoplasm following membrane fusion are probably transported to the nucleus along microtubules to dock with the nuclear pore (51). DNA then enters the cell through nuclear pores and is transcribed. CMV genes are grouped into three families as they are temporally regulated and transcribed at immediate early (IE, α), early (E, β) or late (L, γ) times post infection (106). IE gene expression is independent of *de novo* protein synthesis, i.e. after the host cell is infected, they are transcribed within 0-4 hours in the presence of protein synthesis inhibitors such as cycloheximide. When the virus enters the host cell, some viral tegument

proteins are delivered along with the DNA to the nucleus and one is required to bind to host transcription factors required for IE gene expression. Expression of IE genes is required for E gene transcription. This latter phase occurs between 4-24 hours post infection. L genes are transcribed 12-48 hours post infection. The latter L genes can be further divided into early late (E-L or γ1) or true late (L or γ2), the latter being viral DNA synthesis dependent. E-L transcription may take place prior to viral DNA synthesis but generally is enhanced during the late stages of infection. True L gene expression takes place only after the onset of viral DNA synthesis (28).

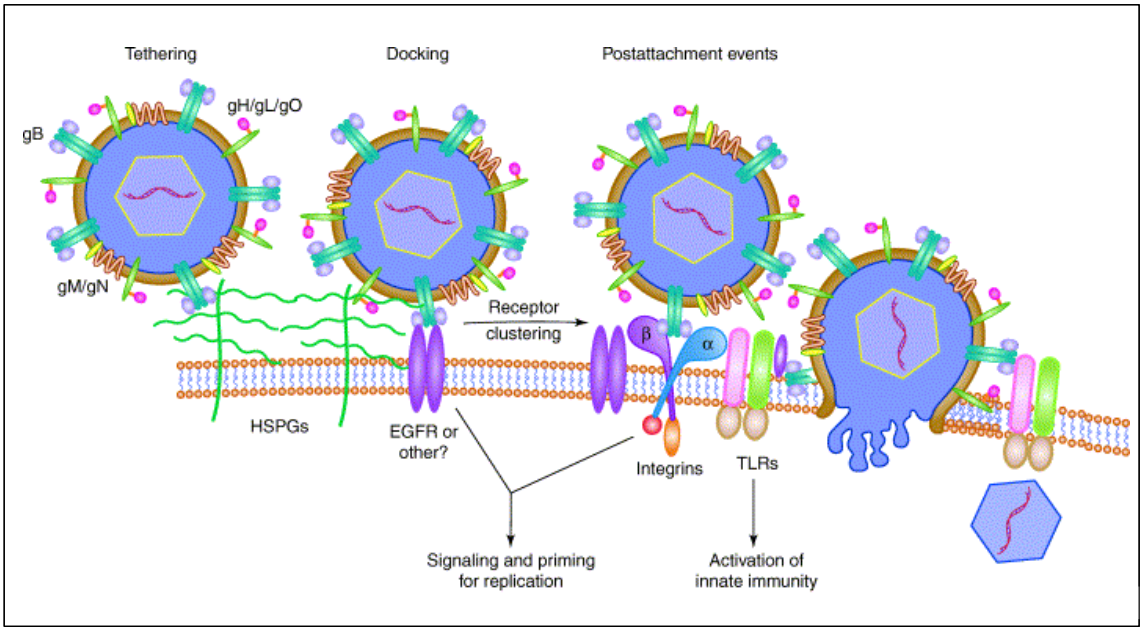


Figure 1.3 Model for HCMV entry. Initial attachment of HCMV in tethering interactions to heparan sulphate proteoglycans (HSPGs) through gM/gN and/or gB glycoproteins. A stable docking step allows gB to cooperate with the epidermal growth factor receptor (EGFR) in HCMV permissive cell types and other receptors in hematopoietic cells. HCMV envelope glycoproteins and cellular integrins allow receptor clustering and thereby activate fusion facilitating internalisation of virion components (33). Abbreviations: HCMV, Human cytomegalovirus and TLRs, Toll-like receptors.

1.6.2.1 Immediate early gene expression

IE genes are transcribed by the host cell machinery, immediately after entry of linearised double stranded viral DNA into the cell nucleus and they are independent of any viral gene expression. Transcription is mediated by the CMV major immediate-early promoter (MIEP) utilising host cell RNA polymerase II and the basal cellular transcription machinery. The MIEP is located downstream from a strong enhancer located between ~-50 and -550bp relative to the transcription start site. Host nuclear transcription factors bind at multiple binding sites on the enhancer and these 16-, 18-, 19- and 21-bp repeats play a central role in the regulation of expression. Two genetic elements designated IE1 and IE2 are under the control of the single MIEP (Figure 1.4). From these regions, at least three IE-RNA transcripts are produced. A single open reading frame (ORF) designated as UL123 initiating in exon 2 and continuing through exons 3 and 4, comprises the IE-RNA transcript, that ultimately encodes IE1 p72 (a phosphoprotein, MW 72 kDa). Two RNA transcripts of 2.25 and 1.7-kb are encoded by IE2. These transcripts are generated through differential splicing mechanisms in the IE2 region. Hence, three exons of IE1 are fused to IE2 region. The 1.7-kb mRNA encodes a 55-kDa protein while the other transcript encodes the 86-kDa (UL-122) protein. IE1 p72 acts as a transactivator of MIEP whereas IE2 p86 acts as a repressor to control expression from the MIEP. However, IE1 and IE2 are transcription factors for E gene promoters (99, 159). The products of the MCMV ie-1(m123) and ie-3(M122) genes are analogous to HCMV IE1 and IE2. These products act as transactivators and repressors respectively under the control of the MIEP promoter (75, 101). MCMV ie-2 encodes IE2 (1.7-kb) which

appears to have no function or sequence homology with the HCMV genome. This gene is transcribed by a separate promoter in the opposite direction to ie-1 and ie-3 (103) and deletion of ie-2 has no effect on virus replication *in vitro* or *in vivo* (27). Cytomegalovirus ie1 and ie2 have no homologues in other members of the herpesvirus family and this is a distinctive feature of CMV.

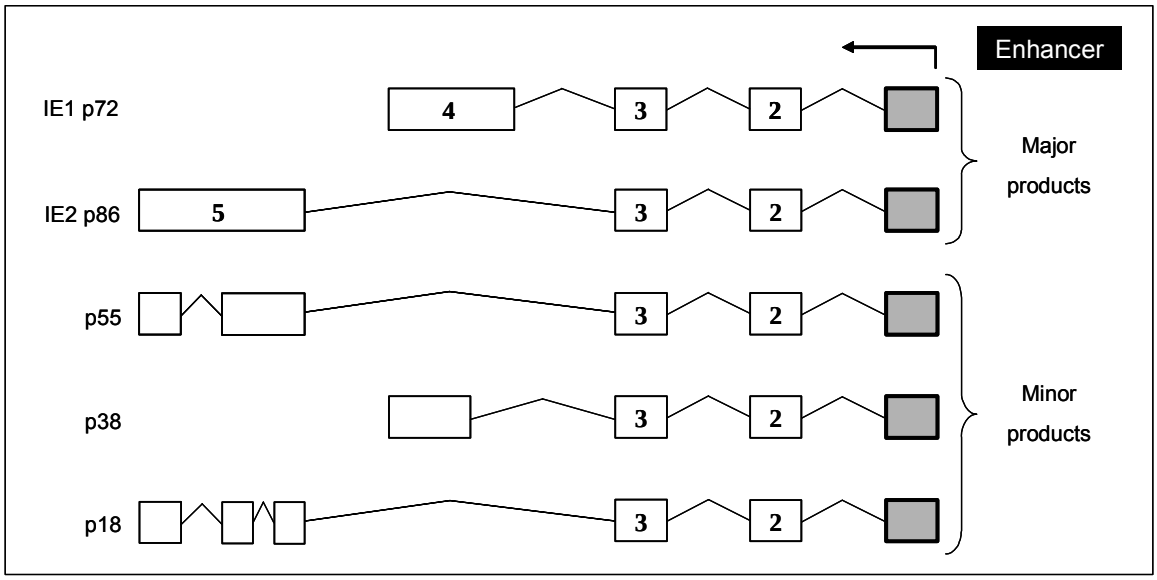


Figure 1.4 Probable organisation of multiple MIE gene products. In HCMV infected cells, IE1 p72 and IE2 p86 appears more noticeable. IE1 p72 is a 491 amino acid protein encoded by exons 2, 3 and 4 whereas IE2 p86 is a 579 amino acid protein corresponding to exons 2, 3 and 5. Minor RNA splice variants produce the p55, p38 and p18 proteins. All the major and minor products contain the same 5'-untranslated region (grey box) and are regulated by the enhancer (black box) (98).

1.6.2.2 Early gene expression

As described above, the products of IE genes, including IE1 and IE2, transactivate E promoters. During early gene expression, the machinery required for DNA replication is formed. These include the DNA polymerase (UL 54), helicase (UL 105), primase (UL70) and the associated proteins required for DNA synthesis (7). E genes are transcribed from all along the CMV genome. The E gene transcription region (UL112-113) lies in proximal vicinity to the IE transcription sites and this region has homology to the e1 transcription region (M112-113) found in MCMV. UL112-113 encodes a family of alternatively spliced RNAs that produce a series of related phosphoproteins (160). Products of both immediate early and early genes are responsible for the initiation of expression of the late gene set (γ1+ γ2) which ultimately produces viral structural proteins.

1.6.2.3 Late gene expression

The final phase of gene expression, designated late (L), begins with the onset of virion DNA replication. It is also accompanied by the synthesis of structural proteins and other proteins responsible for the packaging of the replicated viral DNA genome. E gene products activate the promoters of L genes facilitating the expression of these genes. E gene products are responsible for viral DNA replication but the L proteins are involved in the formation of mature virus particles. Some genes are designated as γ1 because these genes are transcribed initially during or before DNA synthesis and are not true L genes.

All the true L genes belong to the subset γ2. Some E genes, such as UL4 is expressed in both E and L phases of the virus replication cycle (164).

1.6.3 DNA replication and packaging

DNA replication occurs and the progeny virus particles are assembled in the late phase. HCMV DNA replication requires six herpesvirus-conserved replication-fork proteins; the DNA polymerase (UL54), a single stranded binding protein (UL57), polymerase accessory protein (UL44), and a three subunit helicase-primase complex (UL70, UL102, UL105) (106). The products of additional CMV genes (UL84, UL112, UL113, UL114) are also required for optimal replication (7, 126, 137). The unique replication origin (oriLyt) initiates binding of the replication complex and rolling cycle mechanism is followed for DNA synthesis.

HCMV DNA replication starts at approximately 16 hpi and peaks at 60-80 hpi. In the nucleus of permissive cells within 4 hpi, the HCMV genome circularises and replication generates concatemers late in infection. Most replicating viral DNA lacks terminal fragments although they are larger than genome length. Short conserved sequence elements at the genome termini, pac1 and pac2, direct the signal for the cleavage of the concatemers into unit length genomes and packaging of the CMV genome. The packaging proteins bind to pac2 sequences and direct the entrance of cleaved viral genomes into the capsid (95). In HSV-1, the viral genome is packaged into the capsid through a capsid protein encoded by UL6, called the vertex portal protein (113). CMV employs a

similar type of mechanism which is also conserved in bacteriophages. The homologous internal repeats in the HCMV genome permits US and UL regions to invert with respect to each other and therefore DNA is packaged into virions in any of the four isomeric forms. After a unit length linear DNA genome is packaged, mature nucleocapsids accumulate in the nucleus and gather at the inner nuclear membrane prior to budding into the perinuclear space. They also acquire matrix proteins and an envelope in the process.

1.6.4 Egress of viral progeny

Enveloped capsids in the perinuclear space fuse with the outer nuclear membrane and the bare nucleocapsid is released into the cytoplasm. The nucleocapsid again acquires both the matrix proteins and an envelope by budding into the Golgi. It is then transported inside a vesicle towards the cell membrane where the vesicle membrane fuses with cell membrane and the virion is released (66). Thus, it is predicted that the Golgi contributes towards the viral glycoproteins and matrix proteins are acquired in the cytoplasm.

1.7 Innate immunity to CMV

Entry of CMV into the host triggers strong anti-pathogen responses, which includes the activation of the innate immune system. Induction of interferon – α/β takes place after the recognition of virions by Toll-like receptors (TLRs). Over many years, it has been known that CMV elicits interferon-α/β, but the mechanisms of inducing such responses have been elucidated only recently.

Activation of the innate immune system takes place during binding and entry of virions into the host cells. It has been confirmed through transcriptional profiling studies that CMV induces inflammatory cytokines and interferon –α/β (22, 144, 189, 190). Transcriptional up-regulation of innate markers as well as activation of a variety of cell signalling pathways occur rapidly upon infection, including activation of NF-κB and the key transcriptional regulator of interferon-α/β interferon regulatory factor-3 (IRF3) (22, 112, 125, 183-185). The interferon-α/β response to viral infection takes place in two phases, an activation phase followed by an amplification phase (171, 172). The former phase commences with initial recognition of the virus, proliferation of intracellular signals and ends with the secretion of interferon-α/β (157). TLRs are one way in which cells can initiate these processes. Overall, TLRs function as general pathogen recognition receptors (PRRs) that detect and initiate immune responses to numerous pathogens (167). The important effects of TLR activation are inflammatory cytokine secretion, expression of immune co-stimulatory molecules, dendritic cell maturation, and for defined TLRs, the secretion of interferon-α/β (168). All these contribute to control of viral replication and initiate and modulate adaptive immune responses by B and T-cells.

TLRs are type I transmembrane glycoproteins with a cysteine and leucine-rich extracellular domain. They also possess a cytoplasmic tail with structural analogy to the IL-1 receptor, which is known as TIR (Toll/IL-1 receptor) (96). Akira and colleague (3) identified 12 members of the TLR family in mammals and these TLRs recognize pathogen-associated molecular patterns (PAMPs), different types of bacterial products such as lipoproteins, glycolipids,

lipopolysaccharides, peptidoglycans, flagellins and bacterial DNA. Viral DNA and RNA and viral envelope glycoproteins and endogeneous ligands, i.e Hsp60, are also recognized. Stimulation of TLRs activates the adaptor protein MyD88 (myeloid differentiation factor 88) that employs IRAK (IL-1R-associated kinase) and TRAF6 (tumor necrosis factor receptor associated factor 6) (97). Thus, NF-κB and MAPK (mitogen-activated protein kinase) are activated, proinflammatory cytokines are synthesized and co-stimulatory molecules CD80/CD86 are expressed (3). Compton (33) found a range of cells like macrophages, neutrophils and DCs manifest TLR expression. NK cells express TLRs (63) and absence of TLR signalling caused DCs to produce insufficient amounts of type I IFN and only partial NK cell activation (3).

Several groups have demonstrated a central role for TLRs in CMV infection (33, 79, 166). TLR2-dependent early innate immune activation was reported by Compton and colleagues (34). Nonetheless, this finding is in doubt as TLR2-deficient mice showed no defect in their response to MCMV infection (166). They further showed TLR3 signalling in Lps2 mice which have a non-sense mutation in their Trif gene. This gene encodes an essential adaptor molecule, that is required for MyD88- independent signalling downstream of TLR3 and TLR4. These mice were vulnerable to MCMV infection and did not produce type I IFN. Another type of TLR, TLR9, also plays a role in CMV infection. TLR9 recognizes double-stranded DNA unmethylated at CpG motifs (79, 166) and TLR9 knock-out mice showed a severely impaired NK cell response to MCMV infection and insufficient production of cytokines (166). Krug and colleagues (79) found similar results for DCs in that TLR 9-dependent

secretion of IFN-γ and IL-12 was found to be critical for the prevention of MCMV replication by Ly49H+ NK cells. These findings showed a clear role for TLR signalling in NK cell activation and control of CMV infection.

Once activated NK cells are attracted to the site of infection through the synergistic effect of cytokines such as IFN-α/β, IL-2, IL-12, IL-15 and IL-18 (11). Multiple activating receptors come into action to recognize infected cells and in the contrary, the receptors of self MHC class I molecules inhibit their action (81). NK cells also coordinate the regulation of the specific immune response between the innate and the adaptive immune system. NK cell activity is controlled through MHC class I expression and this prevents damage to healthy cells. NK cells have the potential to recognize infected cells expressing low levels of MHC class I molecules and ligands for activating receptors. NK cells process two types of receptors: inhibitory and stimulatory. Both types of receptor are classified into one of two families according to their chemical structures: Ig-like ectodomain receptors and C-type lectin-like domain receptors. NK cells express both types of receptor which may also be expressed on T cells (67, 127). An amino acid sequence motif is found in the cytoplasmic tail of inhibitory receptors, which is denoted as an immuno receptor tyrosine-based inhibitory motif (ITIM). Binding of ligands to those receptors leads to phosphorylation of tyrosines in ITIMs and subsequently NK cell activation is inhibited through activation of the tyrosine phosphatase SHP-1. Stimulatory receptors lack ITIMs but associate with adaptor molecules that contain an activating motif called immunoreceptor tyrosine-based activation motif (ITAM) or an YxxM motif (81).

A range of diverse ligands along with MHC class I molecules bind to activating receptors, though inhibitory NK cell receptors are specific for MHC class I molecules. So far, three types of NK cell receptor specific for MHC class I molecules have been identified and they are: killer cell Ig-like receptors (KIRs) in humans, Ly49 in rodents, and the CD94/NKG2 heterodimer expressed in humans and rodents. KIRs belong to the Ig superfamily and consist of two (2D) or three (3D) Ig domains and a long (L) or short (S) cytoplasmic tail (174). The long cytoplasmic tail contains ITIMs and act as inhibiting receptors whilst KIRs having short cytoplasmic tails are activating receptors that bind with the adaptor molecule DAP12. The Ly49 gene family is polymorphic and polygenic in nature and encodes both inhibitory and activating receptors. MHC class I molecules are directly recognized by both KIRs and Ly49 receptors and thus differentiate healthy cells from infected cells and are subjected to down-modulation of MHC class Ia molecules. The third type, the CD94/NKG2 heterodimer family is specific for MHC class I molecule expression (54).

NK cells undoubtedly play an important role in early control of CMV infection. Still there is some debate whether they are essential for this early control. Most laboratory mouse strains do not exert any NK cell response to CMV. Mouse strains are either susceptible (e.g. BALB/c mice) or resistant (e.g. C57BL/6 mice) to MCMV infection (138). A single dominant locus, designated as Cmv1, located in the NK gene complex on mouse chromosome 6 (40, 46, 138-140), controls the NK cell response and depletes MCMV titres in spleen and lungs. MCMV susceptible ($Cmv1^s$) mice show higher virus titres as NK cells are unable to control the infection at early times. Therefore, depletion of NK cells in

these mice has a negligible effect on virus titres. In contrast, resistant ($Cmv1^r$) mice can inhibit MCMV replication which in turn lowers virus titres and depletion of NK cells leads to high titres. Therefore, $Cmv1^s$ mice are susceptible to MCMV infection while $Cmv1^r$ mice are resistant. The $Cmv1^r$ (Ly49h) gene expresses the Ly49H receptor belonging to the Ly49H family of NK cell receptors (21, 36, 83) and is found on approximately half of the population of NK cells in C57BL/6 mice (151). Non-covalent interactions occur between Ly49H and the adaptor molecule DAP12 and an activation signal is passed into the cell through its ITAM (32, 150). Ly49H differs from other members of the Ly49 receptor family and binds to an MCMV encoded protein, the m157 gene product, while other members bind to MHC class I molecules (8, 152). Similar to a number of MCMV m145 gene family members, the m145 protein has some structural homology to MHC class I molecules. Thus, the Ly49H NK cell activation receptor is expressed in C57BL/6 mice conferring resistance to MCMV infection through the interaction between Ly49H and the m157 protein. Cells infected with a mutant virus in which the m157 gene was deleted, were not recognised by NK cells and thus this virus induced a severe infection *in vivo* (25). Therefore, mice devoid of the Ly49H receptor (BXD-8/Ty mice) and mice lacking $Ly49H^+$ NK cells are susceptible to MCMV infection and, as a result, removal of the m157 gene does not have any effect. Though the Cmv1 host resistance locus primarily controls MCMV infection through the NK cell response in the spleen, recent studies have shown that the Ly49H NK cell receptor can also control viral infection in the lungs (25).

NK cells may kill target cells either directly or indirectly. The direct mechanism employs cytolytic granule (perforin and granzyme inside) exocytosis, the Fas-Fas ligand pathway, the TNF-related apoptosis-inducing ligand (TRAIL) pathway, the membrane TNF-α pathway, and the antibody-dependent cellular cytotoxicity (ADCC) through CD16. Noncytolytic effects comprise the secretion of IFN-γ and TNF-α from NK cells (127).

It has been also found that low numbers of virus specific CD8+ T cells can protect an MCMV infected host. The major immunoevasion mechanism of CMV compromises the signalling via the NK cell receptor NKG2D, which is also a co-receptor on T cells.

1.8 The adaptive immune response

HCMV-specific CD8+- T cell mediated cytotoxicity is observed in virus infected cells isolated from the peripheral blood mononuclear cells (PBMCs) of normal healthy HCMV carriers (179). Identification of the major immediate-early protein as a major T-cell target in MCMV was followed by the discovery of human CD8+-T cells as a potential candidate for recognizing the homologous IE1 protein in HCMV (179). The lower matrix protein pp65, an HCMV T-cell antigen, can also be recognized by CD8+ T-cells from HCMV infected individuals (94, 177). Furthermore, it has been found that six different HCMV proteins expressed by recombinant vaccinia viruses are recognised by CD8+ T cells, but these are regarded as minor antigens (15). Specific virus carriers showed higher CD8+ T cell responses to IE1 proteins and were found to have

much higher frequencies of IE1 specific T-cells. These findings were similar to those CD8+ T cells specific for the pp65 protein, when individual donors were compared for cytotoxic T cell frequencies using HCMV-infected fibroblasts (73).

Increased disease incidence results from impaired T cell immunity during bone marrow transplantation or stem cell transplantation. Removal of reconstituted CD8+ cells in murine models of bone marrow transplantation leads to death and disease in immunocompromised mice can be prevented if reconstituted with CD8+ cells (130).

Bone marrow transplantation in MCMV infected mice initiates the reconstitution of CD8+ T-cells and resolves the productive infection although latency is established with a high level of silenced genomes. The antiviral CD8+T-cells can effectively lyse infected cells in all three temporal stages, i.e. IE, E and L, of viral gene expression (62, 123). These cells persist during the whole life span of such bone marrow transplant recipient mice with little decline in absolute numbers (123).

It is usual that high titres of antibody are able to reduce the prevalence and multiplication of infectious virus. Maternal antibody was thus found to protect the foetus against HCMV infection (16). Similarly, *in vivo* models have shown that neutralising antibodies play a role in the course of infection. Passive immunization with polyclonal antibodies obtained from immune donors was found to give protection prior to challenge infection. Both polyclonal antibodies

raised against MCMV and single envelope proteins expressed from recombinant virus were able to protect (91, 142). Injection of monoclonal antibodies specific for different structural proteins of MCMV also resulted in lower mortality (43). Furthermore, adoptive transfer of antisera to B-cell deficient mice were able to reduce MCMV titres to the same extent as immunocompetent mice and antiviral antibody prevented spread of virus to unaffected organs and confined the recurrent infection to the site of reactivation (68). But all these cases lacked a direct correlation between the level of protection and antibody neutralisation titres *in vitro*. Both ineffectual and potent monoclonal antibodies in regard to neutralisation of MCMV were able to offer similar protection *in vivo*. Although monoclonal antibodies were able to reduce MCMV titres in the livers of BALB/c and C57BL/10 mice showing close correlation with their neutralisation titres *in vitro*, they were still found incapable in reducing MCMV titres in spleen.

1.9 Latency and reactivation

HCMV persists as a lifelong infection in the normal human host without any noticeable clinical symptoms and is maintained in the absence of detectable infectious virus. Thus, it establishes a latent infection (146). It is still unclear how the virus remains in some cells without producing any further virus particles. Virus can reactivate in these cells upon certain external stimuli and produce new viral progeny to infect new cells (106).

The sites and mechanisms of HCMV latency are still poorly defined. Peripheral blood monocytes have been suggested as one site of latency in humans. Analysis of CD34+ bone marrow progenitor cells, precursors of monocytes, has revealed endogenous HCMV without viral IE gene expression (100). Similar results were found in the case of HCMV DNA, which is detectable predominantly in peripheral blood monocytes (PBM) of normal, seropositive individuals but IE gene expression was absent. Clinical isolates of HCMV have the capacity to efficiently infect monocytic cells at different developmental stages but the differentiation state of these cells determines the extent of viral gene expression. In normal individuals persistently infected with HCMV, the bone marrow may act as a reservoir for the virus. Following primary infection, HCMV may infect bone marrow stem cells with the capacity for self- renewal (105). In one study, it was found that granulocyte-macrophage progenitors (GM-Ps), progenitors of monocytes, granulocytes, and dendritic cells, expressed latency-associated transcripts (LATs), whereas mature macrophages, granulocytes, T cells, and B cells lack evidence of these transcripts. The differentiation state of GM-Ps appears critical in dictating whether latency is maintained or is reactivated. The progenitors of dendritic and myeloid lineage cells harbour latent virus in a similar manner to that of cultured GM-Ps (55). Again it was found that arterial endothelial cells harbour latently infected HCMV apart from the role of HCMV in governing the pathogenesis of atherosclerosis (59).

CMV replication in undifferentiated or unstimulated cells *in vitro* was compared with their differentiated or stimulated counterparts to establish a model of

latency and reactivation. In the case of non- differentiated cells, IE gene expression is down-regulated. It can be induced if some agents which initiate differentiation are employed. This result implies that if the major promoter of IE region is induced by host or viral transcription factors, productive infection will develop. So it was inferred by the investigators that reactivation was the predicted outcome of IE gene expression (72, 104). However, most latently infected cells express IE RNA and their protein products insufficiently. As a result E gene expression is not initiated. When reactivation is perturbed, IE gene expression may be up-regulated enabling production of some E gene products. Therefore, threshold levels of E RNAs accumulate in some cells, permitting DNA replication and L gene expression. As a consequence, a few infectious virions initiate infection in new cells leading to lytic cascades and generation of viral particles. In undifferentiated cells, modulator binding factor one (MBF1) apparently binds to the modular section of MIEP and represses transcription. Another repressor that also comes into action is transactivating binding protein (YY1) (86), which binds to the 21-bp repeat element of the MIEP enhancer and represses transcription from this region (76, 147). During lytic infection, virion associated proteins, such as the upper matrix protein (ppUL82), interact with host DNA binding proteins and initiate transcription from the MIEP. It is evident that these proteins are not available to carry out this function in latently infected cells. Acute inflammation and macrophage activation play vital roles in this regard. MIEP has cAMP response elements. Furthermore, prostaglandin E-2(PGE-2) can up-regulate the expression of MIEP, as determined by transient expression of the chloramphenicol acetyltransferase (CAT) gene under the control of the MIEP. PGE-2 induced

cAMP expression and simultaneously produced cytokines such as tumour necrosis factor (TNF)-α and interleukin-1 (IL-1)-β. TNF-α, IL-1 β, IL-6 and IL-10 act additively with PGE-2 in this effect (158). However, TNF-α has also been shown to inhibit CMV replication (5), so there should be a regulatory balance between these effects. There are other pathways in which cytokines may also activate the MIEP. An 18-bp repeat element in this region has four consensus binding sites for NF-kβ and this nuclear factor can activate the CMV promoter in a monocytic cell line and in mouse liver (88). These studies may therefore explain why immunosuppression is associated with CMV reactivation.

Two other factors may control virus reactivation: chromation remodelling and CD8+ cells coupled with virus immunoevasion. Silencing/desilencing of expression takes places by chromatin opening and it leads to reactivation of viral transcription. All essential genes must adopt an open viral genome chromatin structure together with local desilencing at the MIE locus. The dynamic opening and closing provide a suitable environment for the production of essential proteins to start the productive viral cycle. The immune system recognises new expressed membrane proteins or stops reactivating virus by killing infected cells or by inhibitory lymphokines. Specifically, the immunodominant IE1 epitopes are recognised by CD8+ T-cells in the earliest stage of viral transcriptional reactivation (145).

Reactivation from latency has also been investigated in the murine model. Latency is established in many organs and genes are silenced by histone binding to episomal viral DNA. It is estimated that 1 in 60,000 latent genomes

actively transcribe IE1 from the MIEP, as histone loss desilences this part of the genome. Following TNF-α mediated reactivation, expression proceeds to IE3 expression in approximately 1 in 10^4 genomes but still reactivation does not proceed to E or L gene expression without further immunosuppression suggesting that there are further check points mediated by CD8+ T cells. Cytotoxic T cells that recognise the IE1 nonapeptide probably keep the virus from reactivating. Possibly other CD8+ T cells with specificities for E and L gene products provide further checkpoints (145).

It is well established that cytomegaloviruses express gene products that help to evade immune recognition, so called immuno-evasions, and these may play an important role in virus reactivation. Hence, the pp37/40 protein encoded by the MCMV early m152 gene retains MHC class I molecules with bound IE1 peptide in the ER to be directed to lysosomes by the m06 gene products (gp48) and thence degraded. Failure of recognition of the IE1 nonapeptide by CD8+ T cells may allow virus to reactivate. Cells lacking MHC class I expression would be recognised by NK cells but expression of gp34 (the product of the m04 gene) transport MHC class I molecules devoid of IE1 peptide to the cell surface to inhibit NK cell killing. Similarly, mutation in the m157 protein, recognised by NK cells, prevents NK cell killing, while m145, m152 and m155 gene products inhibit expression of the NK cell activating ligands MULT-I, RAE-I and NKG2D respectively (58, 77, 87). Furthermore, the virus encodes an MHC class I homologue (m144) and Fc receptor to further avoid host defences (80, 173).

1.10 Treatment and prevention of HCMV infection

Nucleoside analogues have been used over the years to inhibit viral infections. These nucleoside analogues target viral DNA polymerases or reverse transcriptases in host cells. One nucleoside analogue, ganciclovir (GCV), an acyclic nucleoside analogue of 2'-deoxyguanosine, is used for treatment of HCMV (Table 1.2). It is converted into ganciclovir triphosphate, its active form, by both viral and cellular enzymes. CMV UL97 encodes a protein kinase which catalyzes the initial phosphorylation of ganciclovir, the other two phosphates are added by host enzymes (163). The triphosphate competes with dGTP and inhibits DNA synthesis by the UL 54 encoded viral DNA polymerase. Other antiviral drugs e.g. valganciclovir, foscarnet, cidofovir, acyclovir, fomivirsen, have also been used for the treatment of CMV (12). Their mode of action is more or less similar encompassing the inhibition of viral DNA synthesis.

The nucleoside analogues show detrimental side effects. They have proved to have an unfavourable safety profile, with severe acute and long-term toxicities. Haematologic abnormalities including neutropaenia, anaemia thrombocytopaenia and reproductive toxicity and carcinogenicity are reported on preclinical toxicological studies (12). Poor bioavailabilty and solubility limit the use of nucleosidic anti-viral drugs (38) to treat CMV in solid organ transplant patients and haematopoietic stem cell transplant recipients. There is still some concern as to whether prophylactic or pre-emptive therapy should be used. The cell mediated immune response is reported to be affected in prophylactic therapies (14, 84, 148). A guide line has been established by the

International Herpes Management Forum as to when these anti-viral drugs can be used in either therapies in these high risk patients (129).

The non-nucleosidic drug, foscarnet (FOS), binds to the pyrophosphate binding site and inhibits the activity of the viral DNA polymerase. Then, it blocks the cleavage of pyrophosphate from the terminal nucleoside triphosphate added to the growing DNA. As this drug is administered for the treatment for CMV retinitis in AIDS patients, the major dose-limiting toxicity causes renal impairment which emphasizes the utility of adequate hydration and regular monitoring of serum creatine levels in these patients. Renal impairment can cause mineral and electrolyte abnormalities leading to a number of cardiac or neurologic disorders, including seizures and in extreme cases, death (12). In the patients with failed GCV therapy due to viral resistance, or those who can not be treated with GCV therapy due to dose-limiting neutropenia or leucopenia, FOS is considered as second-line therapy (129). CMV can be treated with fomivirsen, a 21-nucleotide anti-sense RNA. It specifically binds to mRNA expressed from the major immediate-early transcriptional unit of CMV. Ocular inflammation (uveitis) is one of the most frequent adverse effects of fomivirsen (12).

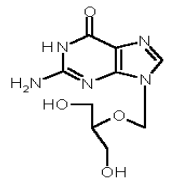
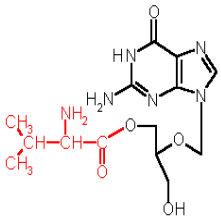
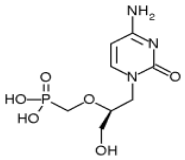
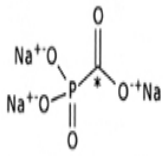
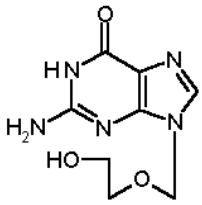
Several anti-CMV drugs are in clinical development, especially for congenitally infected neonates. These drugs are designed to be potent, selective and bioavailable. Marivabir (1-(β-L-ribofuranosyl)-2-isopropylamino-5,6-dichlorobenzimidazole) prevents replication in cell culture (13, 186) by inhibiting viral nucleocapsid egress from the nucleus (78). Other drugs

including BAY 38-4766, GW275175X and Cidofovir esters are presently undergoing clinical trials and are expected to be free from toxicity and other associated problems of the previous described nucleoside analogue drugs (12).

A number of approaches have been taken to develop a suitable vaccine to prevent CMV infection. The attenuated Towne strain was used as a vaccine in both healthy immunocompetent and CMV positive and CMV negative renal transplant patients. In CMV positive patients, a virus-specific cellular immunity was present for over ten years post immunization (122) but in the latter case, i.e. CMV negative recipients, the attenuated virus was unable to induce an immune response capable of preventing re-infection but it did show some decrease in severity of infection (121).

This disappointing finding led to the development of a recombinant subunit vaccine. Naturally infected patients produce a neutralising antibody response to glycoprotein B (gB). Thus, incorporation of this glycoprotein into a baculovirus and its subsequent expression in chinese hamster ovary cell lines enabled it to be a potential candidate for a subunit vaccine (74). Another approach utilized a canarypox virus recombinant expressing gB. This has shown promise in that an antibody response to gB and neutralising antibodies were induced by this vaccine in humans (50). Similarly, DNA plasmids expressing CMV genes (gB, pp65) have shown excellent results in mouse models (116).

Table 1.2 Structure of the drugs approved today for the therapy of HCMV

Structure	Compound	Clinical usage
	Ganciclovir (GCV, DHPG) - guanosine analogue	•Therapy of HCMV disease •preemptive therapy of active HCMV infection •prophylaxis of active HCMV infection and disease in immunosuppressed patients
	Valganciclovir (ValGCV) -guanosine analogue, L-valinester of GCV	•Therapy of HCMV disease •prophylaxis of active HCMV infection and disease in immunosuppressed patients
	Cidofovir (CDV, HPMPC) - acylic cytosine analogue	•Therapy of HCMV disease (second line) • Especially for GCV-resistant HCMV due to UL97 mutation
5`-GCGTTTG CTCTTCTTC TTGCG-3`	Fomivirsen (ISIS2922) – antisense phosphorothioate oligonucleotide	•Therapy of HCMV retinitis in HCMV patients. For GCV- and PFA-resistant HCMV
	Foscarnet (PFA) – pyrophosphate analogue	•Therapy of HCMV diseases • Especially for GCV-resistant HCMV due to UL97 mutation, •prophylaxis of active HCMV infection and disease in immunosuppressed patients
	Acyclovir (ACV) - guanosine analogue	•Therapy of diseases with HSV and VZV • Prophylaxis of active HSV and VZV infections • Prophylaxis of HCMV infections in solid organ transplantation?

1.11 MCMV as model of HCMV infection

CMV is species specific. HCMV can replicate inside human hosts and cause persistent infections. But other animal species are not permissible for HCMV infection. However, MCMV in mice has been proved to be a useful model for HCMV disease. This virus can cause acute, latent and persistent infection of its natural host. Also the pathogenesis of these two viruses is closely related and shows similar clinical syndromes. Sequencing of these two viruses has revealed analogy in genome architecture, expression and function. Analysis of the complete nucleotide sequence of MCMV has depicted that more than 75 ORFs have significant homology to those of HCMV (30, 128). Thus, elucidation of mechanisms of MCMV expression will be useful to provide insight into functions of HCMV in infection and pathogenesis.

1.12 Mutagenesis approaches

Mutagenesis is a powerful tool for studying the function of virus-encoded genes. The viral genome is subjected to mutation and viral mutants are screened in both tissue culture and animals for possible growth defects or functional loss *in vitro* and/or *in vivo*. Several mutagenesis strategies have been adopted over the years for fast, efficient and productive manipulation of the viral genome (23). Chemical mutagens were used for the production of temperature sensitive (ts) mutants around 30 years ago (141) and later this approach was adopted to produce mutants of MCMV (2, 136). Large numbers of mutants can be produced by this method which induces random point

mutations, insertions or deletions but has the disadvantage of being laborious and technically difficult to identify the mutations responsible for the observed phenotype.

Site directed mutagenesis, a method by which targeted mutation of individual genes is achieved, was developed for members of the herpes virus family. This strategy adopts the recombination and repair machinery of bacterial cells together with selectable markers. Though mutation is very much specific, purification still remains laborious and sometimes impossible because the mutant has a poor growth rate as compared to wild type virus (23).

Herpesvirus genomes have been cloned as a series of overlapping cosmid clones facilitating efficient manipulation of the cloned segment. The targeted mutation can be introduced into a cloned sequence and, following transfection of cosmid DNA into the tissue culture cells of choice, a generally homogeneous population of mutant virus is generated by homologous recombination of overlapping DNA segments (42, 71). The advantage of this methodology is the generation of mutant virus only, there is no requirement of selection against wild type virus and it precludes the presence of any foreign DNA (selectable marker) (93). Finding suitable restriction sites and overlapping mutated regions in long cosmids may give rise to difficulty. However, apart from these, many recombination events are necessary for the assembly of the full-length viral genome in the transfected cells.

Cloning vectors have been developed based on replicons of bacterial F plasmids, which are capable of maintaining large DNA fragments of up to 300kbp, named BAC (Bacterial artificial chromosome). In a single BAC, the largest herpes virus genome can be easily cloned and it retains the power of infection in permissive cells. The clone is maintained stably and is easy to handle. This breakthrough led to a new era of mutagenesis of large virus genomes. The methods of genetic engineering in prokaryotes could now be directed to mutate the BAC efficiently. With the help of this technology, alteration of viral genes and their functional analyses can be done more quickly in an efficient manner (23). The MCMV genome was first cloned and maintained as 230 Kbp BAC in *E. coli* (102, 176). Similarly, this technique was employed to clone the other human and animal CMV genomes as infectious BACs in *E. coli* (Table 1.3) (24). The BAC cassette controls the replication of the BAC plasmid in *E .coli* in low copy number. It is inserted into the viral genome by homologous recombination in eukaryotic cells. The introduced viral flanking regions at each end of the BAC cassette govern the recombination event. Recombination deficient *E. coli* is then transformed with circular intermediates of viral genomes containing the BAC cassette. Simultaneously, the viral genome can be subjected to any kind of mutation (deletion, insertion or point mutation) or reversion of the mutation with the methods of bacterial genetics. Following this event in *E. coli*, the viral BAC plasmid can be used to produce infectious virus particles in tissue culture.

Table 1.3 Cytomegaloviruses cloned as BACs in *E. coli*

Virus, Strain	Full length	BAC excisable	Reference
MCMV, Smith	No	No	(102)
MCMV, Smith	Yes	Yes	(176)
MCMV, K181	Yes	Yes	(131)
HCMV, AD169	No	No	(17)
HCMV, AD169	Yes	Yes	(61)
HCMV, AD169	Yes	Yes	(182)
HCMV, Towne $_{ATCC}$	No	No	(92)
HCMV, Towne $_{RIT}$	No	No	(57)
HCMV, Towne $_{LONG}$	No	No	(57)
HCMV, Toledo	No	No	(57)
HCMV, FIX	No	No	(56)
HCMV, PH	No	No	(107)
HCMV, TR	No	No	(107)
GPCMV, 22122	Yes	No	(93)
RhCMV, 68-1	Yes	Yes	(29)

MCMV, Murine cytomegalovirus; HCMV, Human cytomegalovirus
GPCMV, Guinea pig cytomegalovirus; RhCMV, Rhesus Cytomegalovirus

A novel approach, ET recombination, has been employed to modify the BAC plasmids in the host (110, 187). This method is independent of the presence of restriction sites and the size of the DNA molecule to be modified and based on the homologous recombination mediated by RecE and RecT proteins (Figure 1.5). RecE proteins are exonucleases which have 5'-3'activity. It degrades the DNA in 5'-3'direction starting from a double-stranded break. RecT proteins are DNA annealing proteins. These proteins bind to the single stranded DNA and form a recombinogenic proteonucleic filament. A functional interaction between these two proteins catalyzes homologous recombination. Strategies involving ET recombination and two rounds of BAC mutagenesis (24) are illustrated in Figure 1.6. In a two-step approach, a PCR product carrying selectable and counter-selectable marker genes is first introduced at the location to be modified and in the second step replaced by non-selectable DNA carrying mutation (108, 188). This approach can be used to generate a second-round

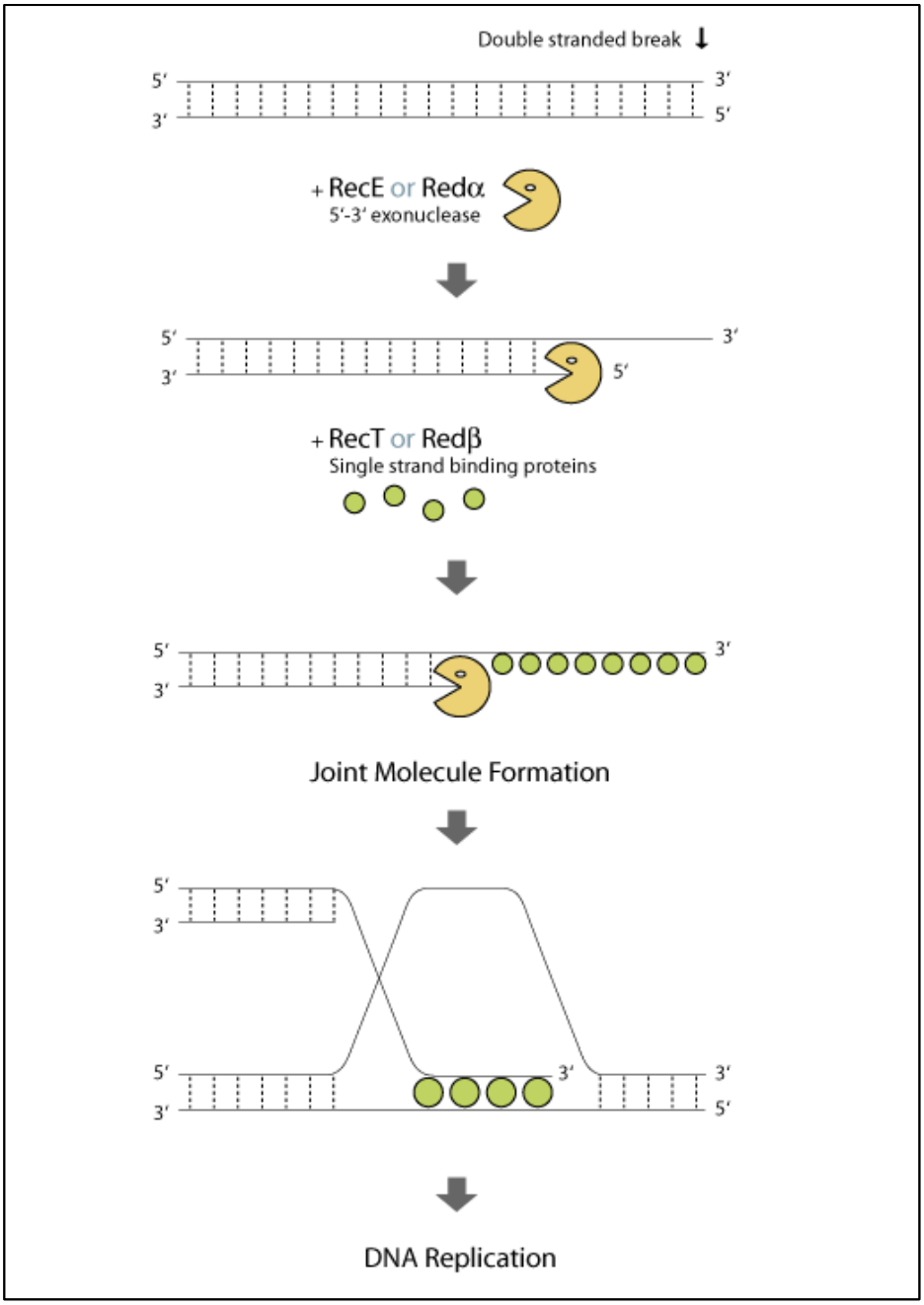


Figure 1.5 Mechanism of Red/ET recombination. Recombinase protein pairs (RecE/RecT or Redα/Redβ) starts double-stranded break repair. Here, RecE (Redα) digest one strand of the DNA from the double-stranded break, leaving the other strand as a 3` protruding end. RecT (Redβ) recognises and coats the DNA overhang to form the protein-nucleic acid filament aligning with homologous DNA. Then the 3` end acts as primer for DNA replication (109).

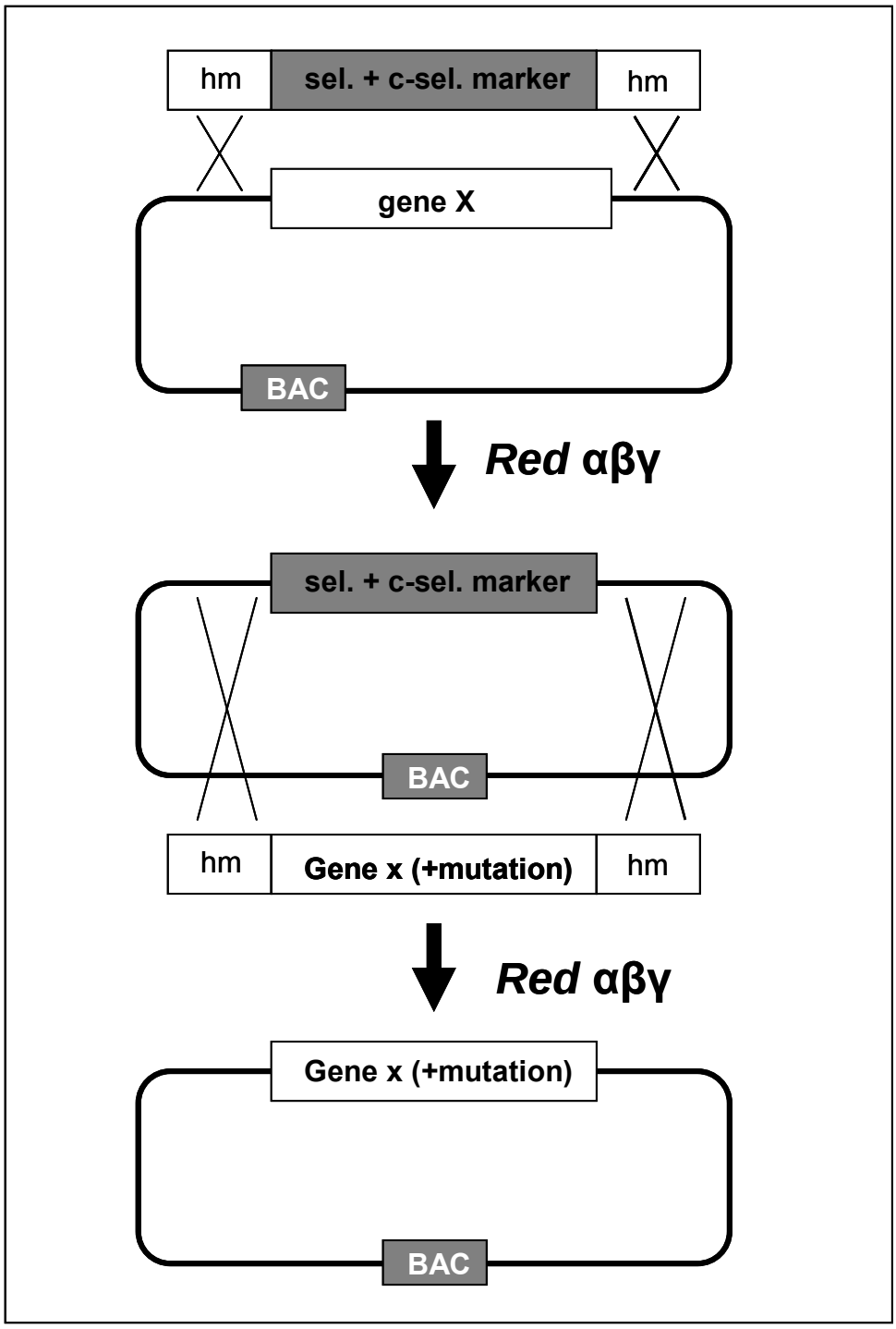


Figure 1.6 BAC mutagenesis using linear DNA fragments. A viral gene (X) is first replaced by a selectable (sel) and a counter-selectable (c-sel) marker cassette flanked by two homology arms (hm). In a second step, the marker cassette is replaced either by the wild type or a mutated gene sequence (24).

product that includes the intended sequence change (s) or insertion of DNA region of interest completely free of any operational sequences used for the engineering. If oligonucleotides and short PCR products are used, absolute ET recombination efficiencies may approach 1 in 100. Hence, the correct recombinants can be confirmed by PCR (111).

1.13 Aim

The aim of the project is to determine the role of the m29 and m29.1 ORFs in MCMV replication both *in vitro* in tissue culture and *in vivo* in immunocompetent and immunodeficient mice. Both ORFs are located in the *Hind*III-B (Figure 1.7) region of MCMV genome. This 26.4kb long MCMV *Hind*III-B region as defined by Rawlinson and colleagues(128) consists of 18 ORFs, of which 11 have HCMV homologues.

While MCMV homologues of HCMV ORFs are actively being pursued by a number of groups, ORFs unique to MCMV with little or no sequence homology to HCMV or other herpesvirus have been relatively neglected despite the fact that several of these (e.g. m04, m06, m152, m155, m157) are functional HCMV homologues involved in immune evasion. Approximately 90 of the MCMV ORFs have no sequence homology with HCMV ORFs but may have important functions for virus replication in tissue culture. More likely, such genes may be dispensable for replication in cultured cells and may function to modulate the interaction between the virus and its host. The role of these genes will never be understood if HCMV homologues only are targeted.

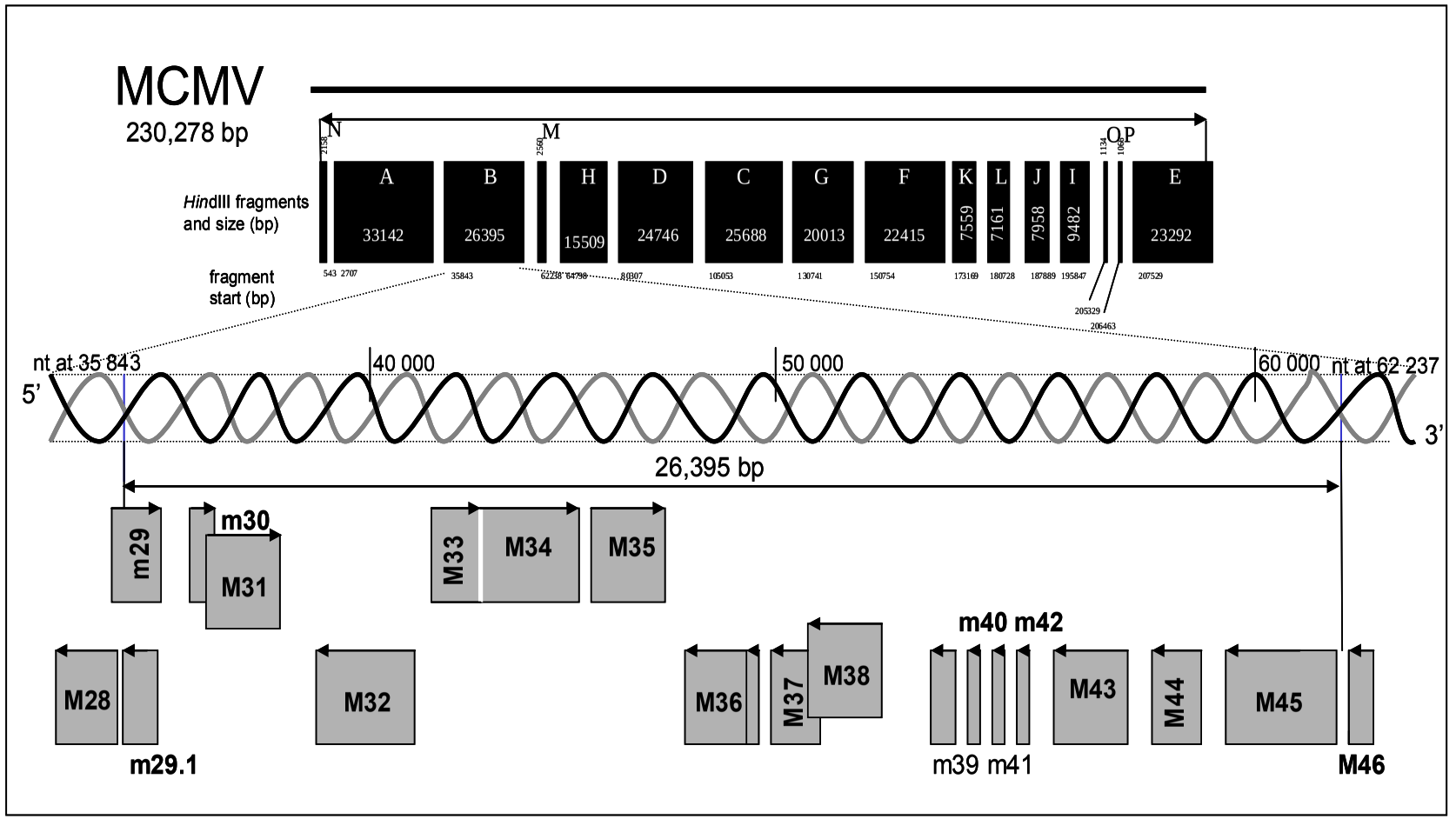


Figure 1.7 A probable map of MCMV and *Hind*III digested MCMV (Smith strain) genome giving emphasis to the *Hind*III-B fragment. The *Hind*III fragments are labelled A to P according to their size. The transcription orientation of the ORFs is represented by an arrow. Abbreviations: MCMV, murine cytomegalovirus and nt, nucleotide. Based on Rawlinson, 1996 (128).

The m29 and m29.1 ORFs were previously mutated by Dr. Melissa Kirby in this Laboratory. These mutants were constructed, using RecE/RecT homologous recombination, by insertion of 1.3 Kb kanamycin cassette at the restriction enzyme site *Bam*HI of m29 and *Sfo*I of m29.1 and confirmed through sequencing. However, she was unable to produce revertants to wt which necessitated a different strategy. Furthermore, early indicators were that the published sequence was incorrect. A selection/counterselection system using the established RecE/RecT recombination method (110, 187) allowed mutants to be selected through kanamycin resistance and a wt revertant produced in a similar manner with selection for streptomycin resistance. As indicated in Figure 1.7, m29 and m29.1 occur on different strands of DNA in overlapping reading frames making it difficult to knock out each gene independently using transposon mutagenesis or insertion of an antibiotic cassette. Using the above approach, it was intended to generate stop codon mutants and revertants of m29 and m29.1 ORFs.

Infectious mutant viruses will be generated following transfection of NIH 3T3 cells with MCMV BAC DNA. The genome of the reconstructed virus will be investigated by sequencing to confirm the desired mutation and by PCR to confirm the presence and absence of the BAC cassette. Both mutant and revertant viruses will be characterised phenotypically *in vitro* by examining replication at high and low MOI in primary mouse embryo fibroblasts and *in vivo* in adult immunocompetent and SCID mice. Temporal expression of the transcripts will be examined and the 5' and 3' end of the transcripts determined

by RACE analysis. Protein will be expressed in *E. coli* and antibodies developed in rabbits to examine whether transcripts are translated.

2. MATERIALS AND METHODS

2.1 Maintenance of cell lines

2.1.1 NIH 3T3 cells

The immortalised mouse embryo fibroblast cell line, NIH 3T3 (CRL-1658), was purchased from the American Type Culture Collection (ATCC) (Middlesex, UK) and propagated in growth medium (GM) comprising Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, Dorset, England) supplemented with 10% (v/v) newborn calf serum (NCS) (Cambrex, Nottingham, UK), 2% L-glutamine (Invitrogen, Paisley, UK) and 2% Penicillin-Streptomycin (Invitrogen, Paisley, UK).

2.1.2 Isolation and maintenance of primary mouse embryo fibroblasts

Mouse embryo fibroblasts (MEF) were aseptically isolated from foetuses of 14-day-old CD-1 mice. Embryos were decapitated, washed twice with 25ml phosphate buffer saline (PBS) (Oxoid, Hampshire, England) and forced through a 20ml syringe to remove foetal membranes and excess fluids. 10ml of Trypsin:EDTA (0.5% trypsin; 5.3mM EDTA) (TE) (Invitrogen, Paisley, UK) was added to the cells, which were shaken and incubated for 10 minutes at 37°C. The digested sample was mixed by rigorous shaking and filtered through a fine

metal sieve. 5ml of NCS and 10ml DMEM was added and the homogenate placed in a universal. After centrifugation at 720xg for 10 minutes at room temperature, the pellet was resuspended in 10ml of GM and seeded into a 162cm^2 tissue culture flask (Corning Incorporated, USA) containing 40ml of GM and incubated at 37°C overnight with 5%CO_2/95% air. The following day GM and non-adherent cells were removed from the flask and attached cells were washed with PBS and 40ml of GM was added. The cells were cultured at 37°C and passaged when the cells were grown until 90-95% confluent.

2.1.3 Subculturing of cells

Cells were subcultured at 90-95% confluence. GM was removed from the 162cm^2 tissue culture flask and the cells washed with 10ml of PBS. The cells were rinsed with 2ml of TE and incubated at 37ºC for 1 minute. The cells were observed using an inverted microscope and, after detachment, 10ml of GM was added to neutralize the effect of TE. These cells were seeded into a 162cm^2 tissue culture flask at a split ratio of 1:2 to 1:6 containing 40ml GM, cultured at 37°C until 80-90% confluent and passaged as required.

2.1.4 Long term storage of NIH 3T3 cells

The cells in 100% cell confluent 162cm^2 tissue culture flasks were trypsinised as described above (section 2.1.3). Trypsinised cells were harvested by centrifugation at 750xg for 10 minutes at room temperature. The cell pellet was resuspended in 3.6ml NCS. 0.4ml of tissue culture grade dimethyl sulphoxide

(DMSO) (Sigma, Dorset, England) was added to the resuspended cells, mixed and the cells were aliquoted (1ml) into cryovials (Nalgene, Hereford, UK). The cryovials were placed in a freezing vessel containing isopropanol and incubated at -80°C. After 24 hours, the cryovials were moved to liquid nitrogen for permanent storage.

2.1.5 Resuscitation of NIH 3T3 cells

Cells stored in liquid nitrogen were defrosted at room temperature and added to a 25cm^2 tissue culture flask (Corning Incorporated, USA). 3ml of GM was added to the flask and incubated at 37ºC. After 24 hours, the GM was removed and fresh GM was added. Cells were incubated for several days until 80-90% confluent. Trypsinised cells were then passaged into a 75cm^2 tissue culture flask (Corning Incorporated, USA) and subsequently into 162cm^2 tissue culture flasks as required.

2.2 Viruses

The reconstructed virus from MCMV smith BAC plasmid (176) was provided by Dr. Melissa Kirby for this study. Two kanamycin cassette insertional mutant viruses of MCMV Smith strain, designated as Kn29 and Kn29.1 in this study, were kindly provided by Dr. Melissa Kirby. Wild type viruses and reconstructed viruses used in this study are shown in Table 2.1.

2.2.1 Transfection of MCMV BAC plasmids into NIH 3T3 cells

ExGen500 *in vitro* transfection reagent (Fermentas, York, UK) and a modified method recommended by the manufacturer was applied. ExGen500 is a sterile non-pyrogenic solution of linear 22 kDa polyethylenimine in water; it belongs to an efficient new class of non-viral, non-liposomal gene delivery reagents. 4x105 NIH 3T3 cells were seeded per well of a 6-well flat bottomed plate (Corning Incorporated, USA) in 3ml of GM 12-16 hours before the transfection. At transfection, the cell monolayers were 70-80% confluent. 150mM NaCl was added to a 1.5ml eppendorf tube containing 5μg BAC plasmid DNA (section 2.4.6) to make a final volume of 300μl. 15.5μl of ExGene500 reagent was added and the solution mixed for 10 seconds. 300μl of the ExGen500/DNA mixture was then added to each well. The control well contained only 150mM NaCl and ExGene500 reagent. The plate was gently rocked back and forth and from side to side to achieve even distribution. The cells were then incubated at 37°C in a gassed incubator for 20-48 hours and then subcultured in a 162cm^2 tissue culture flask at 100% confluence (section 2.1.3). Formation of plaques was monitored using an inverted microscope. If no plaques were present by this time, the cells were passaged and incubated for several weeks at 37°C (section 2.1.3).

Table 2.1 Wild type and reconstructed viruses used in this study

Virus name	Description	Source	Reference
wt	Reconstructed from MCMV Smith BAC	M. Kirby	(176)
Kn29	Reconstructed from mutated MCMV Smith BAC, insertional mutant of m29 gene	M. Kirby	This work
Kn29.1	Reconstructed from mutated MCMV Smith BAC, insertional mutant of m29.1 gene	M. Kirby	This work
Rc29	Reconstructed from mutated MCMV Smith BAC, stop codon mutant of m29 gene	This work	
Rc29.1	Reconstructed from mutated MCMV Smith BAC, stop codon mutant of m29.1 gene	This work	
Rv29.1	Reconstructed from mutated MCMV Smith BAC, revertant of Rc29.1mutant	This work	
K181	mouse passaged, wild-type	CA Mims[2]	(136)
K17A	Wild-type MCMV isolate	GR Shellam[1]	(89)
N1	Wild-type MCMV isolate	GR Shellam[1]	(89)
G4	Wild-type MCMV isolate	GR Shellam[1]	(89)

1. Department of Microbiology, University of Western Australia
2. Department of Microbiology, United Medical and Dental Schools of Guy's and St Thomas Hospital, London.

2.2.2 Plaque purification of BAC derived virus

NIH 3T3 cells were seeded at 1 x 10^6 cells in 3ml of GM into each well of 6-well flat bottomed plates and cultured overnight at 37°C. Harvested supernatants from the transfection reactions were serially diluted 10 fold in GM. Following removal of GM from the 6 well plates, 20μl of individual dilutions were added to a single well. The cells were incubated at 37°C and plaque formation was monitored using an inverted microscope. Several distinct plaques were found in several wells after 4 to 5 days of infection. The GM was removed from these wells and, using the inverted microscope, an area was found that contained a single plaque. A P200 Gilson tip was used to remove the plaque and the surrounding cells which were added to 2ml maintenance medium (MM) (2% NCS, 2% L-glutamine and 2% Penicillin-Streptomycin in DMEM) in a bijou container. 100μl of medium was taken, flushed over the area and added to the

Bijou. A flask of nearly confluent NIH 3T3 cells was prepared for each plaque by removing most of the GM. 2ml MM containing the isolated plaque contents were added to each flask containing cells and incubated for 1 hour at 37°C. A further 2-5ml GM was added so that the monolayer was well covered. The flask was incubated at 37°C until 100% cytopathic effect (CPE) was observed. The viruses were harvested (section 2.2.3) and the cell pellet used for DNA extraction (section 2.2.7). The virus from this stage was designated as 1st passage stock. Several passages were required to recover virus free of the BAC. Aliquots of BAC free plaque purified virus stock were used for generation of viral seed and working stocks.

2.2.3 Virus harvesting

NIH 3T3 cells were seeded in 162cm^2 tissue culture flasks in 40ml of GM and cultured overnight at 37°C. The GM was removed from the flask when the cell confluence was 80 to 100%. 0.2ml of virus was added to the flask, incubated at 37ºC for one hour and then supplemented with 15ml of MM. When the cell monolayer showed 100% CPE the medium was transferred to a centrifuge tube. The cells were then detached using a cell scraper and transferred back to the centrifuge tube. This was then centrifuged at 750xg for 10 minutes at room temperature and the supernatant containing the viral stock was then supplemented with 10% DMSO and stored at -80ºC. The pellet was kept for DNA extraction (section 2.2.7).

2.2.4 Production of viral seed and working stocks

Seed stocks were generated from plaque purified virus (section 2.2.2) once it was demonstrated that the BAC cassette had been eliminated from the virus genome. NIH 3T3 cells were seeded into 162cm^2 tissue culture flask and the virus harvested and stored as described in section 2.2.3. Working stocks were generated from viral seed stock. MEF cells were seeded into 162cm^2 tissue culture flask and the virus harvested and stored as described in section 2.2.3. The virus titre was determined by plaque assay (section 2.2.6).

2.2.5 Growth kinetics of recombinant viruses

MEF cells were seeded at a concentration 1x10^5 cell in 0.5ml of GM into each well of a 24 well tissue culture plate (Corning Incorporated, USA). Cells were allowed to adhere overnight at 37ºC to establish a confluent monolayer after attachment. The cells were then infected at an MOI of either 0.05 (low) or 5.0 (high) plaque forming units (PFU) per cell. After one hour incubation at 37 ºC, the culture medium was removed, the cells washed with PBS, and 900μl of GM added to each well. The cells were incubated at 37ºC and at different time points, three 450μl aliquots were collected from each well and stored at -80°C. When required, the samples were thawed and viral titres were determined by plaque assay (section 2.2.6).

2.2.6 Virus titration

A plaque assay with MEF cells was used to determine viral titres. The amount of virus present in viral stocks (section 2.2.4), tissue culture supernatants (section 2.2.5), is expressed in PFU per ml and virus present in mouse tissue homogenates (section 2.15) is expressed as PFU per tissue.

MEF cells were seeded at $1x10^5$ cells in 0.5ml of GM into each well of a 24 well tissue culture plate. Cells were allowed to adhere overnight at 37ºC. The GM 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 GM] was added to each well. After 5 days incubation at 37ºC, the cells were fixed and stained. 0.5ml of formal saline fixative (4% formaldehyde in PBS) was added directly to each well, removed after 10 minutes and another 0.5 ml of fixative added. Following incubation at room temperature for 30 minutes the fixative was removed and 0.5 - 1.0ml of 0.3% crystal violet solution [0.3% (w/v) crystal violet dissolved in 10% (v/v) methanol in water] added to each well. The plates were left for 1 hour at room temperature. 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.

2.2.7 Isolation of viral DNA from cultured animal cells

Viral DNA was isolated from infected cells using the DNeasy Tissue Kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions. Briefly, virus infected attached and lysed cells were harvested from 162cm^2 tissue culture flasks using a cell scraper and pelleted by centrifugation at 720xg for 10 minutes at room temperature. This was either stored at -20ºC and used subsequently after thawing or used directly. The pellet was resuspended in 200µl PBS. 20µl proteinase K and 200µl buffer Al was added to the sample, mixed thoroughly by vortexing and incubated at 70°C for 10 minutes. To this homogeneous solution, 200µl 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 6000xg for 1 min. The flow-through and collection tube was discarded and the column was placed in a new 2 ml collection tube. 500µl buffer AW1 was added onto the column and the column was centrifuged for 1min at 6000xg. 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 18200xg to dry the DNeasy membrane. After discarding the flow-through and collection tube, the column was placed in a clean 1.5 ml microfuge tube and 200 µl buffer AE was pipetted onto the DNeasy membrane. The column was incubated at room temperature for 1 minute and then centrifuged for 1 minute at 6000xg to collect the elute. The eluted DNA was stored at 4°C.

2.3 Bacterial hosts and plasmids

Stab cultures of wild type MCMV BAC (Smith) plasmids maintained in *E. coli* strain DH10B were kindly supplied as indicated in Table 2.2. MCMV BAC (Smith) plasmid was designated as pSM3fr (176). Plasmid pCR®4Blunt-TOPO (Figure 2.1) was purchased from Invitrogen, Paisley, UK. A linear PCR product of 1945 bp from the MCMV Smith BAC (nt 35241 to nt 37244) (128) was previously cloned into this plasmid by Dr. Melissa Kirby. The modified pCR®4Blunt-TOPO plasmid was designated pCR4B-29 in this study. Plasmid pET28a (Figure 2.2) was provided by Dr. Lynn Dover as DNA and transformed into host bacteria (Table 2.2). A temperature-sensitive plasmid named pRpsl-neo (carrying a kanamycin resistance cassette in the vector backbone) and an ET protein expression plasmid, pKD46 were also provided by Dr. Melissa Kirby. Strains and plasmids used in this study are shown in Table 2.2.

2.3.1 Media for bacterial cultures

Bacterial cultures were grown in Luria-Bertani medium (LB) prepared by dissolving 10gm tryptone, 5gm yeast extract and 10gm NaCl in 800ml distilled water. All culture media were autoclaved for 15 minutes at 121°C and, where required, mixed with appropriate antibiotics (Table 2.2). To make agar plates, 100 to 200ml aliquots of LB supplemented with 15gm/l bacto-agar (L-Agar) were autoclaved, cooled and stored at 4°C. The L-Agar was dissolved by boiling, cooled to 50°C and supplemented with appropriate antibiotics (Table

2.2). In a laminar flow hood, 20-25ml of L-Agar was poured into each petri dish (agar plate) and left to solidify for 20 to 30 minutes.

Table 2.2 Strains and plasmids used in this study

Plasmid name	Host	Selection	Source	Reference
wt MCMV BAC	DH10B	12μg/ml Cm[1]	Koszinowski[5]	(176)
pCR4B-29	TOP10	100μg/ml Carb[2]	M. Kirby	-
pCR4B-29*	XL1-Blue	100μg/ml Carb[2]	This work	
pCR4B-29.1*	XL1-Blue	100μg/ml Carb[2]	This work	
pKD46[4]	TOP10	50μg/ml Carb[2]	M. Kirby	(181)
pRpsL-neo[4]	TOP10	20μg/ml Kn[3]	Gene Bridges	
MCMV BAC-rpsl-neo	DH10B	20μg/ml Kn[3] plus 12μg/ml Cm[1]	This work	
Rc29MCMV BAC	DH10B	12μg/ml Cm[1]	This work	
Rc29.1MCMV BAC	DH10B	12μg/ml Cm[1]	This work	
pET28a	TOP10	25μg/ml Kn[3]		Novagen
pET28a-m29	TOP10	25μg/ml Kn[3]	This work	
pET28a-m29.1	TOP10	25μg/ml Kn[3]	This work	

1. Cm, chloramphenicol
2. Carb, carbenicillin
3. Kn, kanamycin
4. Temperature sensitive origin of replication (30°C)
5. Ludwig-Maximilians-Univesität Műnchen, Germany

2.3.2 Preparation of bacterial cultures and storage

Single colonies isolated from agar plates were transferred aseptically into 5 or 10ml of LB supplemented with appropriate antibiotics (Table 2.2). Bacteria were then grown at 30°C/37°C on a shaker at 200rpm overnight. For storage of bacterial cultures, 500μl overnight cultures were transferred to 2ml cryovials followed by addition of 500μl 80% (w/v) glycerol and stored at -80°C.

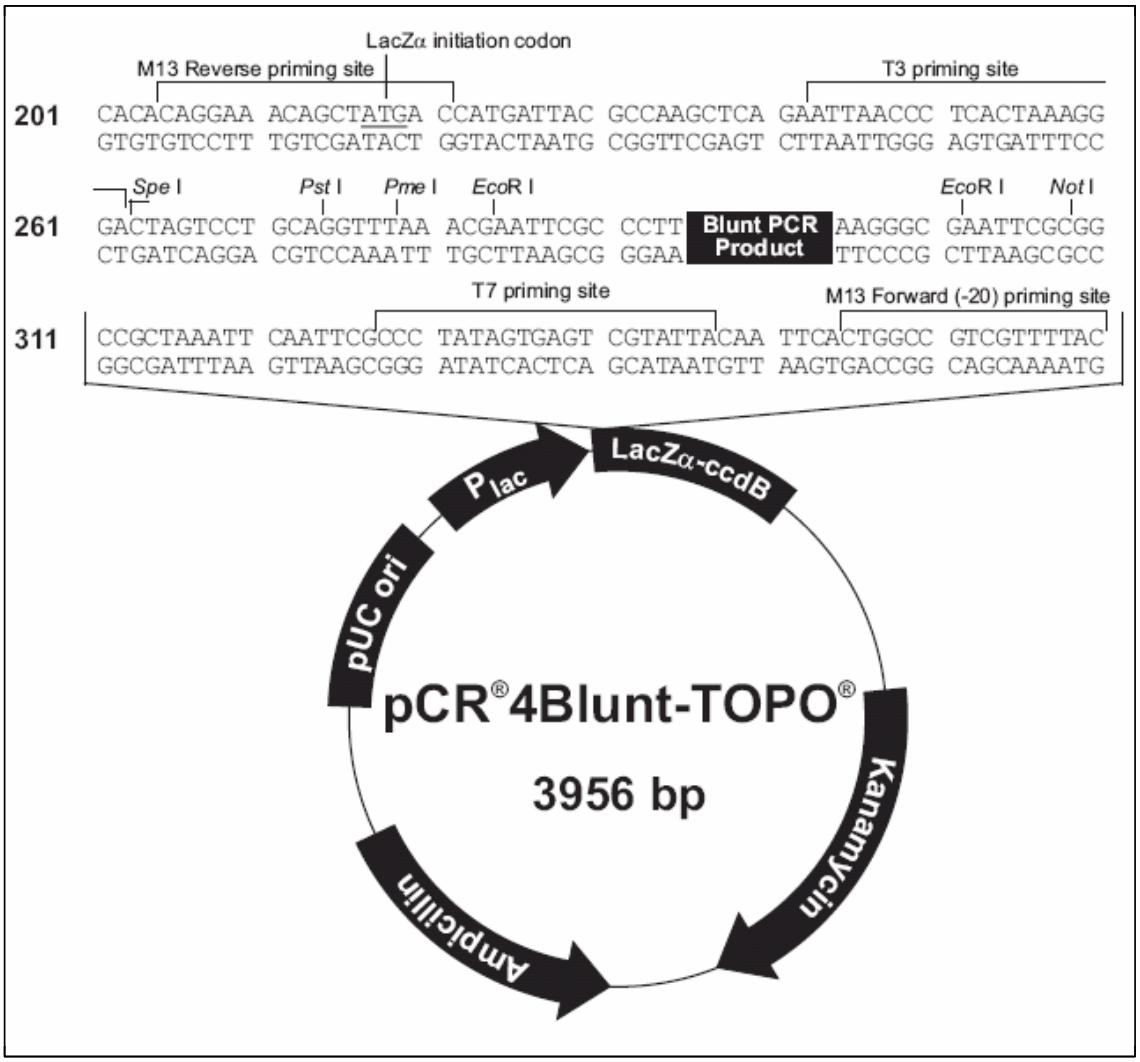


Figure 2.1 Map of the pCR®4Blunt-TOPO vector. A linear 1945 bp DNA fragment containing m29 and m29.1 ORF was cloned between the *EcoR*I sites.

2.3.3 Preparation of chemically competent cells

An aliquot of 2ml overnight culture (section 2.3.2) was added to 100ml of pre-warmed LB broth supplemented with appropriate antibiotics (Table 2.2). Cells were grown at 37°C with shaking until the absorbance at 600_{nm} reached 0.39. Cells were then centrifuged at 750xg for 10 minutes at 4°C, the supernatant discarded and 40ml ice-cold transformation buffer-1 [30mM potassium acetate, 10mM rubidium chloride, 10mM calcium chloride, 50mM manganese chloride,

15% (v/v) glycerol, pH 5.8] added to resuspend the pellet, which was then incubated on ice for 5 minutes. Following another round of centrifugation under the same conditions, the pellet was resuspended in 4ml ice-cold transformation buffer-2 [10mM MOPS {3-(N-morpholino) propanesulfonic acid}, 75mM calcium chloride, 10mM rubidium chloride, 15% (v/v) glycerol, pH 6.5]. The cells were then incubated on ice for 2 hours, dispensed in aliquots (200μl/tube) and frozen in liquid nitrogen prior to storage at -80°C.

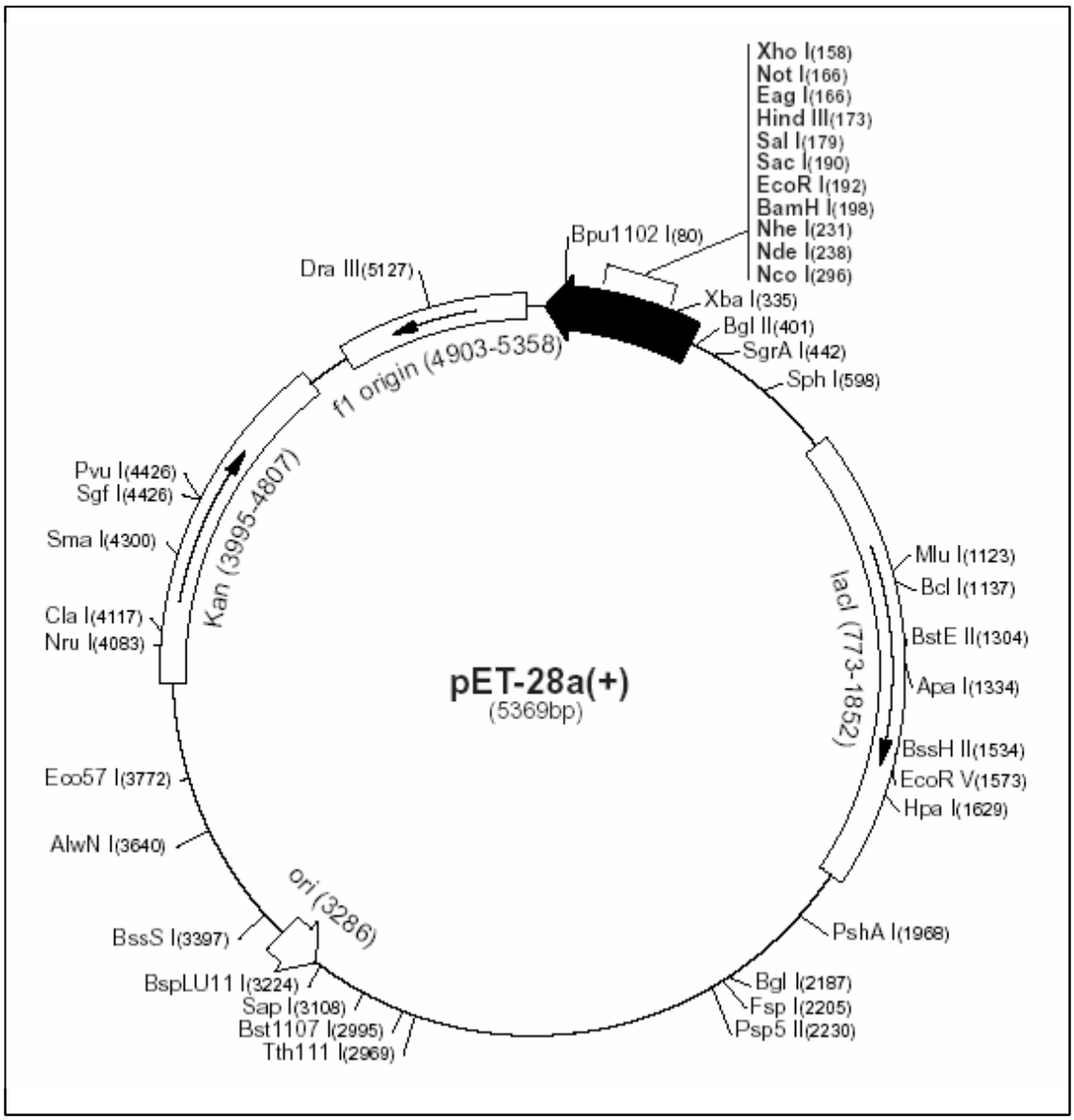


Figure 2.2 Map of the pET28a vector. The m29 and m29.1 gene was cloned between the *Xho*I and *Nco*I sites.

2.3.4 Transformation of plasmid DNA into competent cells

Chemically competent cells (section 2.3.3) were thawed on ice, plasmid DNA (1 to 5ng) added, mixed by tube flicking and incubated on ice for 15 minutes. The cells were then heat shocked at 42°C for 30 seconds followed by incubation on ice for 30 seconds. 800μl of LB broth, pre-warmed to room temperature, were added to the cells and then incubated at 37°C with shaking at 200rpm for one hour. The sample (50-100μl) was then spread on LB agar plate supplemented with appropriate antibiotics (Table 2.2) and cultured at 30 or 37°C overnight.

2.3.5 Preparation of electrocompetent cells

The ET expression plasmid DNA, pKD46 (1 to 5ng), was transformed (section 2.3.4) into chemically competent cells (section 2.3.3) of *E. coli* strain DH10B containing the wild type MCMV BAC (Smith) (176). Following culture overnight at 30°C, a single colony was picked and grown in 5ml LB medium with antibiotics (Table 2.2) overnight with shaking at 30°C. 0.7 ml of overnight culture was then added to 70ml of pre-warmed LB broth supplemented with appropriate antibiotics (Table 2.2), and incubated at 30°C with shaking until the absorbance at 600_{nm} reached 0.1– 0.15. 350μl of 1M L-arabinose was added to the culture to induce ET protein expression. Incubation was continued at 30°C until the absorbance at 600_{nm} reached 0.3– 0.4. To harvest the cells, the centrifuge and rotor was prechilled by centrifuging for 10 minutes at -5°C at 4000xg and then 35ml of cells were centrifuged at 4000xg for 10 minutes at -

5°C and the remainder was kept on ice. The supernatant was discarded and the harvesting step was repeated with the remaining amount of cells. To wash the cells, the pellet was resuspended in 5ml ice cold 10% (w/v) glycerol and a further 25 ml of 10% (w/v) glycerol was added followed by centrifugation as above. The supernatant was discarded and the washing step was repeated twice. The supernatant was poured away and the tube was immediately dried with Kleenex tissue. The cells were resuspended in the remaining liquid (little more than 100μl final resuspended volume). 50μl of cells were transferred into a pre-cooled eppendorf tube and frozen in liquid nitrogen prior to storage at -80°C.

2.3.6 Electroporation

Electrocompetent cells (section 2.3.5) were thawed on ice and DNA solution (100-300ng linear DNA in a maximum volume of 10μl) was added. Electroporation was performed using a 1mm gap ice-cold cuvette and a Bio-Rad gene pulser set to 25μF, 2.3 KV with pulse controller set at 200 ohm. SOC medium (1ml) (2.0gm tryptane, 0.5gm 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 immediately after electroporation. The cells were incubated at 37°C for 75 minutes with shaking and spread on an antibiotic plate for selection.

2.4 Plasmid DNA Isolation

2.4.1 Small scale preparation of plasmid DNA

Overnight cultures (1.5 to 3 ml) grown from a single colony with appropriate selective antibiotics were aliquoted into an eppendorf tube and centrifuged at 750xg at room temperature for 5 minutes. The supernatant was discarded and the pellet was completely resuspended in 100μl of solution-I (25mM Tris pH 8.0; 10mM EDTA ph 8.0; 0.5M sucrose). After resuspension, 200μl of freshly prepared solution-II (0.2M NaOH; 1% SDS) was added to lyse the cells and the tube was inverted several times. The lysed cell suspension was then mixed with 200μl solution-III (117.78gm potassium acetate; 46 ml glacial acetic acid in 400ml distilled water), incubated for 15 minutes on ice and then centrifuged at 15680xg for 15 minutes at room temperature. The supernatant was then transferred to a fresh eppendorf tube containing 500μl isopropanol followed by centrifugation at 15680xg for 10 minutes at room temperature as above. The pellet was suspended in 100μl distilled water and 2μl RNase (10mg/ml) was added followed by incubation at 37ºC for 15 minutes. 100μl distilled water and 200μl phenol-chloroform was then added to the cells followed by centrifugation at 15680xg for 3 minutes at room temperature. The aqueous layer was pipetted into a new tube. This phenol-chloroform extraction was repeated and 20μl 3M sodium acetate and 600μl 100% ethanol was added to the final aqeous layer followed by incubation at -80ºC for 15 minutes. The precipitated DNA was centrifuged at 15680xg for 10 minutes at room temperature, the

pellet washed with 70% ethanol, air dried and resuspended in 50μl distilled water.

2.4.2 Maxi preps

A NucleoBond® PC kit (Abgene, Epsom, UK) was used for the isolation of low-copy number plasmids according to the manufacturer's instructions. An overnight bacterial culture was set up by inoculating 500ml of LB medium (plus antibiotics) with a single colony picked from a freshly streaked plate. The culture was centrifuged at 6000xg for 15 minutes at 4ºC and the pelleted bacterial cells resuspended in 24ml of buffer S1. 24ml of buffer S2 was then added to the suspension which was mixed by gently inverting the tube 6-8 times. The mixture was incubated at room temperature for 5 minutes, when 24ml of pre-cooled buffer (4ºC) S3 was added and the lysate immediately mixed gently by inverting the flask 6-8 times until a homogeneous suspension containing an off-white flocculate was formed. The suspension was then incubated on ice for 5 minutes. For clarification of the lysate, a NucleoBond® folded filter was placed in a small funnel for support and the filter prewetted with a few drops of buffer N2. The bacterial lysate was loaded onto the wet filter and the flow-through was collected. The cleared lysate from the above step was loaded onto the NucleoBond® AX 500 (Maxi) column, previously equilibrated with 6ml of buffer N2, and the column allowed to empty by gravity flow. The column was washed twice with 18ml of buffer N3. The flow-through was discarded and the plasmid DNA eluted with 15ml of buffer N5. 11ml of room temperature equilibrated isopropanol was added to precipitate the eluted

plasmid DNA, which, after careful mixing, was centrifuged at 15000xg for 30 minutes at 4ºC. The pellet was washed with 70% ethanol and centrifuged at 15000xg for 30 minutes at room temperature. After removing ethanol, the pellet was air dried for 10-20 minutes and resuspended in 150μl distilled water.

2.4.3 Midi preps

QIAGEN Plasmid Midi Kit (Qiagen, West Sussex, UK) was used to purify low-copy number plasmids according to the manufacturer's instructions. A single colony from a freshly streaked LB agar plate was picked and inoculated as a starter culture in 5ml LB medium containing the appropriate selective antibiotic (Table 2.2). The culture was incubated overnight at 37°C with vigorous shaking.100ml of LB was inoculated with 500µl of starter culture followed by incubation overnight at 37°C with vigorous shaking. The bacterial cells were harvested by centrifugation at 6000x*g* for 15 minutes at 4°C and the pellets resuspended in 4ml buffer P1. 4ml of buffer P2 was added to the resuspended pellet, mixed thoroughly by vigorously inverting the sealed tube 4–6 times which was then incubated at room temperature for 5 minutes. 4ml of chilled buffer P3 was added, the contents mixed immediately and thoroughly by vigorously inverting the tube 4–6 times, which was then incubated on ice for 15 minutes. The cells were centrifuged at 20,000x*g* for 30 minutes at 4°C, the supernatant containing plasmid DNA removed and centrifuged again at 20,000x*g* for 30 minutes at 4°C. The supernatant was removed and applied to a QIAGEN-tip 100 which had previously equilibrated with 4ml buffer QBT. The QIAGEN-tip was washed twice with 10ml buffer QC and then DNA was eluted

with 5ml buffer QF and precipitated by addition of 3.5ml room-temperature isopropanol. The solution was mixed and centrifuged immediately at 15,000x*g* for 30 minutes at 4°C. The supernatant was decanted carefully and the DNA pellet washed with 2ml of room-temperature 70% ethanol followed by centrifugation at 15,000x*g* for 10 minutes. The supernatant was decanted carefully without disturbing the pellet, which was air dried for 5–10 minutes and the DNA dissolved in 80μl distilled water.

2.4.4 Mini preps

The Wizard plus® SV minipreps DNA purification kit (Promega, Southampton, UK) was used according to the manufacturer's instructions to prepare DNA particularly for sequencing. Briefly, cultures were grown overnight in LB broth with appropriate selective antibiotics (Table 2.2). The overnight culture (10ml) was centrifuged at 15680xg for 10 minutes and the pellet resuspended in 250μl cell resuspension solution. To lyse the cells, 250μl cell lysis solution was added and the tube inverted 4 times to mix. The mixture was incubated for 5 minutes at room temperature after adding 10μl of alkaline protease solution. Neutralization solution (350μl) was then added and the sample centrifuged at 15680xg for 10 minutes. The lysate was then transferred to a spin column inserted into the collection tube and centrifuged for 1 minute. Washing was carried in two steps with 750μl and 250μl of wash solution successively and, in each step, addition of wash solution was followed by centrifugation for 2 min at room temperature. The spin column was then transferred to a sterile 1.5 ml

micro centrifuge tube and centrifuged for 1 minute at 15680xg after addition of 100μl of nuclease-free water.

2.4.5 Small scale BAC plasmid DNA purification

An aliquot of 1.5 ml overnight culture was transferred to a 2.0ml eppendorf tube and centrifuged at 15680xg for 5 minutes at room temperature in a microfuge. The pellet was resuspended in 300μl of resuspension buffer (15mM Tris-HCl, pH 8.0, 10mM EDTA, 100μg/ml RNase), 300μl of lysis solution [0.2N NaOH, 1% (w/v) SDS] added and the tube mixed gently. After 5 minutes incubation at room temperature, 300μl of 3M potassium acetate (pH 5.5) was added; the sample was mixed gently and incubated on ice for 10 minutes. The supernatant was transferred to a fresh tube containing 0.8ml isopropanol and mixed by inversion. After incubation at -80°C for 15 min, the sample was centrifuged at 15680xg for 15 minutes at room temperature, the supernatant removed and the pellet washed with 500μl ethanol followed by centrifugation at 15680xg for 5 minutes at room temperature. Again the supernatant was discarded, the DNA pellet air-dried on the bench for 10-30 minutes. The DNA was resuspended in 50μl distilled water and stored at 4°C.

2.4.6 Large scale BAC plasmid DNA purification

A NucleoBond® BAC kit (Abgene, Epsom, UK) was used for the isolation of low-copy BAC plasmid DNA according to the manufacturer's instruction. The same procedure was carried out as described in section 2.4.2 except that the

NucleoBond® BAC 100 column was used instead of the NucleoBond® AX 500 column.

2.5 Polymerase Chain Reaction

The polymerase chain reaction (PCR) was performed both for screening and cloning purposes using primers and annealing temperatures as indicated in Table 2.3.

2.5.1 Amplification of DNA by PCR

For screening purposes, DNA was amplified using 2x ReddyMix™ PCR Master Mix (Abgene, Epsom, UK). PCR was performed in 50μl reactions as below:

Component	**Amount (μl)**
2x ReddyMix™ PCR Master Mix	25
Forward primer(10μM)(Sigma-Genosys)	3
Reverse primer(10μM)(Sigma-Genosys)	3
DNA (300ng)	--
Distilled Water	to 50

Each PCR reaction was set up as follows:

Initial denaturation	94 °C	2 minutes	1 cycle
Denaturation	94 °C	45 seconds	30 cycles
Annealing	Annealing temperature (Table 2.3)	45 seconds	
Extention	72 °C	1 minute/kb of DNA	
Denaturation	94 °C	45 seconds	1 cycle
Annealing	Annealing temperature (Table 2.3)	45 seconds	
Extention	72 °C	7 minutes	

For cloning purposes, DNA was amplified using Extensor Hi-Fidelity PCR Master Mix (ABgene, Epsom, UK). PCR was performed in 25μl reactions as below:

Component	**Amount (μl)**
Extensor Hi-Fidelity PCR Master Mix	12.5
Forward primer(10μM)(Sigma-Genosys)	1.5
Reverse primer(10μM)(Sigma-Genosys)	1.5
DNA (300ng)	--
Distilled Water	to 25μl

Each PCR reaction was set up as follows:

Initial denaturation	94 °C	2 minutes	1 cycle
Denaturation	94 °C	45 seconds	10 cycles
Annealing	Annealing temperature (Table 2.3)	30 seconds	
Extention	72 °C	1 minute/kb of DNA	
Denaturation	94 °C	45 seconds	
Annealing	Annealing temperature (Table 2.3)	30 seconds	20 cycles (+10s/cycle)
Extention	72 °C	1 minute/kb of DNA	
Denaturation	94 °C	45 seconds	
Annealing	Annealing temperature (Table 2.3)	30 seconds	1 cycle
Extension	72 °C	7 minutes	

Table 2.3 Primers used in this study

Primer name	Forward sequence	Tm(°C)[1]
35610F	GCGGCCACAGGCGGAATCGG	60.0
36371F	CGACGAAGTCATTCATGTCC	60.0
35938F	GGCAAATGGCGAAACCTCCC	60.0
36790F	GCGTGGACGACGGCGCAGG	60.0
35195	GATCAGATCGCCGTGACTCC	60.0
g	GGATACTCAGCGGCAGTTTGC	58°C
b	GCCCGCCTGATGAATGCTC	58°C
RPSL FOR	GGGTGGAGAGGCTATTCGGC	60.0
m29F	CGCATATGGTCATCTCGGAGGAC	60.0
RTm29F	ATCCGCATACCGACAGCTTCC	64.0
RTm29.2F	AACAGAGGGATGGAAGCGCC	60.0
P29F[2]	CATG**CCATGG**GCATGATCCGCATACCG	56.0
P29.1F[2]	CCG**CTCGAG**CTAGATGGTGGTGTTTCTCC	63.0
M29.1FOR	CTAGATGGTGGTGTTTCTCCTGC	58.0
M75F	TGATCATCAGGTTCCTGTCC	56.0
M123F	CAACATGTCCTCCAGAGTC	53.0
GeneRacer™ 5` Primer	CGACTGGAGCACGAGGACACTGA	68.0
GSP29.1F	ACAGGCGAGTGCGTCGCTATCGT	68.0
GSP29F	GCCGATAGGGACACTCCTCACGAA	68.0
m29*F[3]	TAGAAACGCCCACTA**G**TCATACGATCGCACG	65.0
m29.1*F[3]	CGCTAGTATGGAATG**C**TATCTAGCGTGCACC	65.0
ETm29F[4]	***AGGCGACGGAGGTGGGGACGGGCACGGTCGGTTGGAT AACCATCTCCGAGAA***GGCCTGGTGATGATGGCGGGATC	55.0
	Reverse sequence	
36304R	GCGCCGCTCCGAGCGGAAGG	60.0
35722R	CTGGAAGCTGTCGGTATGCG	60.0
f	GGTTACTGGATGGGTACGAG	58°C
36744R	CGACAGGTATCTTCTCACCG	62.3
REV-RPSL	GCCGAATAGCCTCTCCACCC	60.0
m29REV	GATTAATTAACTGCGGATCTGCG	60.0
RTm29R	GGATGAAAGCGAAAGTGGCGG	64.0
RTm29.2R	GATCGGGACCTGGATCTCTC	60.0
P29R[2]	CCG**CTCGAG**TCCATACTAGCGTCT	56.0
P29.1R[2]	CATG**CCATGG**GCTCGCGTACGGTTATGG	63.0
M29.1REV	GATCTCATGGTCAACTTCGCGG	58.0
M75R	GAT GAG ACG CAT CTT GAT CC	56.0
M123R	GATGAGAACCGTGTCTACC	53.0
GeneRacer™ 3` Primer	GCTGTCAACGATACGCTACGTAACG	68.0
GSP29.1R	GCCTGTCGTGGCTCGGACATGAA	68.0
GSP29R	TCC ACT AAC GCC GCT CCT CTG TTG	68.0
m29*R[3]	CGTGCGATCGTATGA**C**TAGTGGGCGTTTCTA	65.0
m29.1*R[3]	GGTGCACGCTAGATA**G**CATTCCATACTAGCG	65.0
ETm29R[4]	***TCCTCCACGCTCGCGTATAAAATAGGTCTCTGCGAGAGT TGCGCTTCAGAC***TCAGAAGAACTCGTCAAGAAGGCG	55.0

1. Annealing temperature of primer
2. Restriction enzyme recognition site incorporated into primers is shown with bold letters
3. Point mutation incorporated into primers for inverse PCR is indicated in bold letter
4. Homology sequence specific to MCMV genome incorporated into primers is shown with bold Italic letters

2.5.2 Inverse PCR

Inverse PCR was used to introduce point mutations into template DNA. Inverse PCR was performed using the QuickChange site-directed mutagenesis kit (Stratagene, Cambridge, UK) as below:

Component	**Amount**
10X Reaction buffer	5.0μl
dNTP mix	1.0μl
Forward primer(10μM)(Sigma-Genosys)	1.3μl
Reverse primer(10μM)(Sigma-Genosys)	1.3μl
DNA (50ng)	--μl
Distilled Water	to 50μl

Then 1μl of *PfuTurbo* DNA polymerase (2.5U/μl) was added to the reactions. Each PCR reaction was set as follows: 1 cycle of 95°C for 2 minutes and 12 cycles of 95°C for 30 seconds, annealing temperature (Table 2.3) for 1 minute and 68°C for 7 minutes.

2.6 Restriction enzyme digestion of DNA

DNA was digested with different restriction enzymes according to the enzyme manufacturer's instructions. Usually digestion was carried out in 20μl of final solution with 1-3μg of DNA, 1μl each of enzyme, 2μl of 10X buffer and, where appropriate, bovine serum albumin, followed by overnight incubation at 37°C. For cloning purposes, digestion was carried out in 50μl of final solution with 3-5 μg of DNA, 2μl each of enzyme and 5μl of 10X buffer. After digestion, samples

were run on an agarose gel (section 2.7) and the samples were stored at 4°C. Restriction enzymes *EcoR*I, *Dpn*I, *Nco*I and *Xho*I (NEB, Herts, UK) were used in this study.

2.7 Agarose gel electrophoresis

Restriction enzyme digest products (section 2.6) and PCR products (section 2.5) were visualized under UV light after resolution by gel electrophoresis through an agarose gel, comprising of 0.8% to 1.0% w/v agarose. Agarose was dissolved in 0.5x TBE buffer (45mM Tris base, 45mM boric acid, 1mM EDTA, pH 8.0). The solution was cooled to 60°C and ethidium bromide was added to a final concentration of 0.5μg/ml and poured into a gel tray containing a comb to produce wells for loading DNA samples. The gel was left to solidify on the bench for 20 to 30 minutes. 50μl of the DNA sample was mixed with 10μl of 5x loading buffer [0.25% bromphenol blue, 15% ficoll type 400 (Pharmacia) in water] before loading into a well of the agarose gel. The gel tank was filled with 0.5x TBE buffer and the gel was run in 100V for 40 minutes. A 1kb DNA ladder (Fermentas, York, UK and NEB, Herts, UK) was used as a marker exhibiting bands ranging from 1 to 10 kb.

2.8 Purification of DNA

After PCR or gel electrophoresis, DNA was purified using the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Bucks, UK) according to the manufacturer's instructions. After purification, all DNA was stored at 4°C.

To purify DNA from PCR products, a GFX column was placed in a collection tube, 500µl of capture buffer was added to the column followed by addition of the sample (up to 100µl). The solution was mixed thoroughly by pipetting up and down 4-6 times. The column was then centrifuged for 30 seconds at 18200xg at room temperature, the flow-through discarded from the collection tube, and the GFX column replaced in the collection tube. 500µl of wash buffer was added to the column, centrifuged at 18200xg for 1 minute, and the flow-through again discarded. The GFX column was transferred to a fresh 1.5ml microcentrifuge tube and 50µl of double distilled water applied to the top of the glass fibre matrix in the column to elute the DNA. After 1 minute incubation at room temperature, the column was centrifuged at 18200xg for 1 minute to recover the purified DNA.

To purify DNA from gels, the gel was placed onto an ultraviolet illuminator to visualise the DNA band of interest. A clean scalpel blade was used to remove the band of interest and the gel slice was transferred to a 1.5ml eppendorf tube. 500µl of capture buffer was added to the eppendorf tube and incubated at room temperature until the agarose was completely dissolved. The sample was then transferred to the GFX column and the remainder of the procedure was as described above.

2.9 Site-directed mutagenesis

The QuickChange site-directed mutagenesis kit (Stratagene, Cambridge, UK) was used to generate a point mutation in the genes of interest using plasmid

pCR4B-29 (section 2.3). The strategy used for site-directed mutagenesis is shown in Figure 2.3. Two synthetic oligonucleotide primers containing a point mutation (Table 2.3) were made according to the manufacturer's instructions. The Wizard®*Plus* purified DNA from plasmid pCR4B-29 (section 2.4.4) was used as template DNA. The oligonucleotide primers, each complementary to opposite strands of the vector, were extended by inverse PCR (section 2.5.2). A mutated plasmid containing a staggered nick was generated by incorporation of oligonucleotide primers. After temperature cycling, 1μl of DpnI was added to the amplification reaction and centrifuged for 1 minute followed by incubation at 37ºC for 1 hour to digest the parental DNA. The XL1-Blue supercompetent cells (Stratagene, Cambridge, UK) were thawed on ice and 50μl was aliquoted to a prechilled Falcon2059 polypropylene tube. 1μl of the DpnI-treated DNA was then transformed into the supercompetent cells as described in section 2.3.4. After incubation, 250μl of cells were transferred to an LB agar plate (section 2.3.1) supplemented with appropriate antibiotics (Table2.2). After overnight culture at 37°C, separate bacterial cultures (section 2.3.2) were prepared from 6 single colonies for the isolation of DNA using Wizard®*Plus* DNA miniprep purification kit (section 2.4.4). DNA from each colony was sequenced (section 2.10) to verify that selected clones contained the desired mutation.

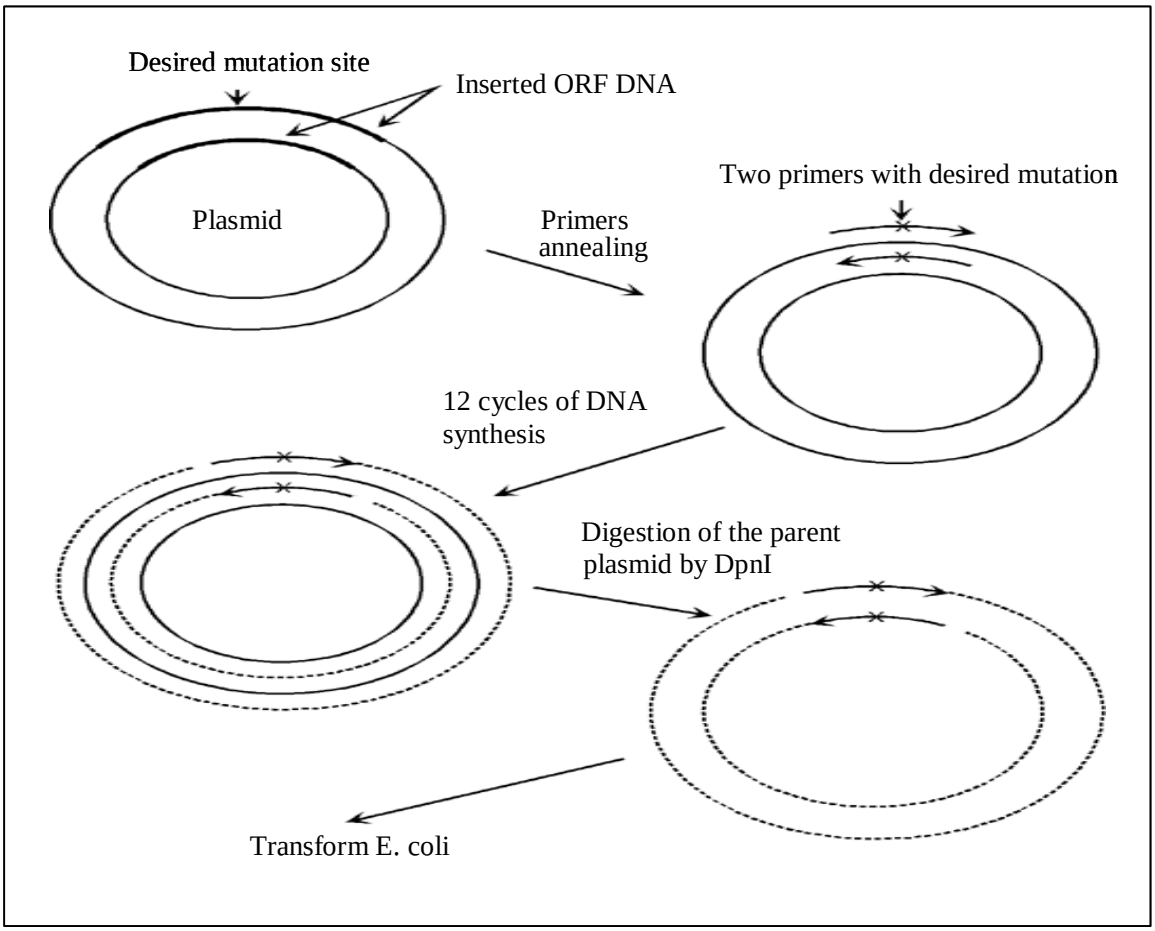


Figure 2.3 Strategy used for site-directed mutagenesis. Two oligonucleotide primers, complementary to each other, containing a point mutation were used to generate the mutated plasmid containing a staggered nick. The parental plasmid DNA was digested by DpnI and the mutated plasmid DNA was transformed into *E. coli* strain XL1-Blue.

2.10 Sequencing

DNA was 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. The concentration of template DNA used in sequencing reactions is shown below:

Template	**Size (bp)**	**Quantity (ng)**
PCR product	100-200	1-3
	200-500	3-10
	500-1000	5-20
	1000-2000	10-40
	>2000	40-100
Plasmid DNA	-	300

The sequencing was carried out in a 10μl reaction volume comprising of 3.2pmol primer, template DNA and sterile water using the University of Birmingham Functional Genomics Laboratory, Plasmid to Profile sequencing reaction (Birmingham, UK). Several primers were used for each gene to be sequenced in order to obtain overlapping sequences in both 5' to 3' and 3' to 5' directions (Table 2.3). The sequences obtained from the ABI 3700 DNA analyser were analysed with Chromas software (version 1.45, Griffith University, Queensland, Australia). The BLAST programme (6) was used to compare obtained sequences with that of the published MCMV Smith strain sequence (accession number U68299) (128).

2.11 ET homologous recombination

A two-step ET homologous recombination was preformed as described previously (110, 187, 188). The strategy used for ET homologous recombination is shown in Figure 2.4.

2.11.1 Inserting the rpsl-neo cassette into MCMV BAC

A linear rpsl-neo PCR product flanked by MCMV homology arms was used in the first step. The rpsl-neo cassette was amplified by PCR (section 2.5) from pRpsl-neo plasmid DNA (section 2.4.2) using primers ETm29F and ETm29R (Table 2.3). After amplification, the PCR products were run on an aragose gel (section 2.7) to confirm that an amplicon of the correct size had been generated. PCR samples were then purified (section 2.8), digested overnight to remove residual template DNA with *Dpn*I (section 2.6) and then purified once again (section 2.8) and used to electroporate (section 2.3.6) cells (section 2.3.5) containing the MCMV BAC (Wagner *et al*., 1999). 100μl of the resultant culture were spread on an LB agar plate containing kanamycin (20μg/ml) plus chloramphenicol (12μg/ml) and an LB agar plate containing chloramphenicol (12μg/ml) only for the control. The plates were then incubated overnight at 37°C. To identify true recombinants, the resulting primary colonies were restreaked in parallel on plates containing 12μg/ml chloramphenicol plus 20μg/ml kanamycin and on plates containing 12μg/ml chloramphenicol plus 80μg/ml streptomycin. The plates were incubated at 37°C overnight to test the function of rpsl-neo cassette. Due to insertion of rpsl-neo cassette, true clones

should not grow on the streptomycin plate, but should grow on the kanamycin plate as the rpsl gene confers streptomycin sensitivity and *neo* gene confers kanamycin resistance. The streptomycin plate was checked and the clones which didn't grow on this plate were identified. Recombinant BAC plasmid DNA was isolated (section 2.4.5) and used for PCR screening (section 2.5) to confirm the MCMV BAC-rpsl-neo clones.

2.11.2 Replacing the rpsl-neo cassette with an oligo carrying the desired point mutation

A restriction enzyme digested linear DNA fragment containing a point mutation was used to replace the rpsl-neo cassette in the MCMV BAC. The ET expression plasmid DNA, pKD46 (1 to 5ng), was transformed (section 2.3.4) into competent cells (section 2.3.3) of the *E. coli* strain DH10B containing the MCMV BAC-rpsl-neo clones (section 2.11.1) and electrocompetent cells were prepared as described in section 2.3.5. Plasmid pCR4B-29 with an insert containing both of overlapping m29 and m29.1 ORFs (1945 bp) (nt 35241 to 37244) (128) was mutated twice separately using QuickChange site-directed mutagenesis kit (section 2.9) to introduce a point mutation at nt position 35,896 in the m29 ORF and at nt position 36,484 in the m29.1 ORF to introduce stop codon mutations at the 5' end of each ORF. These mutated plasmids were digested overnight at 37°C with *EcoR*I (section 2.6) to release a 1945 bp linear DNA fragment. The fragments were separated on an agarose gel (section 2.7), purifed (section 2.8) and used to electroporate (section 2.3.6) bacterial cells containing the MCMV BAC-rpsl-neo clones. 100μl of the resultant culture were spread on an LB agar plate containing streptomycin (80μg/ml) plus

chloramphenicol (12μg/ml) and on an LB agar plate containing chloramphenicol (12 μg/ml) only for the control. The plates were then incubated overnight at 37°C. To identify true recombinants, the resulting primary colonies were restreaked in parallel on plates containing 12μg/ml chloramphenicol plus 80μg/ml streptomycin and on plates containing 12μg/ml chloramphenicol plus 20μg/ml. kanamycin. Due to replacement of the rpsl-neo cassette with mutated DNA, true clones should not grow on the kanamycin plate, but should grow on the streptomycin plate as loss of the rpsl gene confers streptomycin resistance whereas loss of the neo gene confers kanamycin sensitivity. The plates were incubated at 37°C overnight. The kanamycin plate was checked and the clones which didn't grow on this plate were identified. The recombinant BAC plasmid DNA was isolated (section 2.4.5) and used for PCR screening (section 2.5) and sequencing (section 2.10) to confirm the mutated MCMV BAC (Rc29MCMV BAC and Rc29.1MCMV BAC). After confirmation, the recombinant BAC plasmid DNA was isolated again (section 2.4.6) and transfected (section 2.2.1) into NIH 3T3 cells to generate mutant viruses.

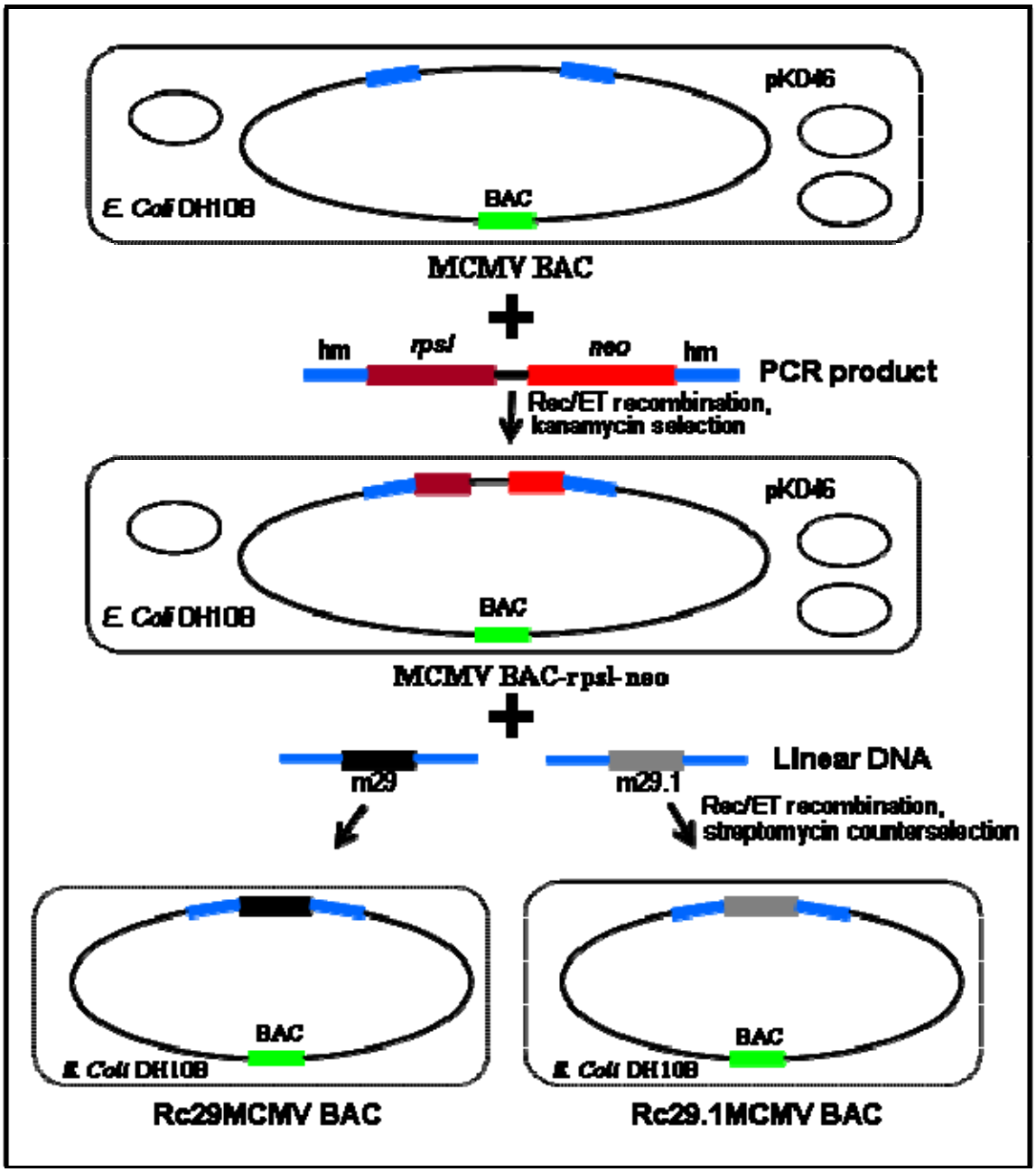


Figure 2.4 Counterselection strategy. The illustration at the top shows *E. Coli* DH10B harbouring the low copy, temperature sensisitve, RecE/RecT expression plasmid, pKD46 and wt MCMV BAC. In the first step, a PCR product of the *neo* and *rpsl* genes flanked by two homology arms (hm) was introduced by selection for kanamycin resistance. In the second step, a restriction enzyme digested linear DNA carrying the desired mutation was used with selection for the loss of rpsl and restoration of streptomycin resistance.

2.12 Cloning

The aim of this section was to clone the m29 and m29.1 genes into the protein expression vector pET28a (Figure 2.2).

2.12.1 Preparation of insert and vector

The DNA fragments (m29 and m29.1 gene) to be ligated were generated from MCMV genomic DNA by PCR. The primers used in the PCR contained restriction enzyme site *Nco*I or *Xho*I at 5' end (Table 2.3). The PCR products and plasmid pET28a were digested with *Nco*I and *Xho*I enzymes (section 2.6), separated on an agarose gel (section 2.7), purified (section 2.8.2) and quantified using Hyper ladder-I (Bioline, London, UK).

2.12.2 Ligation

The m29 and m29.1 inserts were ligated into the pET28a backbone at the common restriction sites (NcoI and XhoI) using T4 DNA ligase (New England Biolabs). The ligation reactions were performed in 20μl volume as follows:

Component	**Amount**
pET28a vector (100ng)	--μl
m29 or m29.1 gene insert (120 ng)	--μl
T4 DNA ligase	1.0μl
10X Ligase buffer	2.0μl
Distilled water	to 20μl

The ligation was carried out overnight at room temperature. 2μl of ligation reaction was transformed into 200μl of competent *E. coli* TOP10 cells (section 2.3.3). 50μl of such cells were plated onto LB agar (section 2.3.1) supplemented with appropriate antibiotics (Table 2.2) and grown overnight at 37°C. Bacterial colonies grown on these agar plates were screened for the presence or absence of the inserted gene by both restriction enzyme digestion (section 2.6) and PCR (section 2.5).

2.13 RNA work

2.13.1 Isolation of total RNA

Total RNA was isolated from MCMV infected NIH 3T3 cells at different time-points using the RNeasy® Mini Kit (Qiagen, West Sussex, UK). NIH 3T3 cells were seeded at $5x10^5$ cells in 3ml GM in each well of a 6 well flat bottomed tissue culture plate. After 12 to 16 hours incubation at 37^oC, GM was discarded and the cells were infected with wt or mutant viruses at an MOI of 1. After 1 hour adsorption at 37^oC, the inoculum was replaced with 2ml of maintenance medium and the plate incubated at 37^oC. At different time-points, the medium was discarded and the cells washed with PBSA. 350μl of buffer RLT with β-mercaptoethanol (10μl mercaptoethanol per 1ml buffer RLT) was added to each well. Lysed cells were homogenised by vigorous pippetting, transferred to a 1.5ml eppendorf tube, drop frozen in liquid nitrogen and stored at -80^oC. Frozen samples were thawed at room temperature and incubated at 37^oC for 10 minutes. Cell lysate (350μl) was transferred onto a QIAshredder column

(Qiagen, West Sussex, UK) and centrifuged for 2 minutes at 16,170xg at room temperature. 350µl of 70% ethanol were added to the lysate, mixed well by pipetting, and up to 700µl of lysate was applied to an RNeasy mini column and centrifuged for 15 seconds at 9,280xg at room temperature. The flow-through was discarded. 700µl buffer RW1 was added to the RNeasy column, which was centrifuged at 9,280xg for 15 seconds. The RNeasy column was transferred to new 2ml collection tube, 500µl of buffer RPE (ethanol added) added and centrifuged for 2 minutes at 15,680xg at room temperature. The RNeasy column was placed into a new collection tube and centrifuged for 1 minute at 15,680xg at room temperature. The RNeasy column was then placed into an RNase free eppendorf, 50µl RNase free water added and the column centrifuged for 1 minute at 9,280xg at room temperature to elute the RNA. The elution step was repeated using the first elutant.

2.13.2 DNA removal from RNA samples

The TURBO DNA-*free*™ Kit (Ambion, Warrington, UK) was used to remove DNA from RNA samples (Section 2.13.1) according to the manufacturer's instructions. 5µl (0.1 vol) of 10X TURBO DNase buffer and 1µl of TURBO DNase (2U/µl) were added to 50µl of RNA sample. The mixture was then incubated for 30 minutes at 37°C, 5µl of DNase inactivation reagent (0.1vol) added and incubated for a further 2 minutes at room temperature with occasional mixing followed by centrifugation for 1.5 minutes at 10,000xg at room temperature. The supernatant was transferred to a new tube and stored at -80^{o}C.

2.13.3 Protein and DNA synthesis inhibition

Inhibition of protein and DNA synthesis was achieved using cycloheximide and phosphonoacetic acid (Sigma, Dorset, UK) respectively. NIH 3T3 cells were seeded at $5x10^5$ cells in 3ml GM in each well of a 6 well flat bottomed tissue culture plate. After 12 to 16 hours incubation at 37°C, GM was discarded and the cells were infected with wt virus at an MOI 1. After 1 hour adsorption at 37°C, the inoculum was replaced with 2ml of MM supplemented with 200μg/ml cycloheximide or 300μg/ml phosphonoacetic acid and incubated for 4 hours or 24 hours at 37°C. RNA was then isolated as described in section 2.13.1.

2.13.4 Reverse transcriptase polymerase chain reaction (RT-PCR)

The reverse transcription reaction was setup as follows:

Component	**Amount (μl)**
Total RNA (1-5 μg)	--
Gene specific primer (GSP) (1μM))(Sigma-Genosys)	2.0
dNTP mix (5mM each)(Abgene)	1.0
RNase free water	to 12

This mixture was heated to 65°C for 5 minutes and then chilled on ice. After a brief centrifugation, 4μl of 10x first-strand buffer (Invitrogen, Paisley, UK), 2μl of 0.1M DTT (dithiothreitol) (Invitrogen, Paisley, UK) and 1μl of RNasin® RNase inhibitor (Promega, Southampton, UK) was added to the mixture. The contents of this tube were incubated for 2 minutes at 42°C and then 1μl of Superscript™ II Reverse Transcriptase (200 units) (Invitrogen, Paisley, UK)

was added. The transcription of RNA to cDNA was performed at 42°C for 50 minutes. Finally, the reaction was stopped by heating at 70°C for 15 minutes. The generated first-strand cDNA was used as template for PCR (Section 2.5.1).

2.13.5 RACE

Rapid amplification of cDNA ends (RACE) was carried out using the GeneRacer™ kit (Invitrogen, Paisley, UK) as recommended by the manufacturer. The protocol used for RACE is shown in Figure 2.5.

2.13.5.1 Dephosphorylating RNA

Total RNA was isolated from wt MCMV infected NIH 3T3 cells (section 2.13.1). To dephosphorylate mRNA, 10µl reactions were prepared as below:

RNA (1 to 5µg)	--
10x CIP buffer	1µl
RNAaseOut™ (40U/µl)	1µl
CIP (10U/µl)	1µl
DEPC water	to 10µl

The samples were mixed gently by pipetting, vortexed briefly and centrifuged to collect the fluid. After incubation at 50°C for 1 hour, the samples were centrifuged briefly and placed on ice. The RNA was then precipitated as described in section 2.13.5.2.

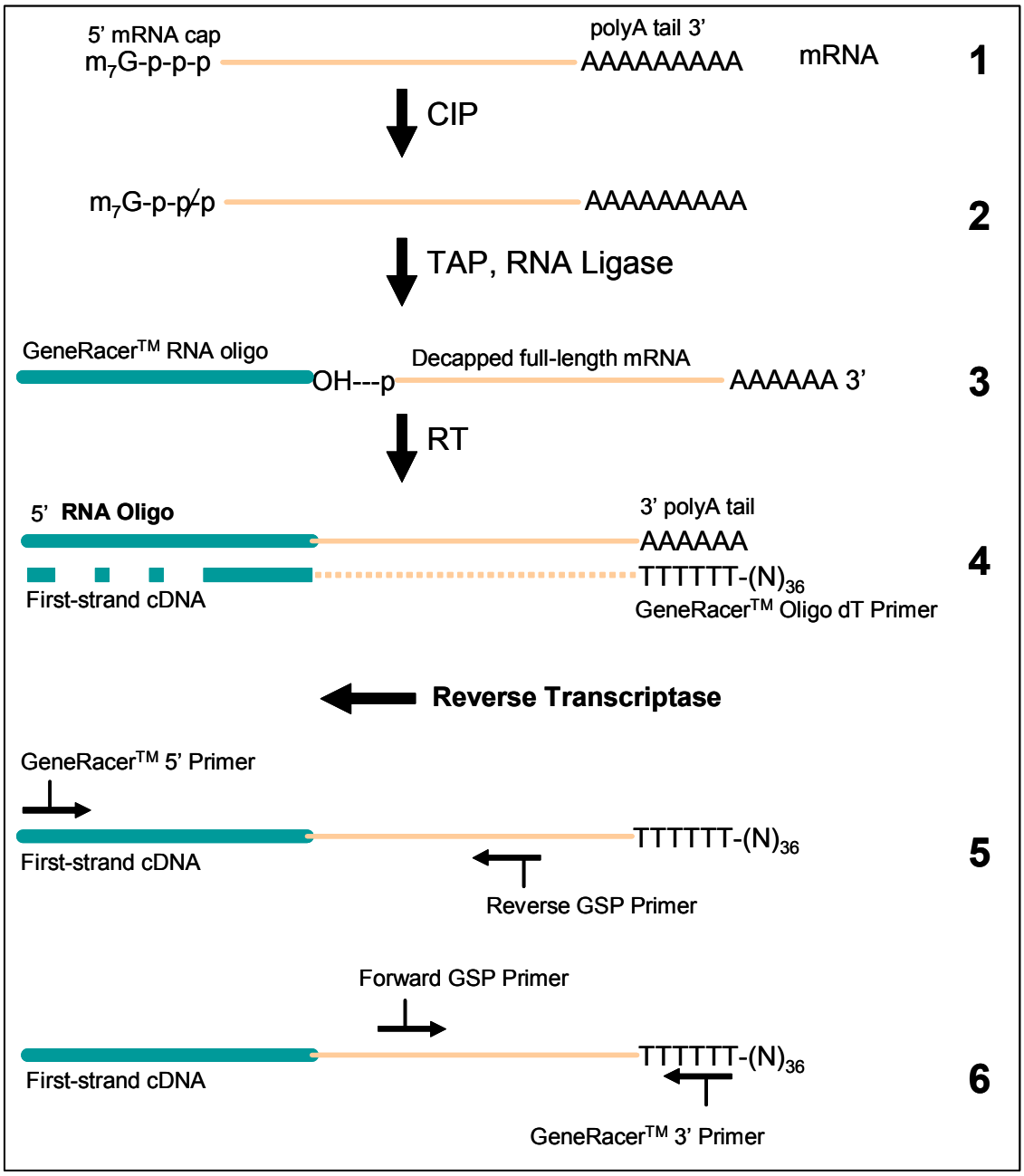


Figure 2.5 Strategy used in RACE. 1. Total RNA is treated with calf intestinal phosphatase (CIP) to remove 5´ phosphate from partial transcripts. **2.** RNA is treated with tobacco acid pyrophosphatase (TAP) to remove the cap from capped mRNA and expose the 5´ phosphate. **3.** The GeneRacer™ RNA Oligo is ligated to the TAP treated mRNA with T4 RNA ligase. **4.** A cDNA template is generated by reverse transcription using SuperScript™ III RT and the GeneRacer™ Oligo dT Primer. **5.** 5´ ends are PCR amplified from these cDNA templates with a primer for the GeneRacer™ RNA Oligo (GeneRacer™ 5´ Primer) and gene-specific primer (GSP). **6.** 3´ ends are PCR amplified from these cDNA templates with a primer for the GeneRacer™ Oligo dT (GeneRacer™ 3´ Primer) and GSP.

2.13.5.2 Precipitating RNA

To precipitate RNA, 90μl DEPC water and 100μl phenol:chloroform were added. The samples were vortexed for 30 seconds, centrifuged at 20,880xg for 5 minutes at room temperature and the aqueous (top) phase transferred to a fresh tube. 2μl mussel glycogen (10mg/ml), 10μl 3M sodium acetate and 220μl 95% ethanol were added, the samples vortexed briefly and then frozen in dry ice for 10 minutes. The RNA was pelleted by centrifugation at 20,880xg for 20 minutes at +4 ^{o}C, the supernatant carefully removed, 500μl of 70% ethanol added, mixed by inversion several times, vortexed briefly and then the contents centrifuged at 20,880xg for 2 minutes. The supernatant was removed carefully, the pellet allowed to air-dry for not more than 1-2 minutes at room temperature and then resuspended in 7μl DEPC water.

2.13.5.3 Removing the mRNA cap structure

To remove the cap structure, the following 10μl reaction was set up:

Dephosphorylated RNA	7μl
10x TAP buffer	1μl
RNAaseOut^{TM} (40U/μl)	1μl
TAP(0.5U/μl)	1μl
DEPC water	to 10μl

The samples were mixed gently by pipetting, vortexed briefly and centrifuged to collect the fluid. After incubation at 37^{o}C for 1 hour, the samples were

centrifuged briefly and placed on ice. The RNA was then precipitated as described previously (section 2.13.5.2).

2.13.5.4 Ligating the RNA oligo to decapped mRNA

The 7μl of dephosphorylated, decapped RNA was added to a tube containing the pre-aliquoted, lyophilized GeneRacer™ RNA Oligo (0.25μg), mixed and centrifuged briefly to collect the fluid in the bottom of the tube. The mixture was incubated at 65°C for 5 minutes to relax the RNA secondary structure and then placed on ice for 2 minutes. 1μl of 10x ligase buffers, 10mM ATP, RNAaseOut™ (40U/μl) and T4 RNA ligase (5U/μl) were added to the tube. The samples were mixed gently by pipetting and centrifuged briefly. After incubation at 37°C for 1 hour, the samples were centrifuged briefly and placed on ice. The RNA was then precipitated as described previously (section 2.13.5.2). The final resuspension in this step, however, was in 10μl DEPC water.

2.13.5.5 Reverse transcribing mRNA

To reverse transcribe the mRNA, GeneRacer™ oligo dT primer (1μl), dNTP mix (1μl) and DEPC water (1μl), were added to the 10μl of ligated RNA, incubated at 65°C for 5 minutes to remove any RNA secondary structure, chilled on ice for 1 minute and centrifuged briefly. 4μl first strand buffer (5x), 1μl DTT (0.1M), 1μl RNAaseOutTM (40U/μl) and 1μl Superscript™ III (200U/μl) were added to the 13μl ligated RNA and primer mixture. The samples were

mixed gently by pipetting and centrifuged briefly. The sample was incubated at 50^{o}C for 1 hour and then at 70^{o}C for 15 minutes to inactivate the reverse transcriptase. This was then chilled on ice for 2 minutes and centrifuged briefly. 1μl RNase H (2U) was added and the reaction mix incubated at 37^{o}C for 20 minutes. Again the samples were centrifuged briefly, diluted in distilled water at 1:20 ratio and used immediately for amplification or stored at -20^{o}C. The control template supplied in this kit was Hela cell total RNA.

2.13.5.6 Amplifying cDNA ends, cloning and sequencing

The 5' ends were amplified by standard PCR (section 2.5.1) using 2μl diluted cDNA from the above step with RNA oligo specific GeneRacer^{TM} 5' primer and reverse GSP and 3' ends were amplified with oligo dT specific GeneRacer^{TM} 3' primer and forward GSP. The PCR products were analysed by agarose gel electrophoresis (section 2.7) and the appropriate sized PCR products were cloned in pGEM®-T Easy vector (Promega, Southampton, UK) and later sequenced (section 2.10) with a vector and GSP.

2.13.6 Northern blot

Northern blotting was carried out using Northern Max® kit (Ambion, Warrington, UK). 20μg total RNA (Section 2.13.1) was precipitated as described as Section 2.13.5.2 and dissolved directly in 20μl formaldehyde load dye. The RNA samples and 5μl of RNA Millennium Markers™ (Ambion, Warrington, UK) were incubated for 15 minutes at 65°C to denature RNA

secondary structure. Samples were then loaded onto a 1% formaldehyde-agarose gel prepared using RNase-free water and denaturing gel buffer (contains formaldehyde). The gel was run at 5V/cm for 3 hours using MOPS gel running buffer (Ambion, Warrington, UK).

RNA was transferred from the gel to a BrightStar-Plus membrane (Ambion, Warrington, UK) by a downward transfer assembly as described in the kit. This is a modified method from Chomczynski (31). Transfer buffer (0.5ml/cm^2 of gel surface) was added to a plastic flat bottomed dish larger than the dimension of the agarose gel to accommodate the transfer buffer and to wet the blotting paper, membrane, and bridge. The membrane and 3MM Whatman filter paper were cut slightly larger than the gel. Three filter paper bridges were cut large enough to cover the area of the gel and to reach across into the transfer buffer reservoir. The blot was set up on a stack of paper towels (~3 cm high), overlaid with 3 pieces of filter paper, 2 pieces of wet filter paper, a wet membrane, the gel, 2 pieces of wet filter paper and 3 filter bridges with one end in the transfer buffer reservoir. The stack was covered with the casting tray to prevent evaporation and a small weight (150–200 g) placed on top of the stack to ensure even contact of all the stack components. After 1.5 to 2 hours transfer, the membrane was rinsed briefly in 1x gel running buffer and crosslinked using a UV crosslinker (UVI Tec, Cambridge, England). The membrane was stained with 0.04% ($^{w}/_{v}$) methylene blue (Sigma, Dorset, UK) in 0.5M sodium acetate pH 5.2 for 5 minutes and then destained in RNase-free water to check that the RNA had transferred successfully.

Membranes containing RNA were prehybridised in ULTRAhyb (10ml/100cm^2 membrane) at 42°C in a Hybaid minioven. After 30 minutes, the radioactive probe (see Sections 2.13.5.1 and 2.13.5.2 below) was added and the hybridisation continued overnight. The hybridised membrane was washed twice at room temperature with low stringency solution # 1 (Ambion, Warrington, UK) for 5 minutes and also washed twice with high stringency solution # 2 for 5 minutes with agitation. The blot was then exposed to X-ray film for autoradiography.

2.13.6.1 Production of random primed DNA probe

Random primed DNA probes were generated using the DECAprime™ II random primed DNA labeling Kit (Ambion, Warrington, UK) according to the manufacturer's instructions. Briefly, PCR template DNAs containing part of the m29 or m29.1 ORF were used in the probe synthesis reaction. Linear template double stranded DNA was diluted in water to a concentration of 1-2.5 ng/µl in a volume of up to 11.5µl. 2.5 µl of 10x Decamer solution was added and incubated at 95-100ºC for 3–5 minutes to denature the DNA. The denatured DNA/decamer mix was then added to the reaction tube in the following amounts:

5X Reaction buffer (–dCTP)	:5µl
[α-32P]dCTP (GE Healthacare)	:5µl
Water	:to 24µl
Exo-klenow	:1µl

The contents of tube were mixed by gentle flicking or pipetting and then incubated for 10 minutes at 37°C. The reaction was stopped by adding 1µl of 0.5 M EDTA. The resultant DNA probe was diluted (~10 fold) with 10mM EDTA, incubated for 10 minutes at 90°C and centrifuged briefly to collect the solution at the bottom of the tube. Approximately 500µl ULTRAhyb was added to the denatured probe and then transferred to the prehybridised blot.

2.13.6.2 Production of single stranded PCR probe

The single stranded PCR probe was generated using the Strip-EZ® PCR kit (Ambion, Warrington, UK) according to the manufacturer's instructions. The reaction was assembled on ice as below:

Components	**Amount**
10X PCR buffer	2µl
10X dNTP solution	2µl
[α-32P] dATP (3000 Ci/mmol, 10 mCi/ml) (GE Healthacare)	2µl
Antisense strand primer (10µM)	2µl
PCR DNA template	2-10ng
BIOTAQ DNA polymerase (5 U/µl) (Bioline, London, UK)	0.3µl
Distilled water	to 20µl

The PCR reaction was set as follows: 1 cycle of 95°C for 30 seconds and 35 cycles of 95°C for 30 seconds, 55°C for 20 seconds and 72°C for 1 minute. 2.2µl of 5M NH_4OAc and 45µl of 100% ethanol were added to the 20µl labelling reaction, incubated at –20°C for 15 minutes and microfuged for 15 minutes at top speed at 4°C. The supernatants were removed carefully, dissolved the pellet dissolved in 50µl of water and stored at –20°C until use.

2.14 Protein work

2.14.1 Preparation of protein samples from bacterial cells

The expression vector, pET28a-m29 and pET28a-m29.1, was used to transform *Escherichia coli* C41 (DE3) cells (Imaxio, France). A single transformant colony was used to inoculate 3ml LB broth containing 25μg/ml Kanamycin and grown overnight at 37°C. Expression cultures were inoculated using 5ml of the starter culture per litre of LB broth at 37°C. Once the cells had grown to A_{600}=0.5-0.7, the m29 and m29.1 protein expression was induced with 1mM isopropyl-1-thio-β-D-galactopyranoside. Growth was continued for a further 4 hours at 37°C when cells were harvested by centrifugation at 4,000xg for 30 minutes at 4°C and stored frozen at -20°C. The thawed cells were resuspended in 50 mM Tris/HCl, 500 mM NaCl pH 7.9 and then lysed using a French Pressure cell at 3000 psi. The resuspension was monitored by SDS-PAGE (section 2.14.3) to check the expression of recombinant protein. The lysate was clarified by centrifugation at 20,000xg at 4°C for 15 minutes and the supernatant was monitored by SDS-PAGE (section 2.14.3) to check the solubility of the recombinant protein.

2.14.2 Preparation of protein samples from infected NIH 3T3 cells

Protein samples were generated from wt, mutant virus and mock infected NIH 3T3 cells. Virus or mock infected cells from 75cm^2 tissue culture flasks were detached using a cell scraper and transferred back to the centrifuge tube. This was then centrifuged at 6,000xg for 10 minutes at room temperature, the cell

pellets lysed in 500μl ice-cold lysis buffer (1% Triton-X-100, 50mM Tris HCL pH 7.5, 150mM NaCl, 1mM EDTA, 10% glycerol, 1mM PMSF and 1μg/ml each of chymostatin, aprotinin, leupeptin and pepstatin) for 15 minutes and the lysates were clarified by centrifugation at 15,000xg for 10 minutes at 4°C; the supernatants were stored at -80°C.

2.14.3 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins using a Mini-Protean 3 gel system (Bio-Rad, Herts, UK) according to the manufacturer's instructions. The following reagents were used for SDS-PAGE:

Resolving gel (15%)

Distilled water	1.1ml
30% acrylamide mix	2.5ml
1.5M TRIS (pH 8.8)	1.3ml
10% SDS	0.05ml
10% ammonium persulfate	0.05ml
TEMED	0.002ml

Stacking gel (4%)

Distilled water	3.025ml
30% acrylamide mix	0.67ml
0.5M TRIS (pH 8.8)	1.25ml
10% SDS	0.05ml
10% ammonium persulfate	0.025ml
TEMED	0.005ml

10x SDS-PAGE running buffer

TRIS	30.3 g
Glycine	144 g
SDS	10 g
Distilled water	to 1 litre

Coomassie stain

Coomassie blue 83	0.1% (w/v)
Methanol	45% (v/v)
Glacial acetic acid	10% (v/v)

Gel de-stain solution

Acetic Acid	70ml
Methanol	250ml
Distilled water	to 1 litre

Proteins were run with 1x SDS PAGE running buffer at 200V until the marker dye reached the end of the gel. The gel with unstained marker (Fermentas) was stained overnight with coomassie blue and destained again in destaining solution and the gel with stained marker (NEB, Herts, UK) was used for western blot analysis (section 2.14.5).

2.14.4 Purification of His-m29.1 protein

The expression vector, pET28a-*m29.1,* was used to transform *Escherichia coli* C41 (DE3) cells (Imaxio, France). A single transformant colony was used to inoculate 50ml LB broth containing 25µg/ml Kanamycin, which was grown to

mid-exponential phase for several hours at 37°C. Expression cultures were inoculated using 5ml of the starter culture per litre of LB broth at 37°C. Once the cells had grow to A_{600}=0.7-0.9, m29.1 protein expression was induced with 1mM isopropyl-1-thio-β-D-galactopyranoside. Growth was continued for a further 4 hours when cells were harvested by centrifugation at 4,000xg for 30 minutes at 4°C and stored frozen at -20°C.

The cells were resuspended in 50 mM Tris/HCl, 500 mM NaCl pH 7.9, lysed using a French Pressure cell at 3000 psi. The lysate was clarified by centrifugation at 27,000xg for 30 minutes at 4°C and the supernatant was loaded directly onto a Ni^{2+}-loaded His-Trap affinity column (GE Healthcare, Bucks, UK) pre-equilibrated with the lysis buffer. After loading, the column was washed with 10 column volumes of 50mM Tris/HCl pH7.9 containing 500mM NaCl, and the protein was eluted with a gradient of increasing imidazole concentration in the same buffer. Elution was monitored by SDS-PAGE. Fractions containing pure protein were pooled and stored at -20°C.

2.14.5 Western blot

Western blots were performed to monitor the presence of m29 and m29.1 protein in virus infected cells. The proteins (section 2.14.2) separated by electrophoresis in a polyacrylamide gel were transferred to a PVDF membrane (Bio-Rad, Herts, UK) by transverse electrophoresis in western blot buffer (Glycine 14.4g, Tris 3g, Methanol 400ml and distilled water up to 1 litre) at 100V for 1 hour and 20 minutes. After blocking in 5% non-fat dried milk in TBS

buffer (Tris 3.03g, NaCl 8g, Tween 20 500μl pH 8 and distilled water up to 1 litre) for 1 hour, blots were washed three times (each time for 15 minutes) in TBS buffer. Membranes were incubated with primary antibody (section 2.14.6) in 5% non-fat dried milk in TBS buffer (dilution of 1:300) for 1-2 hours. Membranes were washed three times in TBS buffer and incubated overnight with peroxidase conjugated anti-rabbit IgG (Sigma, Dorset, England) in 5% non-fat dried milk in TBS buffer (dilution of 1:1000). Membrane was washed three times and immersed in developer solution [16ml PBS, 4ml 4-chloro-1-naphthol (4mg/ml) (Sigma, Dorset, England) and 30μl H_2O_2] until the colour developed and was then finally washed in cold water to stop the reaction.

2.14.6 Generation of antibodies to m29 and m29.1 proteins

A synthetic peptide (CDRDTPHEQRSGVSG) from m29 was synthesised (Biogenes, Berlin, Germany). Two rabbits were immunised with the synthetic peptide to raise polyclonal antibodies. The immunisation of each animal was carried out according to the following protocol (Biogenes, Berlin, Germany):

Day	**works**	**Serum (ml)**
0	pre-serum/ immunisation	1.5
7	boost	--
14	boost	--
28	boost	--
35	bleeding	20
49	boost	--
63	bleeding	20
77	boost	--
91	final bleeding	75

The m29.1 protein (Section 2.14.4) was supplied to Biogenes (Berlin, Germany) to raise polyclonal antibodies by immunising two rabbits. The immunisation protocol was undisclosed.

2.15 Viral growth studies in animals

BALB/c (4 weeks old) and CB17 SCID (6 weeks old) mice were obtained from the Biomedical Services Unit at the University of Birmingham and housed 4 animals/cage in a positive pressurized flexible film isolator one week before the experiment. The food and water were available *ad libitum*. The experiment was carried out in accordance with home office project licence PPL 40/2422 and personal licence PIL 40/8120. One hundred and twenty eight BALB/c mice were arranged into 4 groups of 32 animals and each group was inoculated intraperitoneally with 50µl of MM containing 10^4 PFU of wt, Rc29, Rc29.1 and Rv29.1 viruses. Sixty four CB17 SCID mice were arranged into 2 groups of 32 animals and each group was inoculated intraperitoneally with 50µl of MM containing 10^4 PFU of wt or Rc29.1 viruses. At 3, 7, 10, 14, 21, 28, 35 and 42 days post-infection, 4 animals were sacrificed and dissected aseptically to remove salivary glands, liver, kidneys, heart, spleen and lungs which were frozen at -80°C. Once thawed, tissues were homogenised in 1ml (spleen) or 2ml (all other tissues) of GM using a homogeniser (Pro 200, Monroe, USA). Homogenised tissues were centrifuged at 720xg for 10 minutes at 4°C and the supernatants stored at -80°C. The frozen samples were thawed at room temperature and titres were determined by plaque assay (section 2.2.6).

3. RESULTS

To characterize the role of the m29 and m29.1 ORFs in MCMV replication, the genes were mutated to introduce a point mutation to produce a stop codon and mutant viruses were analyzed for their growth properties both *in vitro* and *in vivo*.

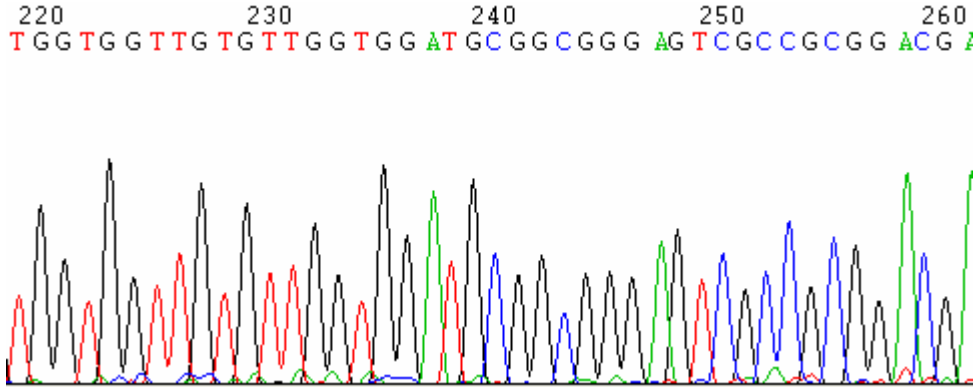
3.1 Newly identified ORFs

The wild type virus reconstructed from MCMV Smith BAC (176) was previously sequenced over the m29 and m29.1 ORFs region by Dr Melissa Kirby in our laboratory. She identified a sequence discrepancy to the published MCMV genome (128) in our Smith strain MCMV BAC. An extra nucleotide, a guanine residue, at nucleotide (nt) position 36,198 (Figure 3.1) changed the predicted ORFs for m29 and m29.1. To confirm this, the MCMV Smith strain (Birmingham), the K181 strain (Birmingham) (136) and natural wild type isolates (N1, K17A and G4) (89) were sequenced (Figures 3.1, 3.2 & 3.3). The predicted protein of the newly identified ORF for m29 is 242 amino acids, 85 amino acids shorter than that published (128) (Figure 3.4) whereas the m29.1 protein is 210 amino acids, 27 amino acids longer than the published sequence (Figure 3.5). A single base change (G to C) was also found at nt position 35,944 (128) in MCMV Smith (Birmingham), K181, K17A, G4 and N1 (89) (Figure 3.2). A further cytosine insertion at nt position 37263, as identified by

Dr Melissa Kirby, in our Smith strain MCMV BAC which also alters the predicted ORF for m30. To confirm this, the MCMV Smith Strain (Birmingham) and natural wild type isolates (N1, K17A and G4) were sequenced (Figure 3.6), although it was not an objective of this study. This insertion alters the ORF such that the stop codon in m30 no longer exists and the predicted protein continues into the published M31 ORF. All sequences were deposited in the EMBL database under the following accession numbers:

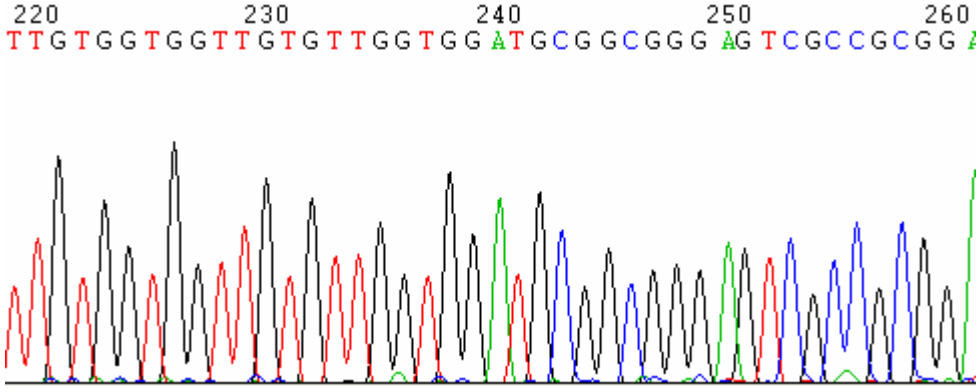
Strain	**Gene name**	**Accession numbers**
K181 Birmingham	m29	AM491340
K17A Birmingham	m29	AM491341
Smith Birmingham	m29	AM491342
Smith BAC Germany	m29	AM491343
N1 Birmingham	m29	AM491344
G4 Birmingham	m29	AM491345
K181 Birmingham	m29.1	AM491346
Smith Birmingham	m29.1	AM491347
Smith BAC Germany	m29.1	AM491348
K17A Birmingham	m29.1	AM491349
N1 Birmingham	m29.1	AM491350
G4 Birmingham	m29.1	AM491351
K17A Birmingham	m30	AM600907
G4 Birmingham	m30	AM600906
N1 Birmingham	m30	AM600908
Smith BAC Germany	m30	AM600910
Smith Birmingham	m30	AM600909

Smith BAC



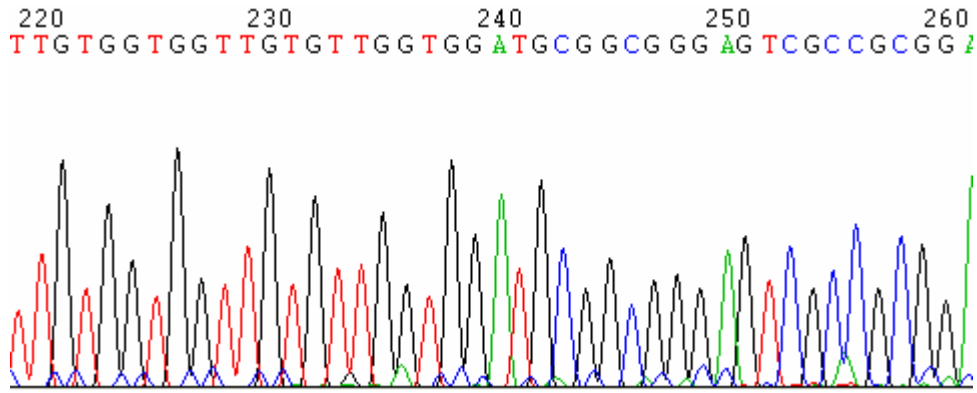

```
35938F 1       TGGTGGTTGTGTTGGTGGATGCGGCGGGAGTCGCCGCGGACG 42
               ||||||||||||||||||||| ||||||||||||||||||||
MCMV     36178 TGGTGGTTGTGTTGGTGGAT-CGGCGGGAGTCGCCGCGGACG 36218
```

K181




```
35938F 1    TTGTGGTGGTTGTGTTGGTGGATGCGGCGGGAGTCGCCGCGG 42
            ||||||||||||||||||||| ||||||||||||||||||
MCMV 36175  TTGTGGTGGTTGTGTTGGTGGAT-CGGCGGGAGTCGCCGCGG 36215
```

K17A




```
35938F  1    TTGTGGTGGTTGTGTTGGTGGATGCGGCGGGAGTCGCCGCGG  42
             ||||||||||||||||||||| ||||||||||||||||||
MCMV  36175  TTGTGGTGGTTGTGTTGGTGGAT-CGGCGGGAGTCGCCGCGG  36215
```

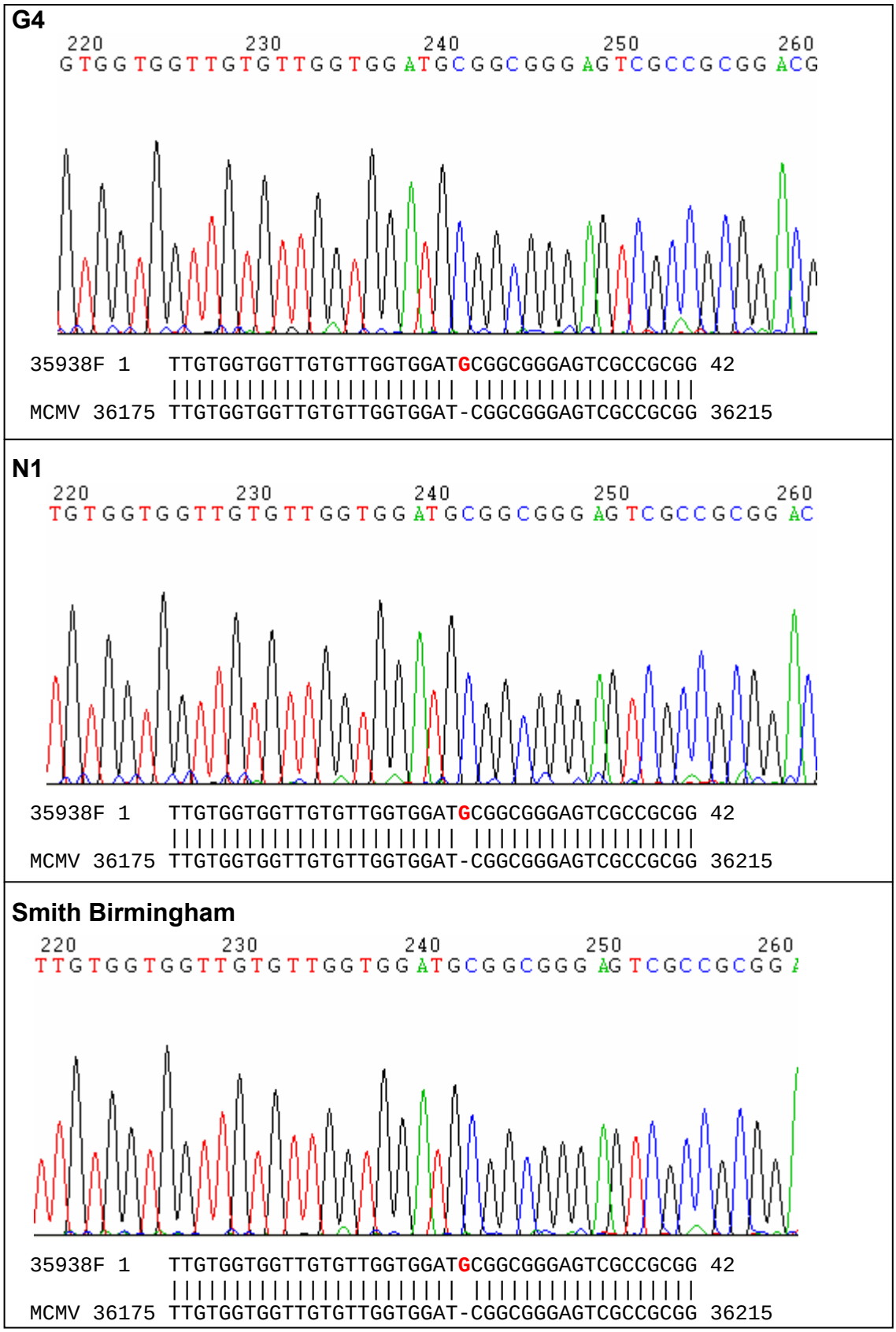


Figure 3.1 Sequencing over extra nucleotide (nt) region in MCMV Smith BAC, K181, K17A, G4, N1 and Smith Birmingham using primers 35938F.

The extra nt Guanine (G) is indicated in red. The gap in the published Smith strain MCMV genome (accession no. U68299) is shown with a dash.

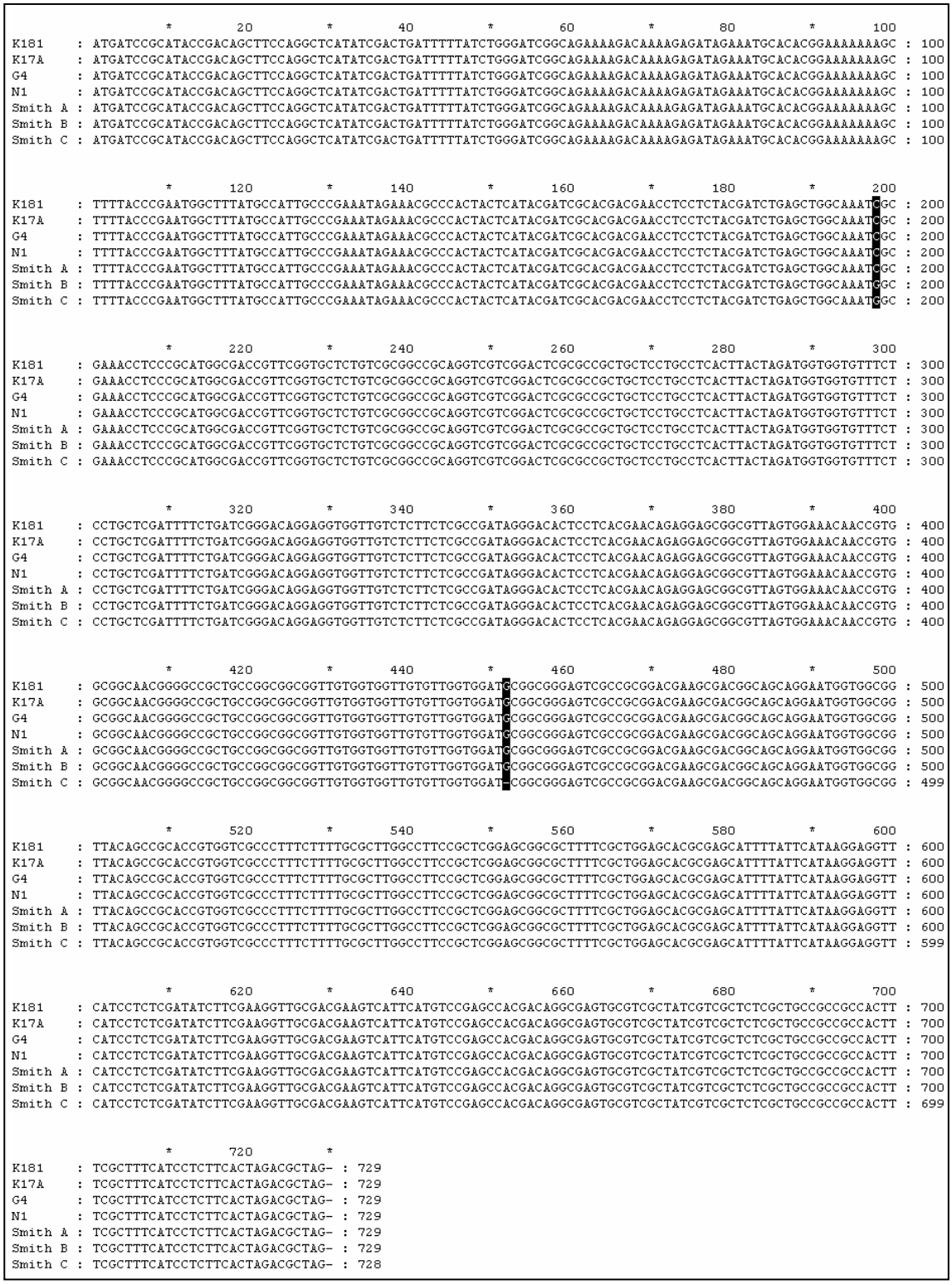


Figure 3.2 Nucleotide sequence alignment of MCMV K181 (Birmingham), K17A, G4, N1, Smith (Birmingham) (A), Smith BAC (B) and Smith published sequence (C) over the m29 region (nt 35747 to 36474) (accession no U68299).

Nucleotides which differ from the published sequence (accession no U68299) are shown in the red box and the extra nucleotide in all viruses is shown with the highlights.

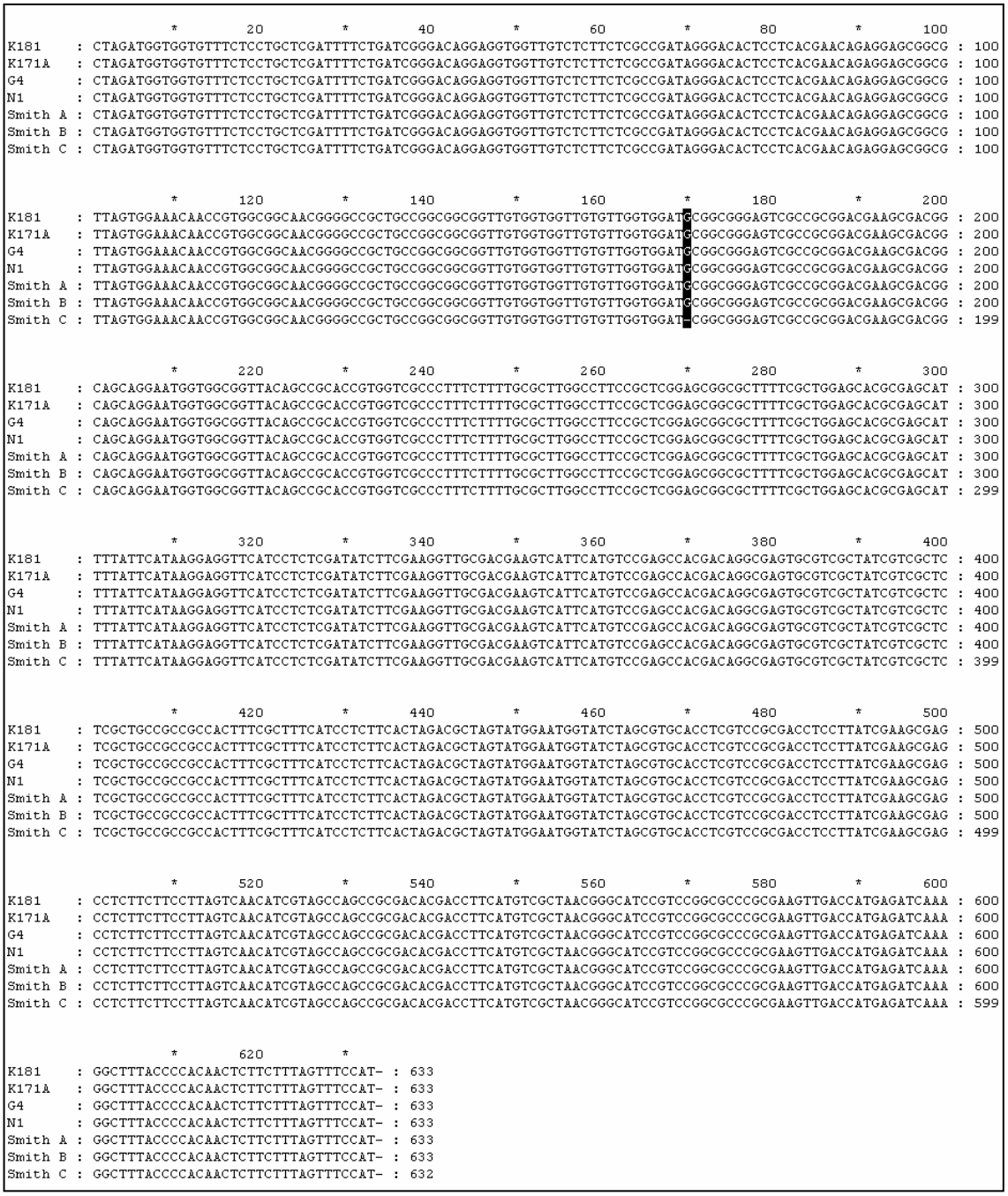


Figure 3.3 Nucleotide sequence alignment of MCMV K181 (Birmingham), K17A, G4, N1, Smith (Birmingham) (A), Smith (BAC) (B) and Smith published sequence (C) over m29.1 region (nt 36029 to 36660) (accession no U68299). The extra nucleotide is shown by highlights.

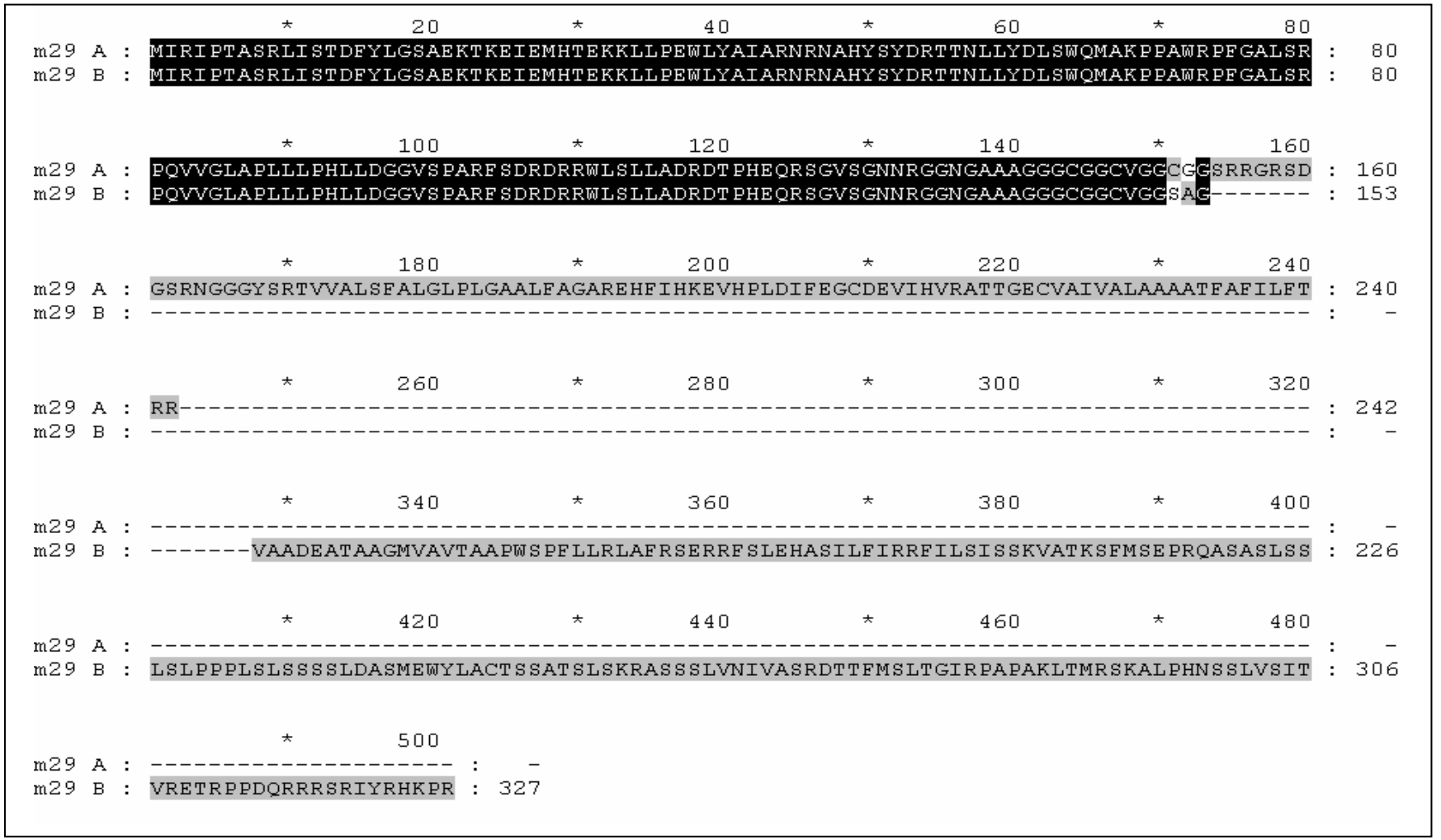


Figure 3.4 Amino acid sequence alignment of predicted protein of the newly identified ORF for m29 (A) and for the published m29 (B) (accession no. U68299)

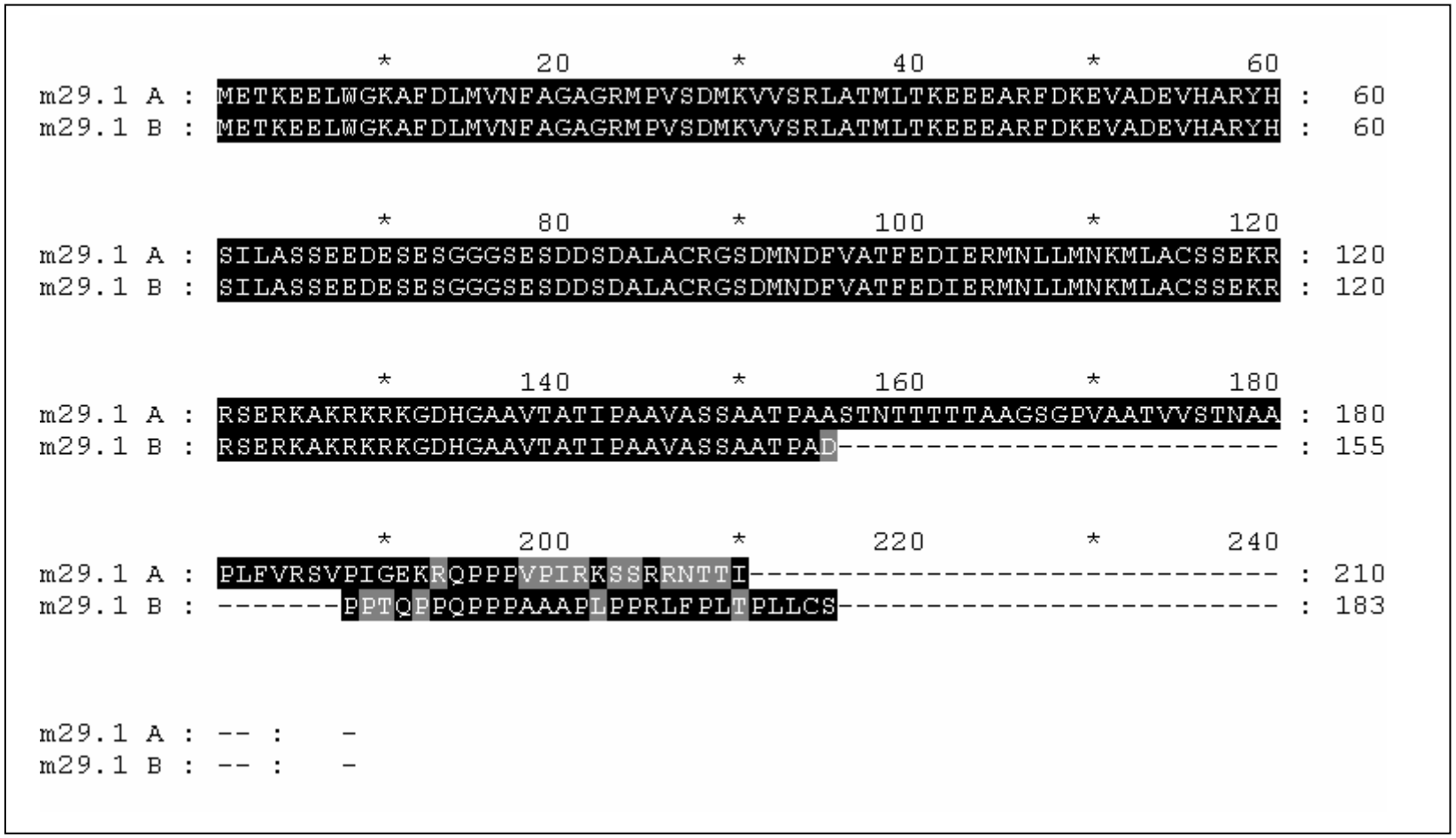


Figure 3.5 Amino acid sequence alignment of predicted protein of the newly identified ORF for m29.1 (A) and for the published m29.1 (B) (accession no. U68299)

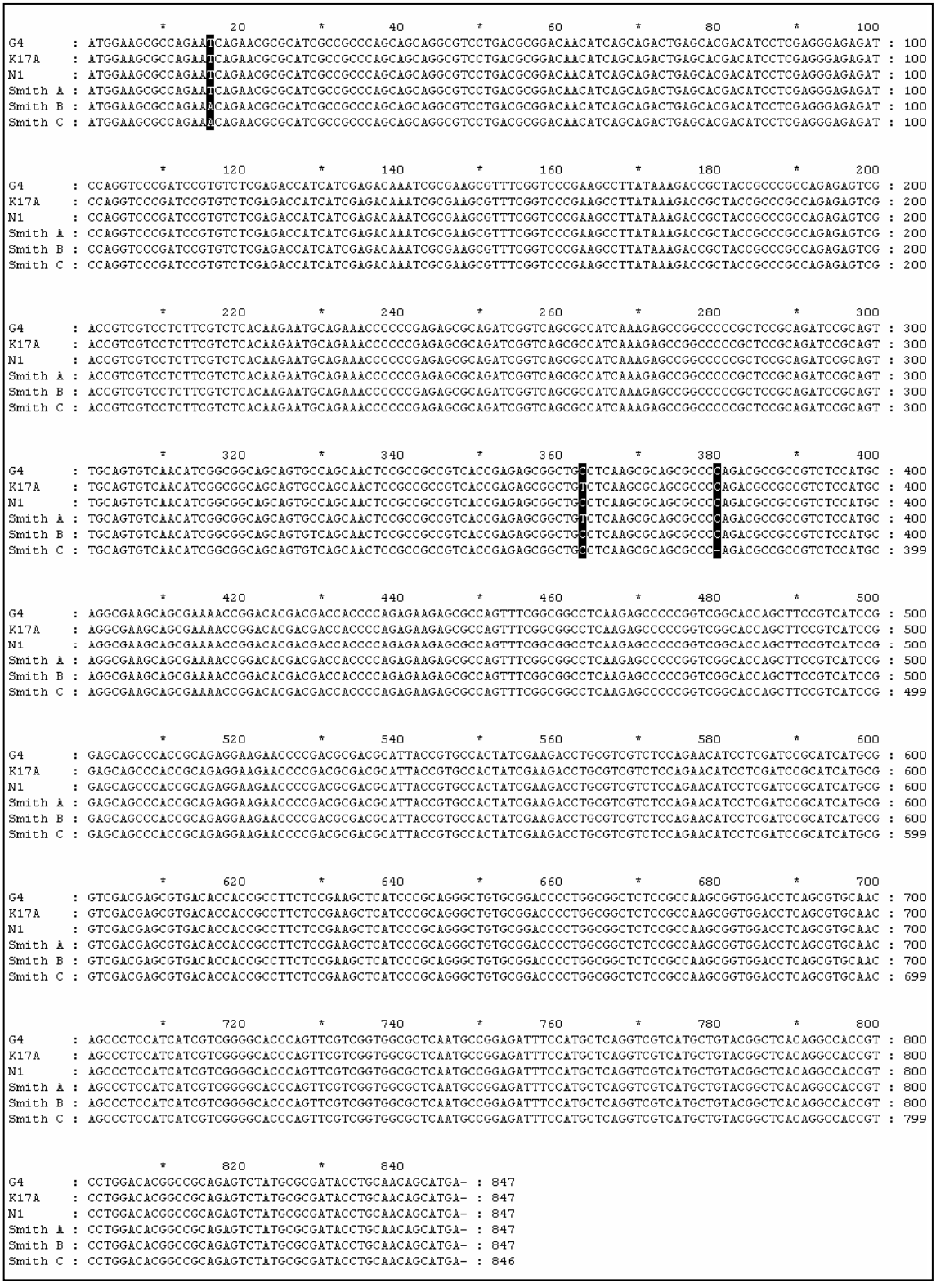


Figure 3.6 Nucleotide sequence alignment of MCMV Smith (Birmingham) (A), G4, K17A, N1, Smith BAC (B) and Smith published sequence (C) over the m30 region (nt 36884 to 377429) (accession no U68299).

Nucleotides which differ from the published sequence (accession no U68299) are shown in the highlights.

3.1.1 Amino acid sequence comparison of the newly identified genes with other CMV proteins

Computation was performed using BLAST network service (6) to test whether the newly identified m29 and m29.1 genes are homologous with any other CMV proteins. Neither of these predicted proteins was found to have significant homology to any recorded proteins in the database.

3.2 RT-PCR for viral gene expression in wt MCMV infected cells

3.2.1 Detection of m29 and m29.1 gene expression

The expression of m29 and m29.1 transcripts was detected in wt MCMV infected NIH 3T3 cells by RT-PCR. Total RNA was isolated from infected and mock infected cells at different times post-infection. Gene specific primers (GSP) RTm29R and M29.1FOR (Table 2.3) were used in generating cDNA to detect the m29 and m29.1 transcripts respectively.

Primers RTm29F/RTm29R and M29.1FOR/M29.1REV were designed to amplify a 708bp and 596bp RT-PCR product from m29 and m29.1 ORFs respectively. The whole MCMV genomic DNA was used as a positive control for the PCR reaction and in the negative control, the reverse transcriptase was omitted to confirm that the isolated RNA was free from DNA contamination. Amplification of a 708bp (Figure 3.7) and a 596bp (Figure 3.8) product

confirmed the expression of m29 and m29.1 respectively in wt MCMV infected NIH 3T3 cells.

The m29 gene specific transcript was first detected 3 hours post-infection and for all tested samples at subsequent times up to at least 24 hours post-infection (Figure 3.7). However, a RT-PCR product of approximately 300bp (Figure 3.7) was also detected in all tested and mock infected samples. It was expected that this product might come from a host gene i.e. a mouse gene. This 300bp product was sequenced to confirm this (data not shown). The m29.1 gene specific transcript was first detected at 2 hours post-infection and detected in all tested samples at subsequent times (Figure 3.8).

3.2.2 Detection of newly predicted m29.2 gene expression

Brocchieri and colleagues (20) predicted a new ORF, named m29.2, on the complementary strand of the MCMV genome just prior to the m29.1 ORF, which had not been predicted during the previous sequence analysis (128). To confirm the expression of this newly identified m29.2 ORF in wt MCMV infected NIH 3T3 cells, RT-PCR was performed. Total RNA was isolated from infected and mock infected cells at 24 hours post-infection. The GSP RTm29.2F (Table 2.3) was used to generate cDNA and primers RTm29.2F and RTm29.2R were used to amplify a 121bp RT-PCR product from the m29.2 gene. The whole MCMV genomic DNA template was used as a positive control for the PCR and in the negative control, the reverse transcriptase was omitted to confirm that the isolated RNA was free from DNA contamination. Amplification of a 121bp

(Figure 3.9) product confirmed the expression of m29.2 gene in wt MCMV infected NIH 3T3 cells.

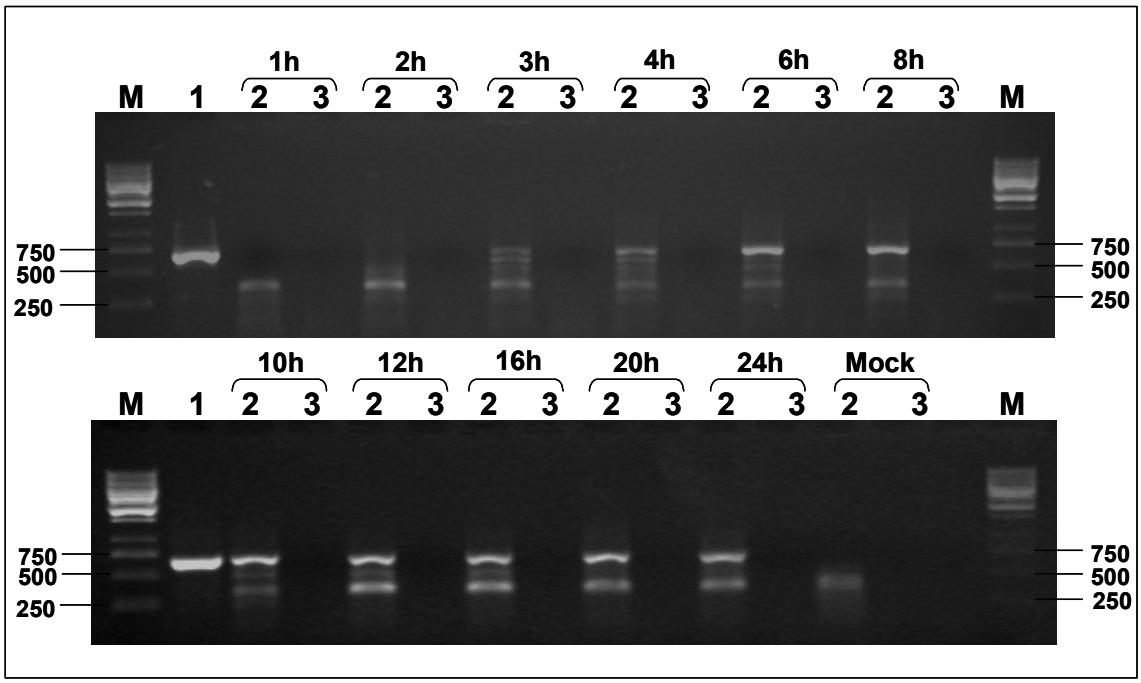


Figure 3.7 RT-PCR for m29 gene expression. Total RNA was isolated from wt MCMV and mock infected NIH 3T3 cells at different time-points post-infection. An RT-PCR product of 708bp (lane 2) indicates the presence of the m29 gene specific transcript at 3, 4, 6, 8, 10, 12, 16, 20 and 24 hours post-infection. A PCR product of approximately 300bp (RT+) indicates the presence of a non-specific transcript also seen in mock infected cells. MCMV genomic DNA was used as a positive control (lane 1) and reverse transcriptase was omitted in the negative control for the reverse transcription reaction (lane 3). The size of the markers from the 1kb ladder (Fermentas) are shown on the right and the left margins in bp.

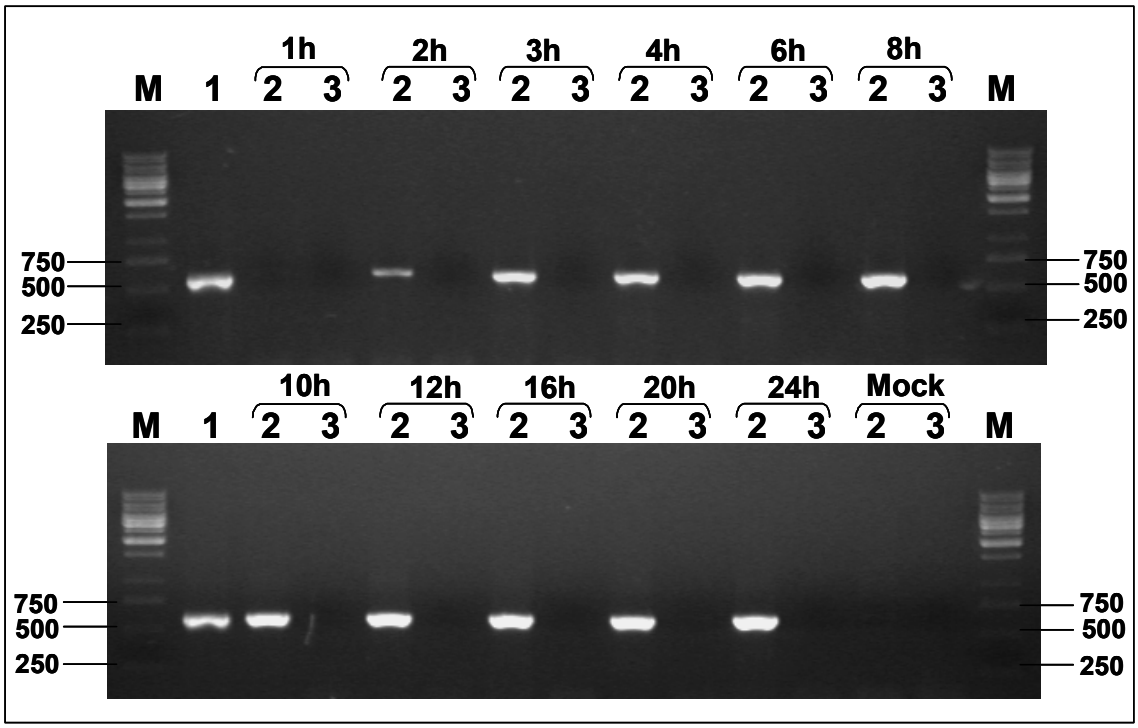


Figure 3.8 RT-PCR for m29.1 gene expression. Total RNA was isolated from wt MCMV and mock infected NIH 3T3 cells at different time-points post-infection. An RT-PCR product of 596bp (lane 2) indicates the presence of the m29.1 gene specific transcript at 2, 3, 4, 6, 8, 10, 12, 16, 20 and 24 hours post-infection. MCMV genomic DNA was used as positive control (lane 1) and reverse transcriptase was omitted in the negative control for the reverse transcription reaction (lane 3). The size of the markers from the 1kb ladder (Fermentas) are shown on the right and the left margins in bp.

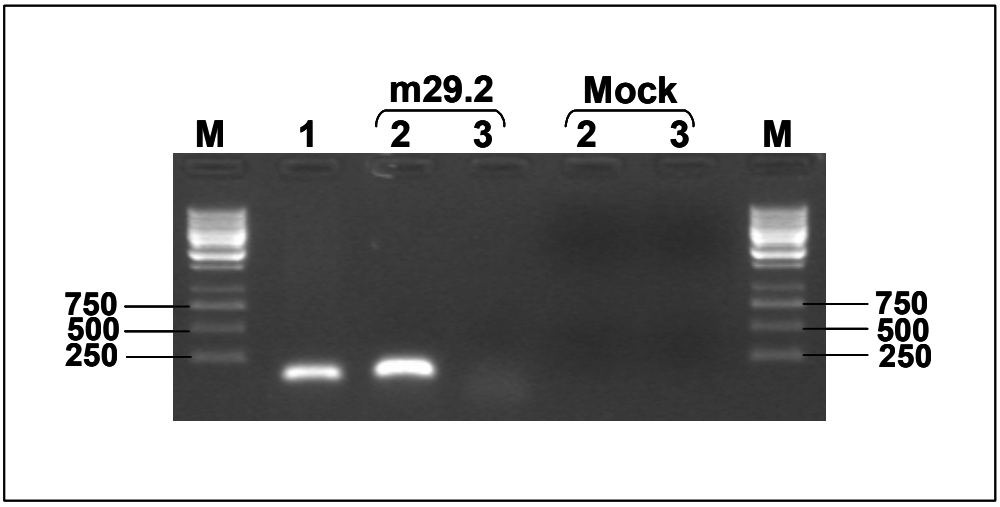


Figure 3.9 RT-PCR for m29.2 gene expression. Total RNA was isolated from wt MCMV and mock infected NIH 3T3 cells at 24 hours post-infection. The RT-PCR product of 121bp (lane 2) indicates the presence of the m29.2 gene specific transcript. MCMV genomic DNA was used as positive control (lane 1) and reverse transcriptase was omitted from the negative control for the reverse transcription reaction (lane 3). The size of the markers (M) from the 1kb ladder (Fermentas) are shown on the right and the left margins in bp.

3.2.3 Classification of m29 and m29.1 gene specific transcripts in wt MCMV infected cells

Results from Section 3.2.1 indicated that the m29 and m29.1 genes are transcribed in wt MCMV infected NIH 3T3 cells at different time post-infection. Another RT-PCR experiment was performed using RNA isolated from wt MCMV infected NIH 3T3 cells treated with protein and DNA synthesis inhibitors to categorise the observed temporal expression of m29 and m29.1 genes into IE, E or L kinetic classes. IE genes do not require *de novo* protein synthesis for their expression. Therefore, IE genes are expressed in virus infected cells treated with cycloheximide, a protein synthesis inhibitor. L genes are

essentially expressed after the onset of viral DNA synthesis (106). Thus, viral transcription is restricted to IE and E gene expression in virus infected cells treated with phosphonoacetic acid, a DNA synthesis inhibitor.

MCMV gene m123(ie1/3), M112/113(e1) and M75 have been previously classified as IE, E and E-L genes respectively and these were chosen as internal controls to check the inhibitory conditions used for the kinetic class experiments. Total RNA was isolated from wt MCMV infected NIH 3T3 cells treated with cycloheximide or phosphonoacetic acid at 4 hours and 24 hours post-infection respectively and Oligo(dT) was used in generating cDNA. The whole MCMV genomic DNA template was used as a positive control and the reverse transcriptase was omitted from the negative control. Primers were designed to bind either side of an intron for genes m123(ie1/3) and M112/113(e1), which have been reported to have spliced transcripts (128). The m123(ie1/3) spliced transcript, but not the M112/113(e1) transcript, was detected in wt MCMV infected, cycloheximide treated cells at 4 hours post-infection (Figure 3.10.A). The e1 transcript was detected in phosphonoacetic acid treated cells (data not shown). The 500bp M75 gene specific product was expressed in the presence and absence of phosphonoacetic acid but was always less prominent in phosphonoacetic acid treated cells compared to untreated cells harvested at 24 hour post-infection (Figure 3.10.B); this is because transcription of the M75 gene is an E/L transcript, its synthesis beginning before the viral DNA synthesis but decreasing in the presence of the DNA inhibitor. From both experiments it was confirmed that the inhibitory

conditions used were suitable for examining the kinetic class of the m29 and m29.1 ORFs.

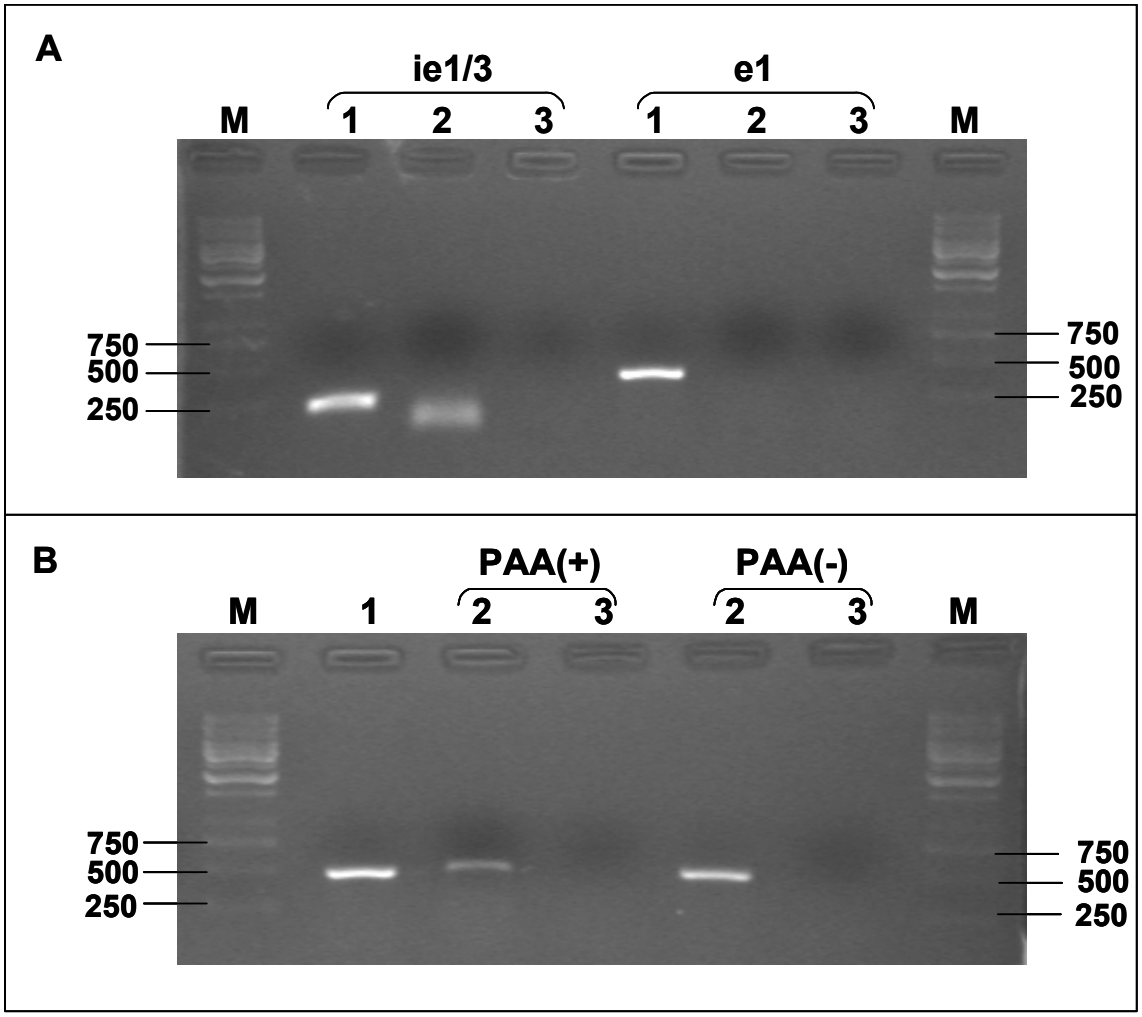


Figure 3.10 RT-PCR to check the inhibitory conditions used in kinetic experiments. Total RNA was isolated from wt MCMV and mock infected NIH 3T3 cells treated with cycloheximide (A) at 4 hours post-infection and treated with (+) or without (-) phosphonoacetic acid (PAA) (B) at 24 hours post-infection. A. The RT-PCR product of 113bp indicates expression of the ie1/3 spliced transcript (lane 2) and no product indicates that e1 does require protein synthesis for its expression (lane2). B. The RT-PCR product of 500bp indicates the expression of M75 gene in the presence and absence of PAA. MCMV genomic DNA was used as the positive control (lane 1) and reverse transcriptase was omitted from the negative control for the reverse transcription reaction (lane 3). The size of the markers (M) from the 1kb ladder (Fermentas) are shown on the right and the left margins in bp.

The kinetic class of m29 and m29.1 were determined using the same primer pairs that successfully amplified the 708 and 596bp products respectively at different times post-infection (Section 3.2.1). Total RNA was isolated from wt MCMV infected NIH 3T3 cells treated with or without either cycloheximide or phosphonoacetic acid at 4 hours and 24 hours post-infection respectively and the GSP was used in generating cDNA. A transcript produced from the m29 gene was not detected by RT-PCR in wt MCMV infected cells treated with cycloheximide at 4 hours post-infection, but was detected at this time in non-treated cells. In contrast, the m29 transcript was detected in wt MCMV infected phosphonoacetic acid treated cells at 24 hours post-infection (Figure 3.11). In contrast, the m29.1 transcript was detected in wt MCMV infected cells treated with cycloheximide at 4 hours post-infection but was also detected in cells treated with phosphonoacetic acid at 24 hours post-infection (Figure 3.12). Three independent experiments confirmed the same results indicating that the m29 gene belongs to E and the m29.1 gene to IE viral kinetic class.

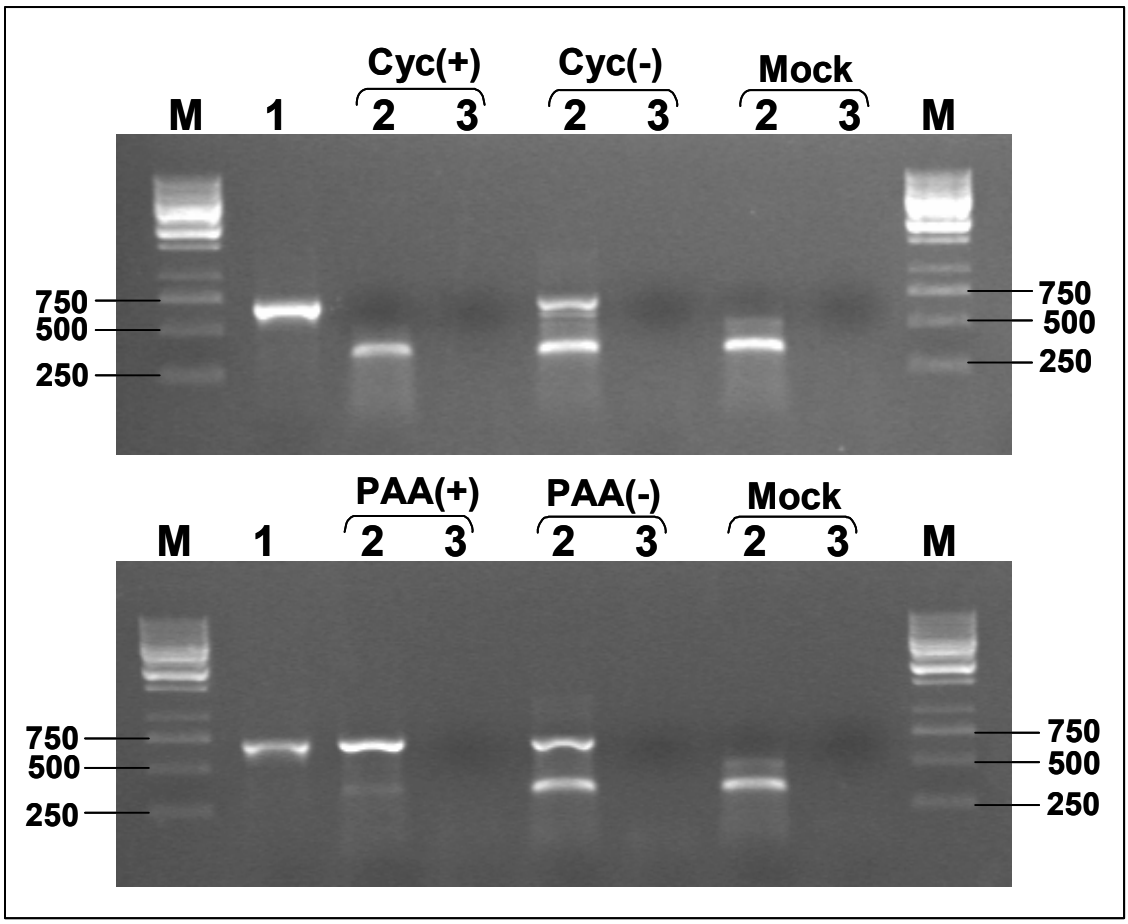


Figure 3.11 RT-PCR for m29 gene classification. Total RNA was isolated from wt MCMV and mock infected NIH 3T3 cells treated with (+) or without (-) cycloheximide (Cyc) at 4 hours post-infection and or cells treated with (+) or without (-) phosphonoacetic acid (PAA) at 24 hours post-infection. RT-PCR product of 708bp (lane 2) indicate the presence of the m29 transcript in cells untreated with cycloheximide and cells treated with/without phosphonoacetic acid. The PCR product of approximately 300bp (RT+) indicates the presence of the non-specific transcript. MCMV genomic DNA was used as the positive control (lane 1) and reverse transcriptase was omitted from the negative control for the reverse transcription reaction (lane 3). The size of the markers from the 1kb ladder (Fermentas) are shown on the right and the left margins in bp.

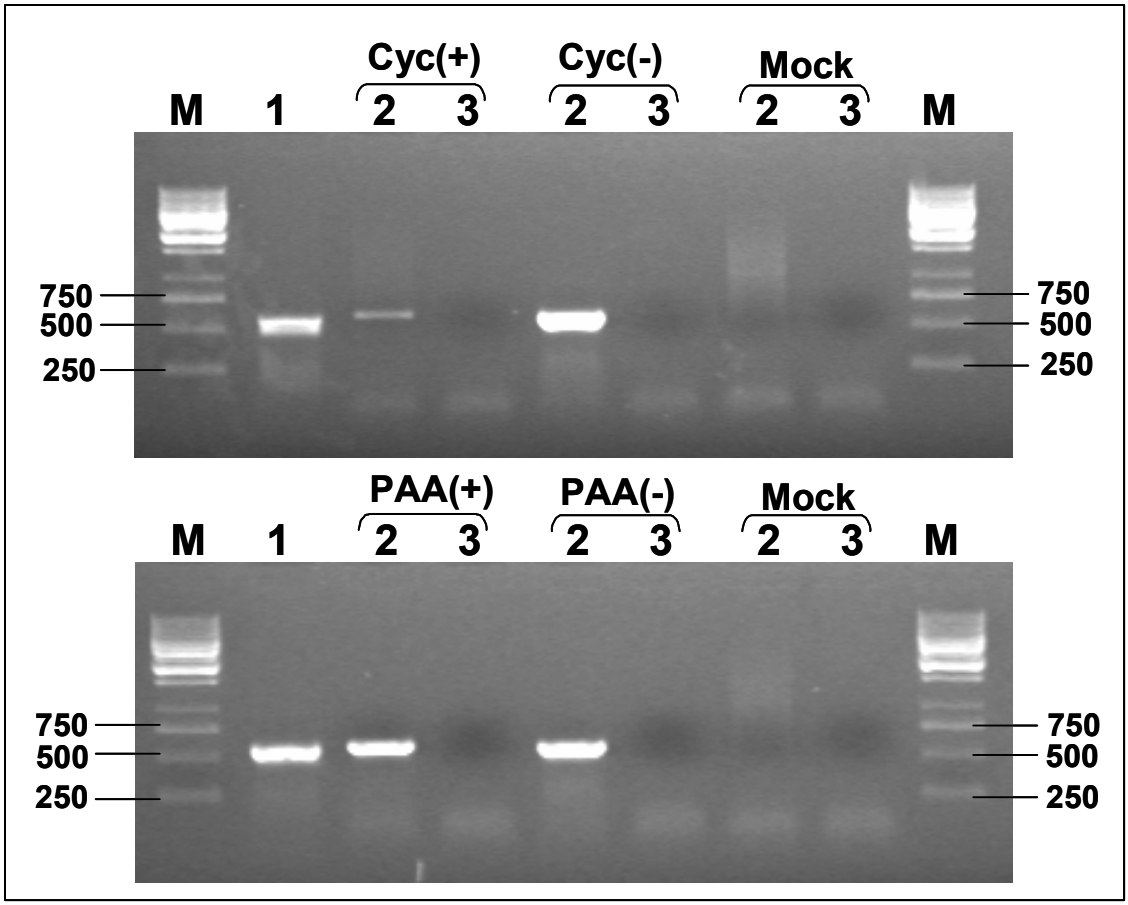


Figure 3.12 RT-PCR for m29.1 gene classification. Total RNA was isolated from wt MCMV and mock infected NIH 3T3 cells treated with (+) or without (-) cycloheximide (Cyc) at 4 hours post-infection and or cells treated with (+) or without (-) phosphonoacetic acid (PAA) at 24 hours post-infection. RT-PCR product of 596bp (lane 2) indicate the presence of the m29.1 transcript in cells treated with or without either cycloheximide or phosphonoacetic acid. MCMV genomic DNA was used as the positive control (lane 1) and reverse transcriptase was omitted from the negative control for the reverse transcription reaction (lane 3). The size of the markers from the 1kb ladder (Fermentas) are shown on the right and the left margins in bp.

3.3 BAC mutagenesis

For the production of mutant viruses, the MCMV genome cloned as a BAC, was modified by targeted mutagenesis in *E. coli* strain DH10B. Incorporation of a point mutation into the viral genome was performed by homologous recombination between the MCMV BAC and a fragment containing the corresponding gene with the introduced point mutation. The fragments for homologous recombination were made by PCR and then cloned into the plasmid vector.

3.3.1 Stop codon mutants of m29 and m29.1

For the production of stop codon mutants of m29 and m29.1, firstly, point mutations were introduced into m29 or m29.1 by site-directed mutagenesis (Section 3.3.1.1) and these were then introduced into the corresponding gene of the MCMV BAC using ET recombination (Section 3.3.1.2).

3.3.1.1 Site-directed mutagenesis of m29 and m29.1

Genetic analysis of DNA viruses is complicated by the presence of many overlapping ORFs. The genome of the MCMV has ORFs on both strands like that of other dsDNA viruses. The m29 and m29.1 ORFs are overlapping and coded on opposite strands of the viral genome (128), thus making it impossible to delete one ORF without affecting the other. To overcome this problem, a stop codon mutation was introduced into each ORF in independent mutant

viruses near to their 5' ends so that the mutation did not affect ORFs on the complementary strand.

Inverse PCR amplification using primers m29*F and m29*R (Table 2.3) on template pCR4B-29 DNA containing both overlapping ORFs (Section 2.3) incorporated a point mutation (C to G) at nt position 35,896 (128) in the m29 gene which results in the conversion of an existing tyrosine codon (TAC) to a stop codon (TAG) (Figure 3.13.A). The resulting construct was designated m29*pCR4B. A similar round of inverse PCR was carried out to incorporate a point mutation (C to G) at nt position 36,484 (128) in the m29.1 gene using primers m29.1*F and m29.1*R (Table 2.3) on same pCR4B-29 template DNA resulting in the conversion of an existing tyrosine codon (TAC) to a stop codon (TAG) (Figure 3.13.B). The resulting construct was designated m29.1*pCR4B. Finally, the mutations were confirmed by sequence analysis (data not shown). The stop codon mutation was designed to interrupt protein synthesis, but not to interrupt mRNA synthesis of each gene.

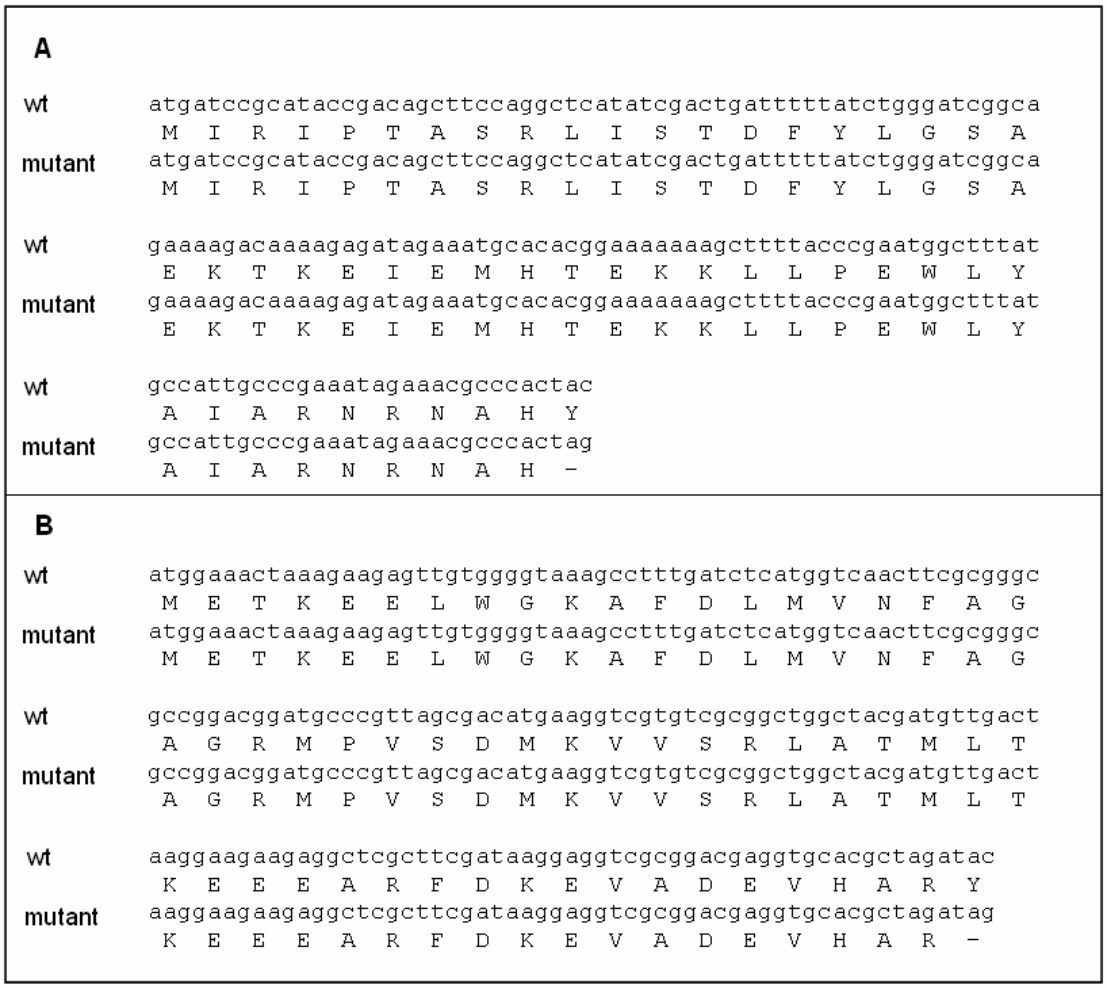


Figure 3.13 Amino acid sequence alignments of the wt m29 protein and of the truncated mutant m29 protein (A) and of the wt m29.1 protein and of truncated m29.1 protein (B)

3.3.1.2 ET recombination

Two-step ET recombination (187, 188) was carried out to produce m29 and m29.1 stop codon mutants and their revertant to wild type viruses.

In the first step, a linear DNA fragment (1.3kb) containing a streptomycin sensitivity and kanamycin resistance cassette rpsl-neo flanked by homology arms identical in sequence to either side of the targeted locus on the MCMV BAC (Section 2.11.1) was amplified by PCR using primers ETm29F and ETm29R (Table 2.3). The ETm29F primer was 75 nt in length and consisted of 52 nt upstream of the m29 ORF (nt 35556 to 35607) and 23 nt of the 5' end of the pRpsl-neo cassette. Similarly, the ETm29R primer was 75 nt in length and consisted of 51 nt upstream of the m29.1 ORF (nt 36749 to 36799) and 24 nt of the 3' end of the pRpsl-neo cassette. Using ET recombination the PCR product containing the pRpsl-neo cassette was introduced into the MCMV BAC genome replacing the m29/m29.1 ORFs. A mutant was selected through kanamycin resistance and streptomycin sensitivity and the resulting construct was designated MCMV BAC-rpsl-neo. PCR screening was performed to check the correct MCMV BAC-rpsl-neo construction with primer sets, m29F/REV-RPSL and RPSL FOR/m29REV (Table 2.3). Primers m29F and m29REV bind to the MCMV BAC viral sequence whereas primers RPSL FOR and REV-RPSL bind to rpsl-neo cassette (Figure 3.14.A). The PCR products of 954bp with primer set m29F+REV-RPSL and of 1138 bp with primer set RPSL FOR+m29REV indicated the presence of the rpsl-neo cassette in the correct position and orientation in the MCMV BAC (Figure 3.14.B). The positive control shows the 1065bp product amplified from the MCMV genomic DNA template

with primers m29F and 36304R. Primer 36304R binds to the region of the MCMV BAC genome which has been replaced by the rpsl-neo cassette (Figure 3.14.B). Thus, no product was amplified in the negative control where the MCMV BAC-rpsl-neo DNA template was used and primers m29F and 36304R (Figure 3.14.B).

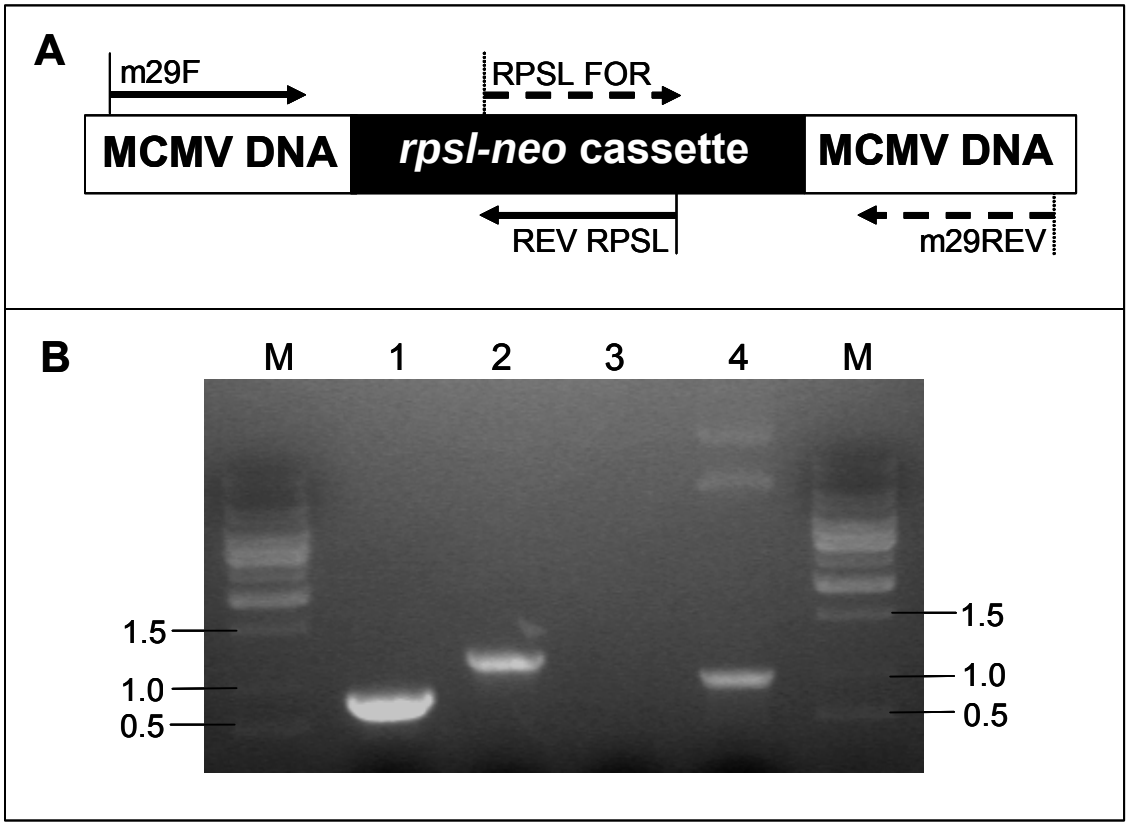


Figure 3.14 PCR confirmation of the correct integration of the rpsl-neo cassette into the MCMV BAC genome. A. The position of the primers m29F and m29REV binding to the MCMV BAC genome and primers RPSL FOR and REV RPSL binding to rpsl-neo cassette. B. The m29F/REV RPSL PCR product of 954bp (lane 1) and RPSL FOR/m29REV PCR product of 1138bp (lane 2) indicate the presence of rpsl-neo cassette in the correct position in the MCMV BAC genome. Lanes 3 and 4 are negative and positive controls respectively. The size of the markers (M) from the 1kb ladder (NEB) are shown on the right and the left margins in kb.

In the second step, the rpsl-neo cassette in the MCMV BAC was replaced by a restriction enzyme digested linear DNA fragment containing the point mutation. Plasmids m29*pCR4B and m29.1*pCR4B (Section 3.3.1.1) were digested by EcoRI to release the mutated m29 and m29.1 gene fragment, respectively. The rpsl-neo cassette was replaced by the relevant mutated ORF through ET recombination and selection for streptomycin resistance and kanamycin sensitivity (Section 2.11.1). The resulting constructs were designated Rc29MCMV BAC and Rc29.1MCMV BAC. PCR screening was performed to check the correct insertion and orientation with primer sets m29F/36744R and 36371F/m29REV. All primers bind to MCMV BAC viral sequence. The PCR products of 1505bp with primer set, m29F+36744R and of 814bp with primer set, 36371F+m29REV indicated the replacement of rpsl-neo cassette in MCMV BAC (Figure 3.15). The positive control shows the 954bp product amplified from the MCMV BAC-rpsl-neo DNA template with primers m29F and RPSL-REV. Primer RPSL-REV binds to the rpsl-neo cassette which has been replaced by the mutated MCMV sequence. Thus, no product was amplified in the negative control where the MCMV genomic DNA template was used and primers m29F and RPSL-REV.

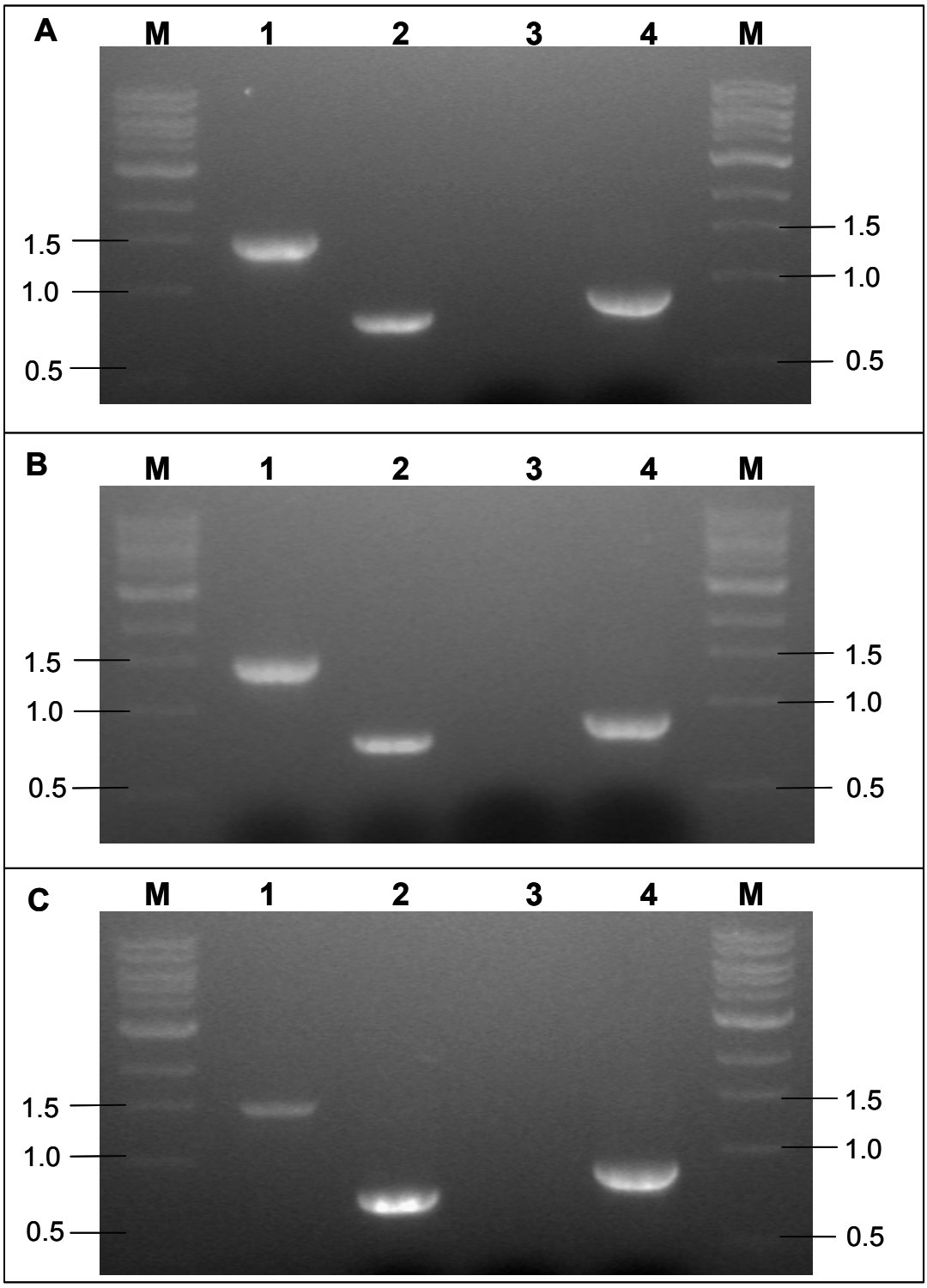


Figure 3.15 PCR confirmation of the replacement of the rpsl-neo cassette by the restricted fragment carrying the required mutation in Rc29MCMV BAC (A), Rc29.1MCMV BAC (B) and Rv29.1MCMV BAC (C) genome.

The m29F/36744R PCR products of 1505bp (lane 1) and 36371F/m29REV PCR products of 814bp (lane 2) indicate the replacement of rpsl-neo cassette in MCMV BAC. Lanes 3 and 4 are negative and positive controls respectively. The size of the markers (M) from the 1kb ladder (NEB) are shown on the right and the left margins in kb.

To rescue the m29.1 mutant to wt, a two step ET recombination was performed as descried above. Firstly, the rpsl-neo cassette was inserted into the mutant Rc29.1MCMV BAC DNA and secondly, the rpsl-neo cassette was replaced by linear wt DNA sequence. Revertant BAC DNA was identified by PCR (Figure 3.15) and designated Rv29.1MCMV BAC.

3.3.2 Partial sequencing of MCMV BAC DNA

DNA isolated from the Rc29MCMV BAC, Rc29.1MCMV BAC and Rv29.1MCMV BAC was sequenced and identified a C to G mutation at nt position 35,896 in the m29 gene in the Rc29MCMV BAC genome and a C to G nt position 36,484 in the m29.1 gene in the Rc29.1MCMV BAC (Figure 3.16). The Rv29.1MCMV BAC DNA was found to be similar to MCMV wild type DNA (data not shown).

3.4 Production of mutant viruses

Following extraction of MCMV BAC plasmid DNA using a commercial kit (Section 2.4.2), it was transfected into NIH 3T3 cells using ExGen500 transfection reagent (Section 2.2.1). Plaques were detected in Rc29MCMV BAC and Rc29.1MCMV BAC DNA transfected cells after 8 days and in Rv29.1MCMV BAC DNA transfected cells 7 days post-transfection. The Rc29MCMV BAC, Rc29.1MCMV BAC and Rv29.1MCMV BAC derived virus were designated Rc29, Rc29.1 and Rv29.1 virus respectively.

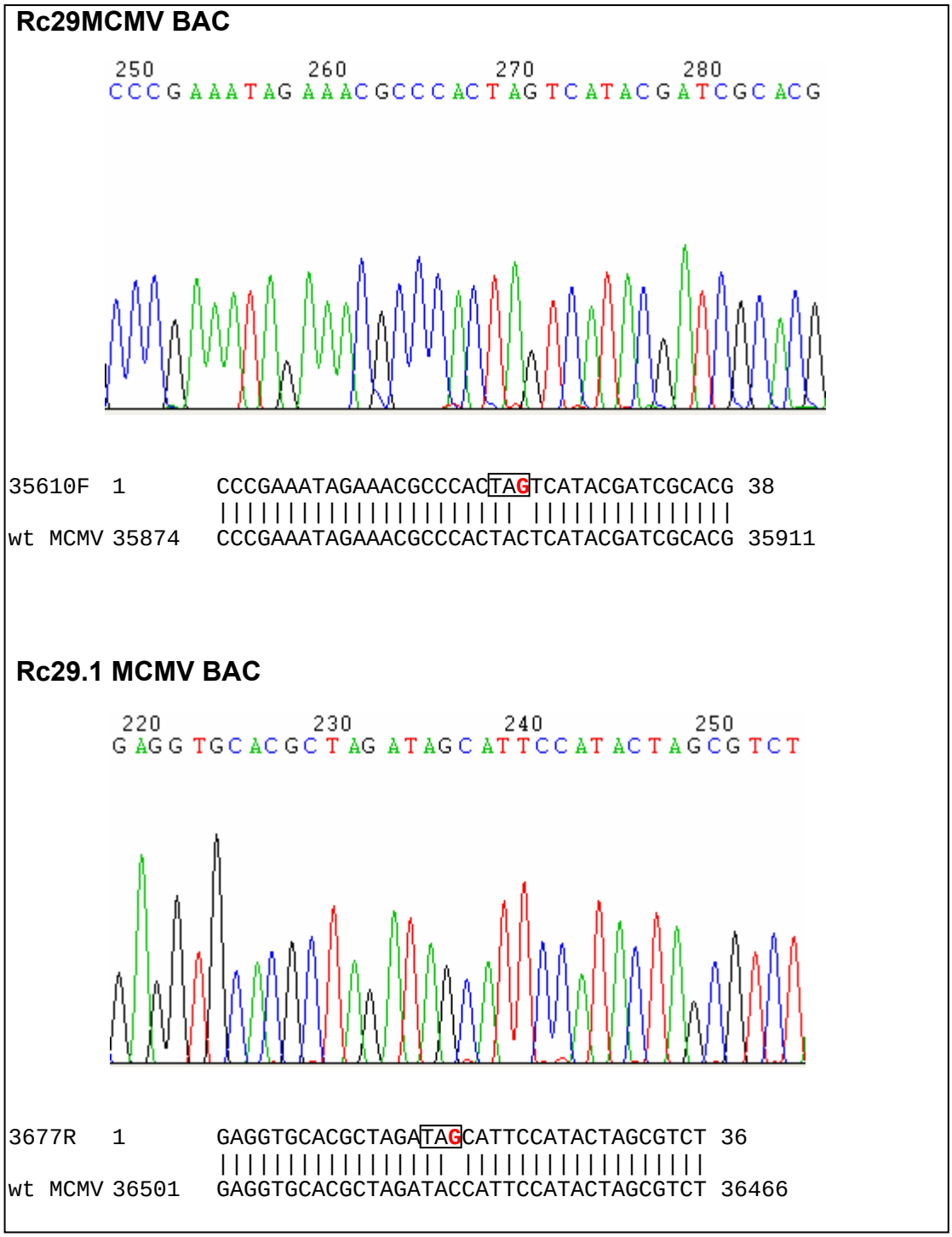


Figure 3.16 Sequencing over the point mutation introduced into the m29 and m29.1 genes of the corresponding MCMV BAC DNA using the 35610F and 3677R primers respectively.

The nucleotide (nt) of the single point mutation is denoted in red and the corresponding stop codon is indicated in the box. The wild-type viral DNA sequence corresponds to the published Smith strain MCMV genome (accession no. U68299).

Two further mutant viruses, kn29 and kn29.1, in which a 1.3kb kanamycin cassette had been inserted into the *BamH*I enzyme restriction site of the m29 gene and the *Sfo*I site of m29.1 gene respectively, were kindly supplied by Dr. Melissa Kirby in our laboratory.

3.4.1 Virus passaging

The BAC cassette was excised from mutant viruses by passaging in tissue culture cells (176). Once transfected into NIH 3T3 cells, the virion containing supernatants were used to infect new monolayers of NIH 3T3 cells and distinct plaques were observed (Section 2.2.2). Viruses from this stock were referred to as first passage virus. The mutant viruses Rc29, Rc29.1 and revertant virus Rv29.1 were passaged 4, 2 and 4 times respectively, when the BAC was shown to have been lost. The mutant virus kn29 was passaged once whereas kn29.1 was passaged twice. Viral DNA was extracted at all stages of passage and analysed for the presence of the BAC (Section 3.4.4) or kanamycin cassette (Section 3.4.3) by PCR.

3.4.2 Partial sequencing of Rc29 and Rc29.1 to confirm the presence of the mutation

DNA extracted from working stocks of the Rc29 and Rc29.1 viruses was analysed by PCR and confirmed the presence of the mutation at nt positions 35,896 and 36,484 in Rc29 and Rc29.1 viruses respectively (data not shown).

3.4.3 Confirmation of the kanamycin cassette insertion in kn29 and kn29.1 viral genomes

DNA extracted from working stocks of the kn29 and kn29.1 viruses was subjected to PCR screening. Primers 35610F and 36304R bound to the MCMV genome as shown in Figure 3.17.A and the 2kb PCR amplified from kn29 viral DNA indicated the presence of the kanamycin cassette in kn29 viral DNA (Figure 3.17.C). In the positive control, wt MCMV DNA was used with same primers and 700bp product indicated the absence of the kanamycin cassette in wt MCMV genome (Figure 3.17.C). Similarly, using primers 36371F and 36744R (Figure 3.17.B) The PCR product of 1.5kb indicated the presence of the kanamycin cassette in the kn29.1 viral genome (Figure 3.17.C) and the 370bp product indicated of its absence in the wt MCMV genome.

3.4.4 Excision of BAC Cassette from the mutant viruses

To remove the BAC from virus, the MCMV BAC must be passaged in tissue culture cells (176). DNA extracted from mutant viruses at each passage was PCR screened with two sets of primers, f/g and b/g, which demonstrated excision of BAC cassette (Figure 3.18.A). If excision of the BAC has occurred, a f/g PCR product of 590bp would be generated; without excision a 7kb product would be generated. The PCR conditions used did not allow the 7kb product to be amplified. The f/g PCR product of 590bp implied the efficient excision of the BAC (Figure 3.18.B and Figure 3.19).

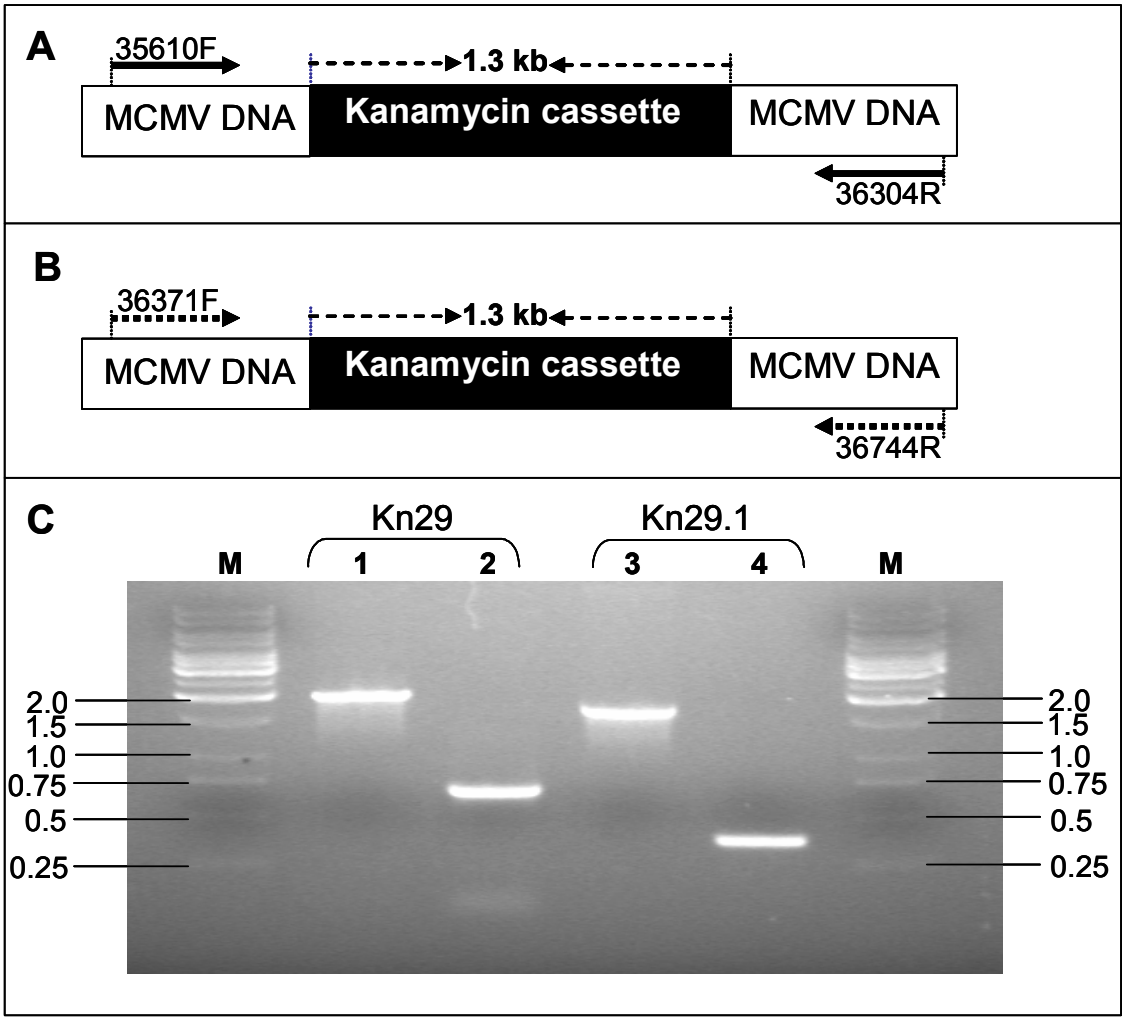


Figure 3.17 PCR screening for the presence of the kanamycin cassette in kn29 and kn29.1 viral DNA. The location of the primers 35610F and 36304R binding to kn29kn viral DNA are shown with the black arrows (A) and of the primers 36371F and 36744 R binding to the kn29.1 viral DNA are shown with black broken arrows (B). The 35610F/36304R PCR product of 2kb (lane 1) and 36371F/36744R PCR product of 1.5 kb (lane 3) indicate the presence of kanamycin cassette in Kn29 and Kn29.1 viral DNA respectively (C). Lanes 2 and 4 are positive controls (C). The sizes of the markers (M) from the 1kb ladder (Fermentas) are shown on the right and the left margins in kb.

PCR with primer set g/b was performed to confirm the result of the PCR screen with primer set f/g. Primer b bound to the BAC sequences as shown in Figure 3.18.A. Amplification of a 1950bp PCR product indicated the presence of a BAC containing genome whereas the lack of an amplified product demonstrated loss of the BAC (Figure 3.18.B and Figure 3.19).

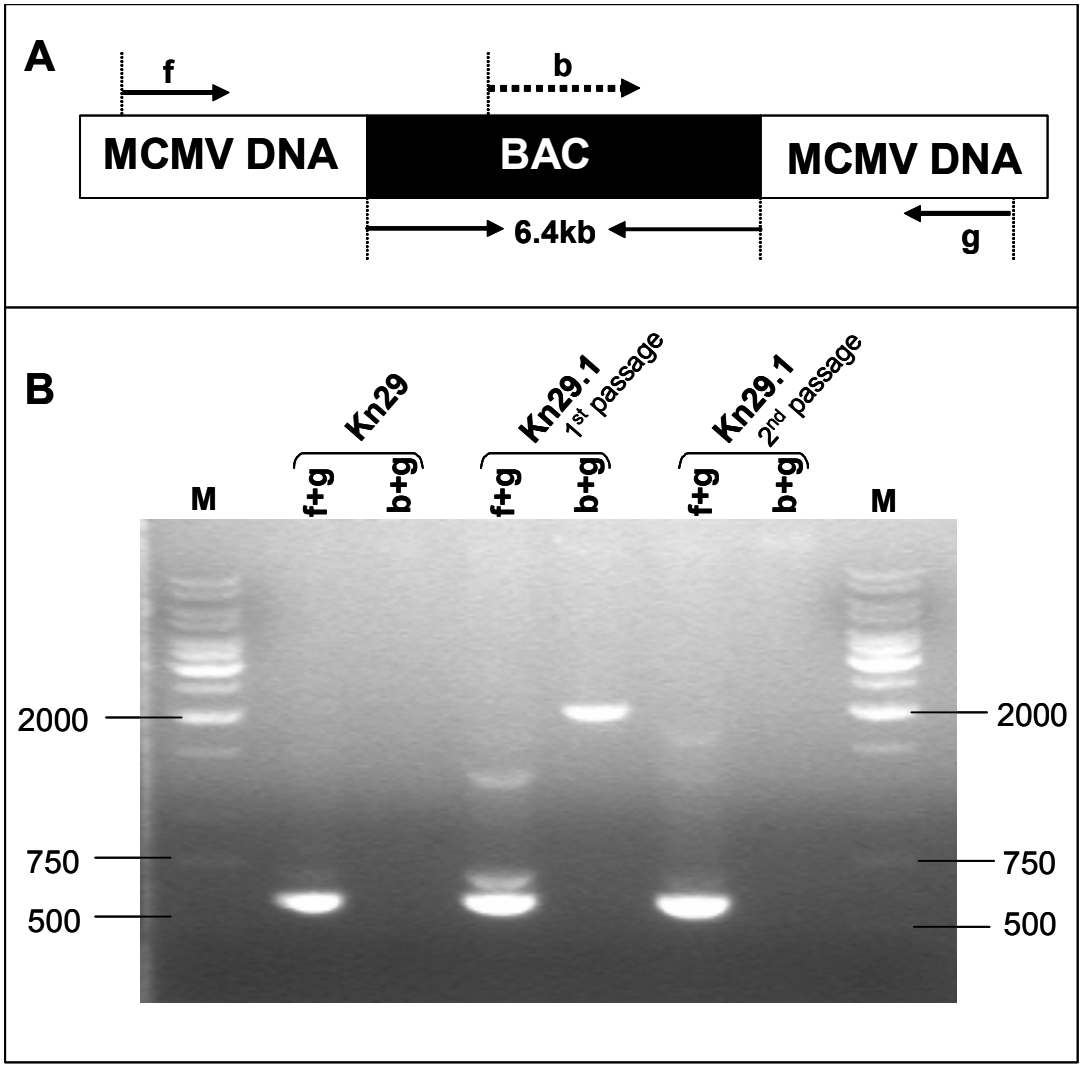


Figure 3.18 PCR screening for the absence of the BAC in viral DNA extracted from mutant virus Kn29 and Kn29.1 (A). The location of the primers g/f binding to the MCMV DNA and the primer b binding to the BAC are shown with black and broken arrow respectively. (B). The f/g PCR product of 590bp and no product with primers b/g indicate the loss of the BAC from the MCMV genome in kn29 and kn29.1 2nd passage virus. Again, the b/g PCR product of 1,950bp indicates the presence of the BAC in MCMV genome of kn29.1 1st passage virus. The size of markers (M) from the 1kb ladder (NEB) are shown on the right and the left margins in bp.

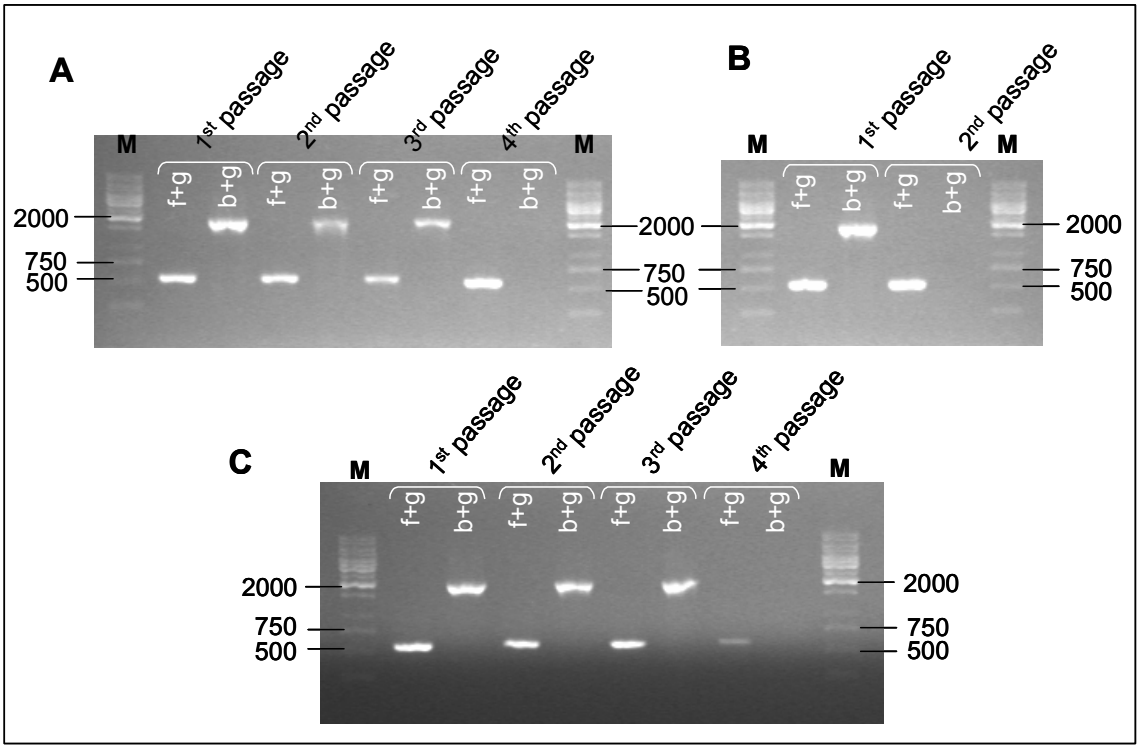


Figure 3.19 PCR screening for the absence of the BAC in viral DNA extracted from mutant virus Rc29 (A), Rc29.1 (B) and revertant virus Rv29.1 (C). The f/g PCR product of 590bp and lack of product with primers b/g indicate the loss of the BAC from the MCMV genome. The b/g PCR product of 1,950bp indicates the presence of the BAC in the MCMV genome. The Size of markers (M) from the 1kb ladder (Fermentas) are shown on the right and the left margins in bp.

The BAC was excised from the kn29, kn29.1, Rc29, Rc29.1 and Rv29.1 viral genomes after one, two, four, two and four passages respectively. The BAC had previously been excised from the wt MCMV BAC genome by Dr. Melissa Kirby.

3.5 RT-PCR analysis of MCMV gene expression in mutant virus infected cells

3.5.1 Detection of m29 and m29.1 gene expression in Rc29, Rc29.1 and Rv29.1 infected NIH 3T3 cells

To determine whether, as expected, ORFs m29 and m29.1 were expressed in mutant virus infected cells, total RNA was isolated from Rc29, Rc29.1, Rv29.1 and mock infected NIH 3T3 cells 24 hours post-infection and analysed by RT-PCR. Figures 3.20.A and B show expression of the m29 and m29.1 transcripts respectively in Rc29, Rc29.1 and Rv29.1 virus infected NIH 3T3 cells. Thus, the stop codon mutation in mutants Rc29 and Rc29.1 appeared not to disrupt transcript expression from either m29 or m29.1 ORFs.

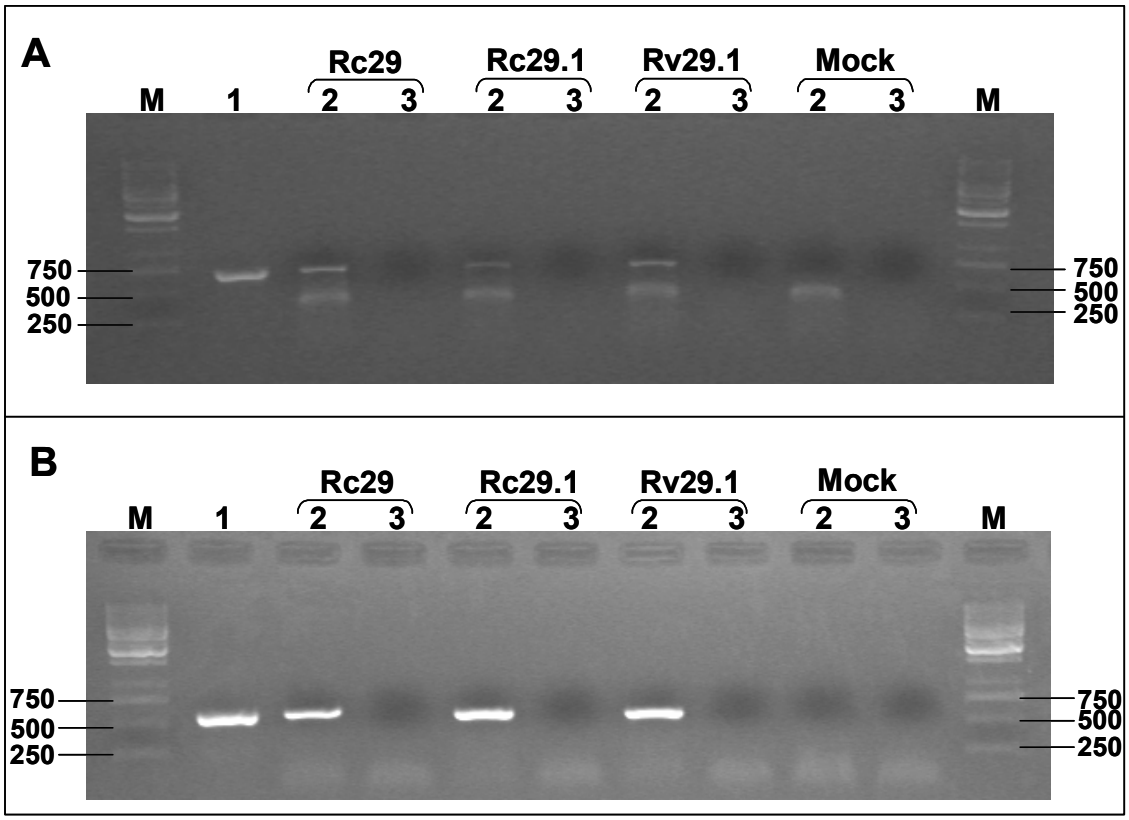


Figure 3.20 RT-PCR for m29 (A) and m29.1 (B) gene expression in Rc29, Rc29.1 and Rv29.1 virus infected NIH 3T3 cells. Total RNA was isolated from Rc29, Rc29.1, Rv29.1 and mock infected NIH 3T3 cells at 24 hours post-infection. A product of 708bp (A) or 596bp (B) in lanes 2 indicate expression of the m29 or m29.1 transcripts. MCMV genomic DNA was used as a positive control (lane 1) and reverse transcriptase was omitted from the RT reaction in the negative control (lane 3). The size of the markers (M) from the 1kb ladder (Fermentas) are shown on the right and the left margins in bp.

3.5.2 Detection of m29 and m29.1 gene expression in Kn29 and Kn29.1 infected NIH 3T3 cells

Because of the position of the kanamycin cassette at the 5' end of the m29 ORF in kn29 and the m29.1 ORF in kn29.1, it was anticipated that transcription would be disrupted. To examine this, total RNA was isolated from Kn29, Kn29.1 and mock infected NIH 3T3 cells 24 hours post-infection and subjected to RT-PCR analysis.

The m29 gene specific transcript was detected in kn29.1 virus infected cells and a minor transcript producing a product approximately similar to this size was also detected in kn29 virus infected cells (Figure 3.21.A). Two additional and more abundant products of approximately 1.5 and 2.0kb were also detected in kn29 virus infected cells (Figure 3.21.A) suggesting that the transcripts contained part or all of the kanamycin cassette. Similarly, the m29.1 gene specific transcript was detected in kn29 virus infected cells and a very low abundant product of a similar size was detected at kn29.1 virus infected cells (Figure 3.21.B). However, the most abundant product was approximately 2.0kb again suggesting the transcript contained some of the kanamycin cassette. Thus, normal transcripts do not appear to be expressed in either kn29 or kn29.1 virus infected cells but they do produce aberrant transcripts which could produce truncated protein products. For this reason, these mutant viruses were not studied further.

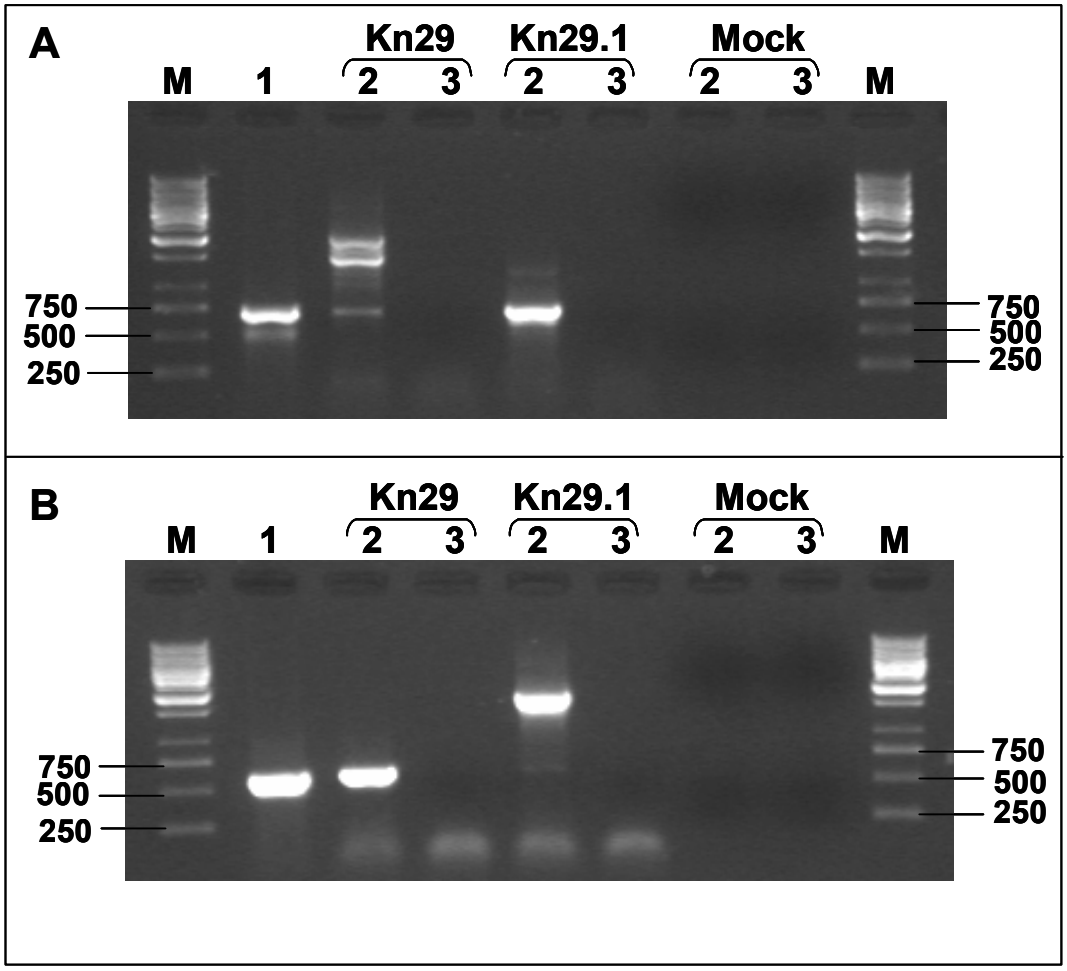


Figure 3.21 RT-PCR for m29 (A) and m29.1 (B) gene expression in Kn29 and Kn29.1 mutant virus infected NIH 3T3 cells. Total RNA was isolated from Kn29, Kn29.1 and mock infected NIH 3T3 cells at 24 hours post-infection. A product of 708bp (A) or 596bp (B) in lanes 2 indicate expression of the m29 or m29.1 transcripts. MCMV genomic DNA was used as a positive control (lane 1) and reverse transcriptase was omitted from RT reaction in the negative control (lane 3). The Sizes of the markers (M) from the 1kb ladder (Fermentas) are shown on the right and the left margins in bp.

3.6 Northern blot

The RT-PCR experiment confirmed the presence of m29 and m29.1 gene specific transcripts in wt and mutant virus infected cells (Section 3.2 and 3.5.1). To examine the size of these transcripts, northern blots were performed. Total RNA isolated from the wt, Rc29, Rc29.1 and Rv29.1 was hybridised to radiolabelled DNA probes, generated previously either by random priming (Section 2.13.6.1) or linear PCR (Section 2.13.6.2). Despite several attempts, no RNA species was detected in the wt or mutant virus infected cells.

3.7 Detection of MCMV protein in virus infected NIH 3T3 cells

To detect MCMV protein in infected cells, polyclonal antibodies were developed against bacterially expressed purified protein or synthetic peptide and finally the antibodies were used in a western blot experiment.

3.7.1 Expression of m29 and m29.1 protein in bacterial cells

The m29 and m29.1 protein encoding region was amplified by PCR using primer sets P29F/P29R and P29.1F/P29.1R (Table 2.3) from MCMV genomic DNA. Each product was purified and cloned into plasmid pET28a between *Xho*I and *Nco*I restriction sites (Section 2.12.1). The resulting constructs were designated pET28a-m29 and pET28a-m29.1. The expression vectors, pET28a-m29 and pET28a-m29.1 were transformed into *Escherichia coli* C41 (DE3) to produce m29 and m29.1 protein. Protein expression was induced by

isopropyl-1-thio-β-D-galactopyranoside. The expression of proteins was confirmed by SDS-PAGE. The bacterial expression of pET28a-m29.1 resulted in the production of m29.1 protein as shown in Figure 3.22. Despite several attempts, no protein was found to be expressed by bacteria containing pET28a-m29. The m29.1 protein was purified using Ni^{2+}-loaded His-Trap affinity column and supplied to Biogenes (Berlin, Germany) to raise polyclonal antibodies by immunising two rabbits using an undisclosed protocol.

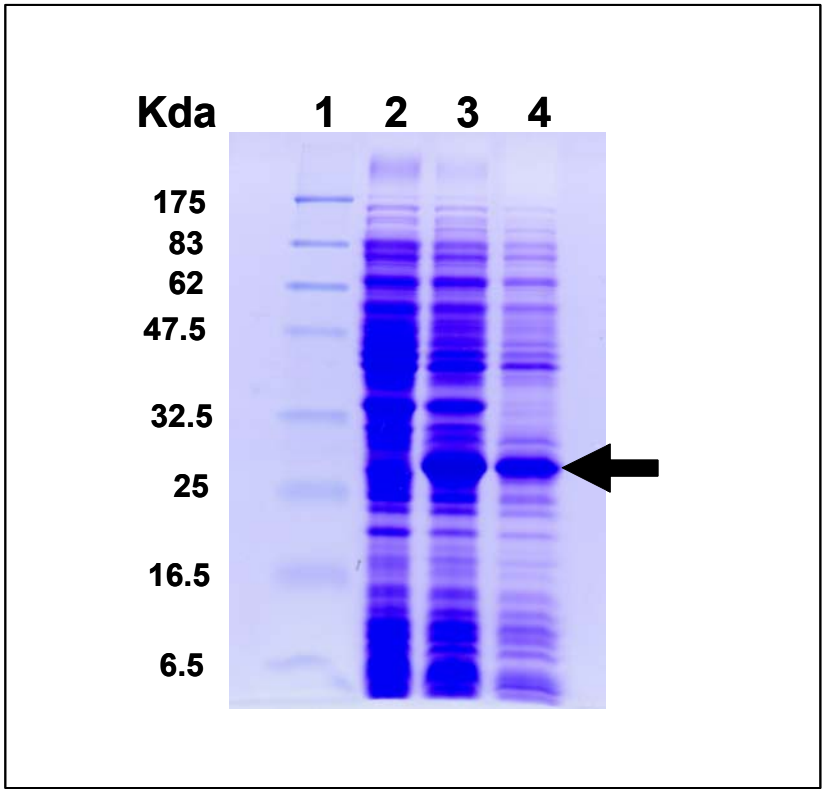


Figure 3.22 SDS-PAGE followed by coomassie blue gel staining showing the results of the pET28a-m29.1 expression. Lane 1, broad range molecular weight protein marker; lane 2, sample before IPTG induction; lane 3, m29.1 protein after IPTG induction; lane 4, soluble fraction; m29.1 protein is indicated by the black arrow.

3.7.2 Production of synthetic peptide from m29

To try to raise an antiserum to the m29 protein an alternative approach was used. Using the published Smith MCMV sequence several synthetic peptides with likely antigenic potential were designed by Biogenes (Berlin, Germany). One of these was selected (CDRDTPHEQRSGVSG) as it was encompassed in both the predicted amino acid sequence of the published Smith MCMV and the new ORF depicted by the new sequence as shown in Figure 3.4. The peptide was synthesised by Biogenes and used to immunise two rabbits using the protocol described in Section 2.14.6.

3.7.3 Western blot

To determine whether translation from the m29 and m29.1 transcripts was disrupted due to the incorporation of a stop codon at the 5' end of the ORFs, proteins were isolated from wt, Rc29, Rc29.1, Rv29.1 and mock virus infected NIH 3T3 cells 24 hours post-infection and separated on SDS-PAGE. Western blot analysis was carried out using polyclonal antibodies raised in rabbits. The anti-m29.1 antibodies detected a protein of approximately 28 to 30 kDa in wt and Rc29 virus infected cells whereas no protein was detected in Rc29.1 virus or mock infected cells (Figure 3.23). Thus, the mutation in Rc29.1 appeared to stop translation from the m29.1 transcript, whereas the m29.1 translation was restored in Rv29.1. The antibodies raised to the synthetic peptide failed to detect any protein in wt virus infected cells (data not shown). Thus it was not

possible to determine whether the m29 protein was expressed in Rc29 virus infected cells.

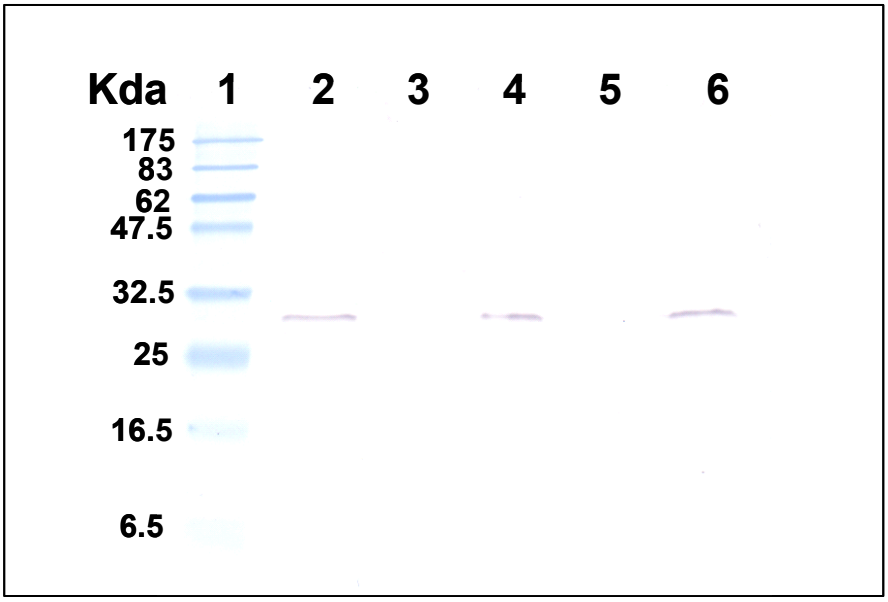


Figure 3.23 Western blot analysis of m29.1 protein. Proteins were isolated from wt (lane 2), Rc29.1 (lane 3), Rv29.1 (lane 4), mock (lane5) and Rc29 (lane 6) infected cells. Lane 1 shows broad range molecular weight protein marker.

3.8 Growth of mutant viruses in tissue culture

To determine whether the mutant viruses had any growth defects *in vitro*, the growth rates of the mutant viruses were studied in MEF cells. MEF cells were infected with mutant viruses at both low and high MOIs. Supernatants from the infected cells were collected at different times post-infection and titrated on MEF cells (Section 2.2.6). The experiments were repeated twice and each experiment was carried out with three replicates.

3.8.1 Replication of Rc29 virus in tissue culture

At an MOI of 5.0, no significance differences were found in growth rates between Rc29 and wt virus (Figure 3.24). At 2 days post-infection, viral yields in Rc29 virus infected cells were significantly decreased compared to wt virus ($p<0.05$) but not at any other time point (Figure 3.24). At an MOI of 0.05, Rc29 and wt virus grew similarly on MEF cells at 1 and 2 days post-infection, but from 3 to 7 days post-infection, Rc29 virus produced a significantly ($p<0.05$) lower yield that wt virus although these differences were small (~ 1 $\log_{10}$PFU/ml) (Figure 3.24).

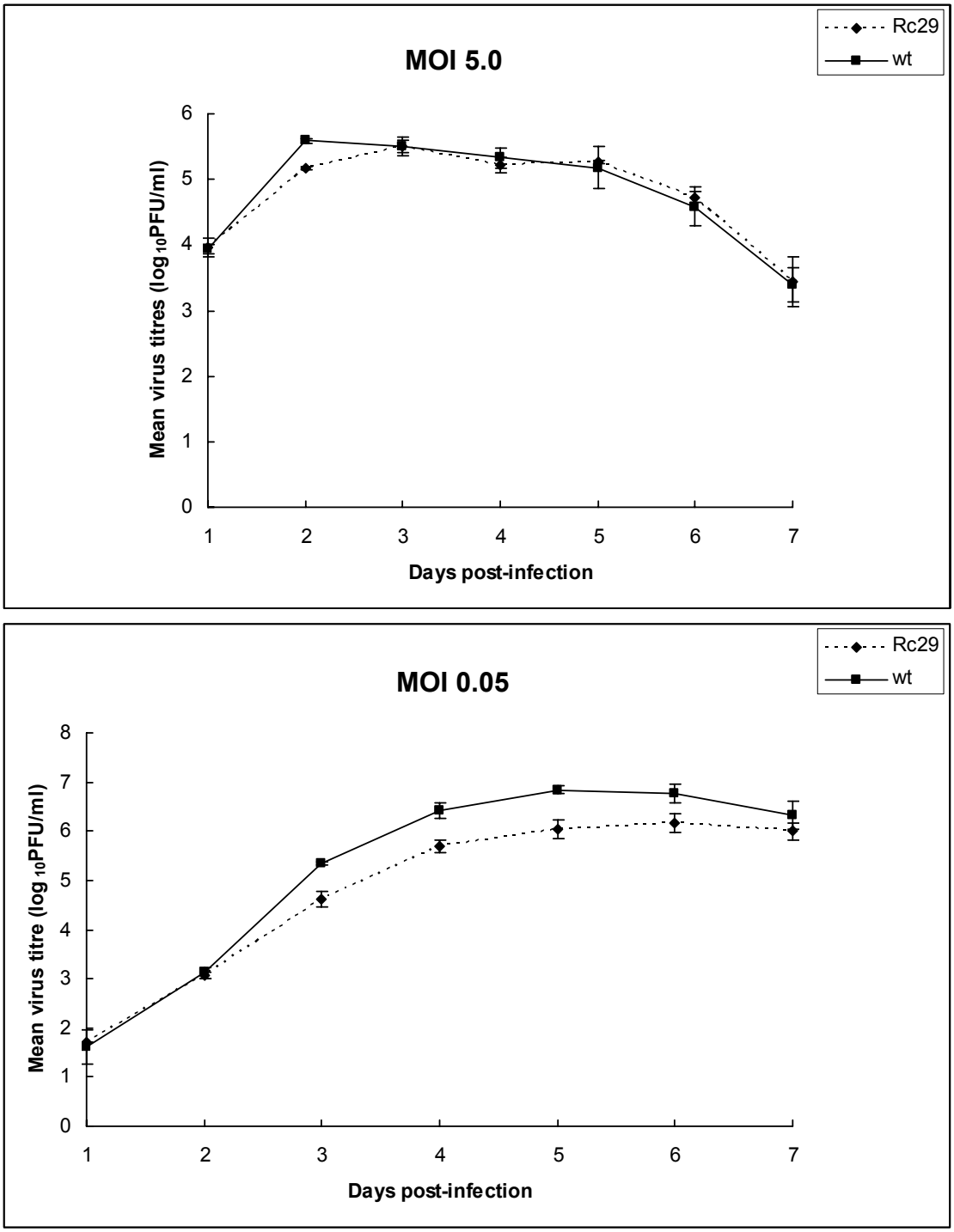


Figure 3.24 Replication of Rc29 virus in tissue culture. MEF cells were infected with Rc29 and wt viruses at a MOI of either 5.0 PFU or 0.05 PFU per cell. At 1, 2, 3, 4, 5, 6 and 7 days post-infection, cells and culture media were collected. Values represent the means of three replicates ± standard deviation. wt, wild type

3.8.2 Replication of Rc29.1 and Rv29.1 in tissue culture

At the high MOI (5.0) virus yields of Rc29.1 were initially reduced by 2-3 log_{10}PFU/ml at days 1-4 post-infection (Figure 3.25). These differences were smaller at later time points but at all times Rc29.1 virus yields were significantly ($P<0.05$) lower than those of wt virus. In contrast, virus yields of wt and revertant Rv29.1 viruses were not significantly different at any time point (Figure 3.25). Similar, but smaller differences were evident at the low MOI (0.05) (Figure 3.25). Interestingly, Rc29.1 virus produced smaller plaques (Figure 3.26) suggesting that this virus had a defect in release or produced fewer particles per cell.

3.9 RACE analysis of the m29.1 transcript

The MCMV immediate early region produces two alternative spliced transcripts that encode the ie1 protein pp89 and the 88 kDa ie3 protein (101). MCMV ie1 mutant virus showed slower growth and formed smaller plaques than the parental virus in MEF cells (102). The m29.1 gene belongs to the IE class of genes (Section 3.2.3) and Rc29.1 virus showed a phenotype similar to MCMV ie1 mutant virus. Thus, it was possible that m29.1 ORF might produce a spliced transcript. To examine this, a 5' and 3' RACE analysis (as an alternative approach to northern blot) of mRNA extracted from wt virus infected cells was performed.

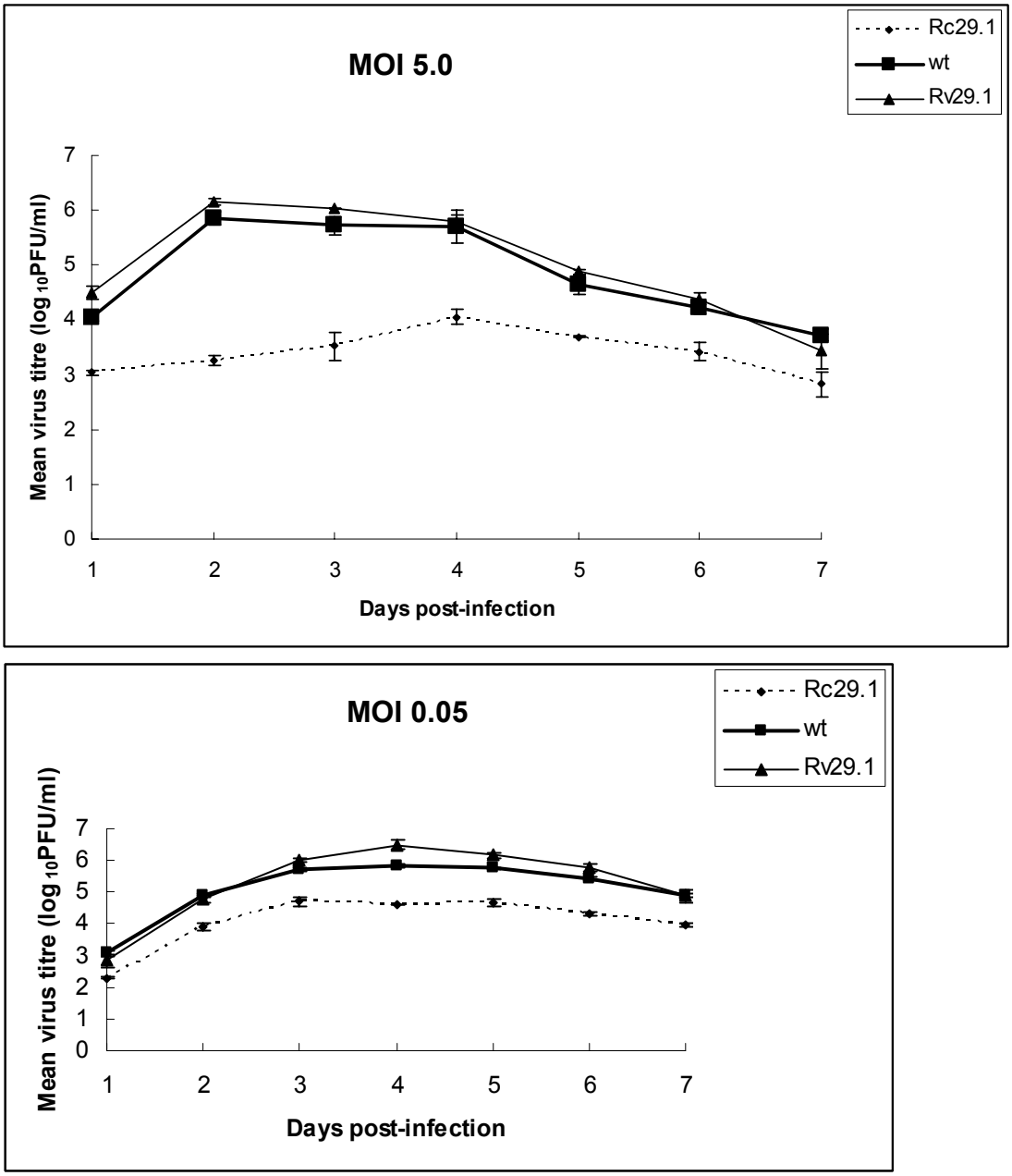


Figure 3.25 Replication of Rc29.1 virus in tissue culture. MEF cells were infected with Rc29.1, Rv29.1 and wt viruses at a MOI of either 5.0 PFU or 0.05 PFU per cell. At 1, 2, 3, 4, 5, 6 and 7 days post-infection, cells and culture media were collected. Values represent the means of three replicates ± standard deviation. wt, wild type

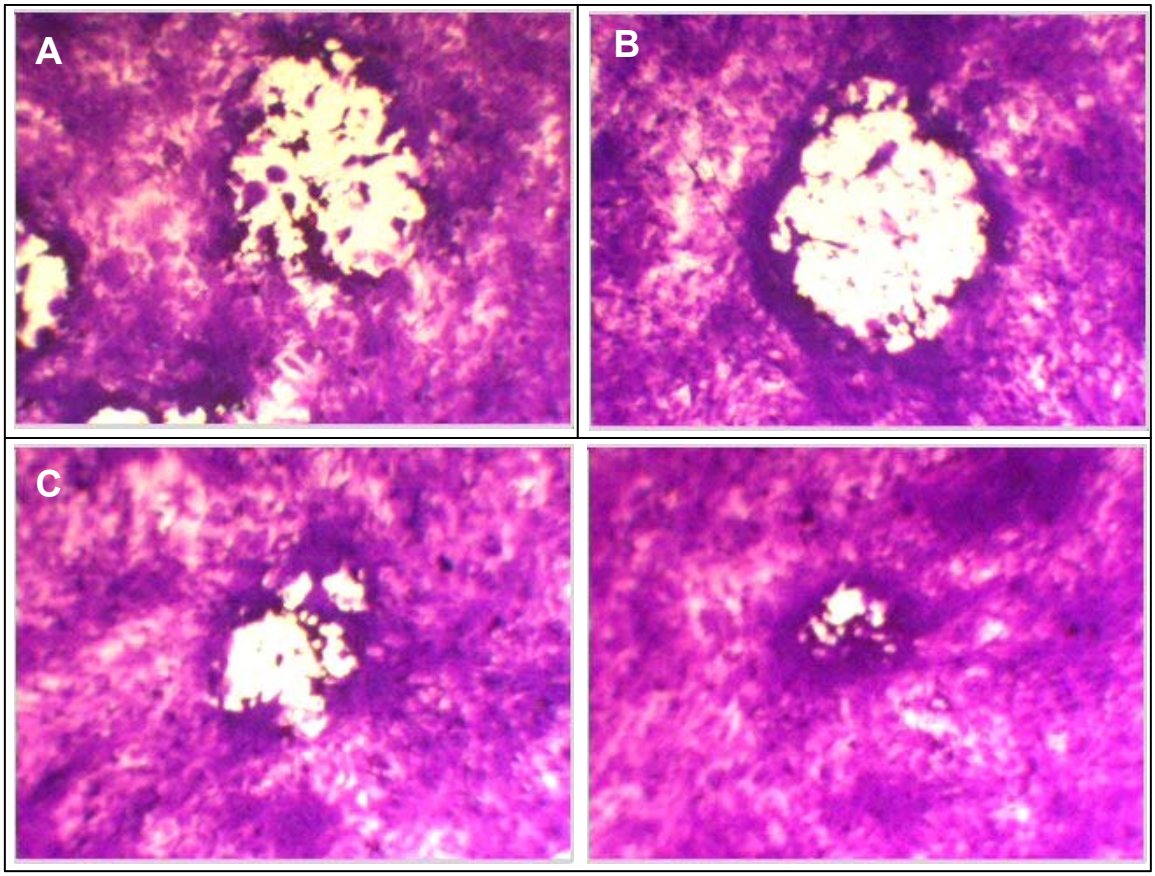


Figure 3.26 Plaque size of wt (A), Rv29.1 (B) and Rc29.1 viruses (C). The viruses were grown and titrated on MEF cells. The images were taken using an inverted microscope attached to a digital camera at 60x magnification.

A RNA Oligo ligated cDNA library was generated by oligo(dT) priming of total RNA isolated from NIH 3T3 cells 24 hours post-infection. Specific PCR amplification of the 5' end of m29.1 cDNA was carried out with GeneRacer^{TM}5` primer homologous to the RNA oligo in combination with an m29.1 specific primer (GSP29.1F) (Table 2.3). Analysis of the PCR products by agarose gel electrophoresis revealed one major band of approximately 500bp, which was cloned into plasmids. Sequencing of six independent clones revealed that the start site for transcription initiation was at nt 36,752, 92bp upstream from the previously predicted initiator methionine (128) (Figure 3.27). Sequencing of a

seventh clone revealed that the transcription start site could, with low frequency, be also at nt 36,754, 94bp upstream from the previously predicted initiator methionine. Both transcription start sites were preceded by an appropriate TATA sequence at position 36,784 (Figure 3.27).

Specific PCR amplification of the 3' end of m29.1 cDNA was carried out with GeneRacer^{TM}3` primer homologous to oligo(dT) in combination with an m29.1 specific primer (GSP29.1R) (Table 2.3). Analysis of the PCR products by agarose gel electrophoresis revealed one major band of approximately 2000bp, which was cloned. Sequencing of eight independent clones confirmed an AAUAAA polyadenylation signal at nt 34359, followed by a poly(A) tag at nt 34342 (Figure 3.27).

Analysis of the MCMV genomic sequence (128) down stream of the m29.1 ORF suggests that the next ORF, designated M28, may be contained within the m29.1 mRNA, since the first AAUAAA polyadenylation signal is located 3` to the M28 ORF (Figure 3.27). Comparison of the sequence of eight independent clones with the MCMV genomic sequence revealed that only one cDNA clone lacked an 123-nt genomic sequence located between M28 and m29.1 ORFs. Inspection of the MCMV genomic sequence reveals splice donor and acceptor sites flanking the 123-nt intron, which are predicted in Figure 3.27. Thus, RACE analysis suggests that the m29.1 ORF produces an ~2.4kb transcript and a low abundance spliced transcript from which a 123bp intron had been removed.

Figure 3.27 RACE analysis of the m29.1 transcript. The location and genome coordinate of the 5` end of the m29.1 mRNA is shown by arrows and the TATA box is shown in bold and underlined. The location of the AATAAA polyadenylation signal and of the poly(A) of the 3` end of the m29.1 mRNA are shown by a box and broken arrow, respectively. The nucleotide sequence is shown with the location of 123-nt intron identified by RACE analysis indicated in lowercase letters. The stop codon is shown in grey highlights. The region of MCMV genomic DNA containing the m29.1 and M28 is presented by bold and grey letters respectively.

3.10 RT-PCR for M28 and m29.2 gene expression in Rc29.1 infected cells

The m29.2 ORF is encoded on the complementary strand of the MCMV genome just prior to m29.1 (20) and has been shown to be expressed in wt virus infected NIH 3T3 cells (Section 3.2.2). The above RACE results suggested that the M28 ORF may be contained within the m29.1 mRNA (Section 3.11). Thus, it is possible that the introduction of a point mutation into the m29.1 ORF of Rc29.1 might affect the expression of these two ORFs. In addition, it is important to check that construction of mutant viruses does not affect ORFs downstream and upstream of the modification. To examine this, total RNA was isolated from Rc29.1 and mock infected NIH 3T3 cells 24 hours post-infection and analysed by RT-PCR. The GSP 35195F (Table 2.3) was used to generate cDNA and primers 35195F and 35722R were used to amplify a 559bp RT-PCR product from the M28 gene. The m29.2 expression was examined as described in Section 3.2.2.

The whole MCMV genomic DNA template was used as a positive control and the reverse transcriptase was omitted in the negative control to confirm that the isolated RNA was free of DNA contamination. Amplification of a 559bp and a 121bp (Figure 3.28) product confirmed the expression of M28 and m29.2 gene respectively in Rc29.1 infected NIH 3T3 cells. Thus, the point mutation in the m29.1 region of Rc29.1 virus appeared not to disrupt expression of the M28 or m29.2 gene.

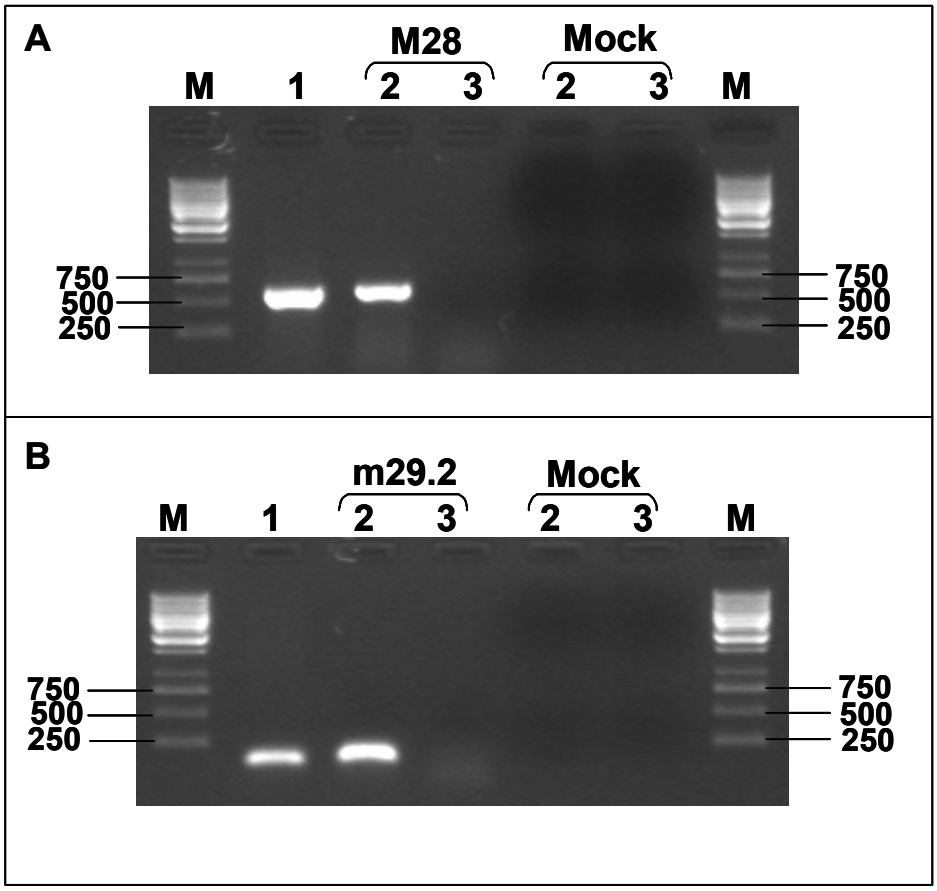


Figure 3.28 RT-PCR for M28 (A) and m29.2 (B) gene expression in Rc29.1 infected NIH 3T3 cells. Total RNA was isolated from Rc29.1 virus and mock infected NIH 3T3 cells 24 hours post-infection and analysed by RT-PCR. Products of 559bp or 121bp (lane 2) indicate the presence of the M28 or m29.2 gene specific transcript, respectively. MCMV genomic DNA was used as the positive control (lane 1) and reverse transcriptase was omitted from the negative control (lane 3). The size of the markers (M) from the 1kb ladder (Fermentas) are shown on the right and the left margins in bp.

3.11 Replication of mutant viruses in immunocompetent animals

To determine whether disruption of translation of the m29 and m29.1 ORFs significantly affects viral replication *in vivo*, BALB/c mice were injected intraperitoneally with 10^4 PFU of Rc29, Rc29.1, Rv29.1 and wt viruses. Salivary glands, lungs, spleens, hearts, livers and kidneys were harvested 3, 7, 10, 14, 21, 28, 35 and 42 days post-infection. In Rc29, Rc29.1, Rv29.1 and wt infected mice, no virus was detected in lungs, spleens, hearts, livers or kidneys at any time post-infection. In Rc29 infected mice, virus was first detected 7 days post-infection in salivary glands and the peak titre was found at 21 days post-infection (Figure 3.29). No significance difference was found in virus titres between Rc29 and wt virus in this tissue except at days 7 and 10 post-infection, when Rc29 virus titres were significantly higher than those of wt virus ($p<0.05$) (Figure 3.29). In contrast, in Rc29.1 infected mice virus was not detected until 10 days post-infection in salivary glands and yields peaked at 21 days post-infection (Figure 3.30). These yields were lower (~1 log10 PFU/tissue) and significantly different ($p<0.05$) from wt. Unlike wt virus, titres decreased significantly at 28 days post-infection and no virus was detected 35 and 42 days post-infection (Figure 3.30). The titres of revertant virus Rv29.1 were similar to those of the wt virus (Figure 3.30). Thus, these results provide evidence to suggest that m29 and m29.1 ORFs are dispensable for viral replication *in vivo* but the m29.1 ORF is important for optimal viral growth.

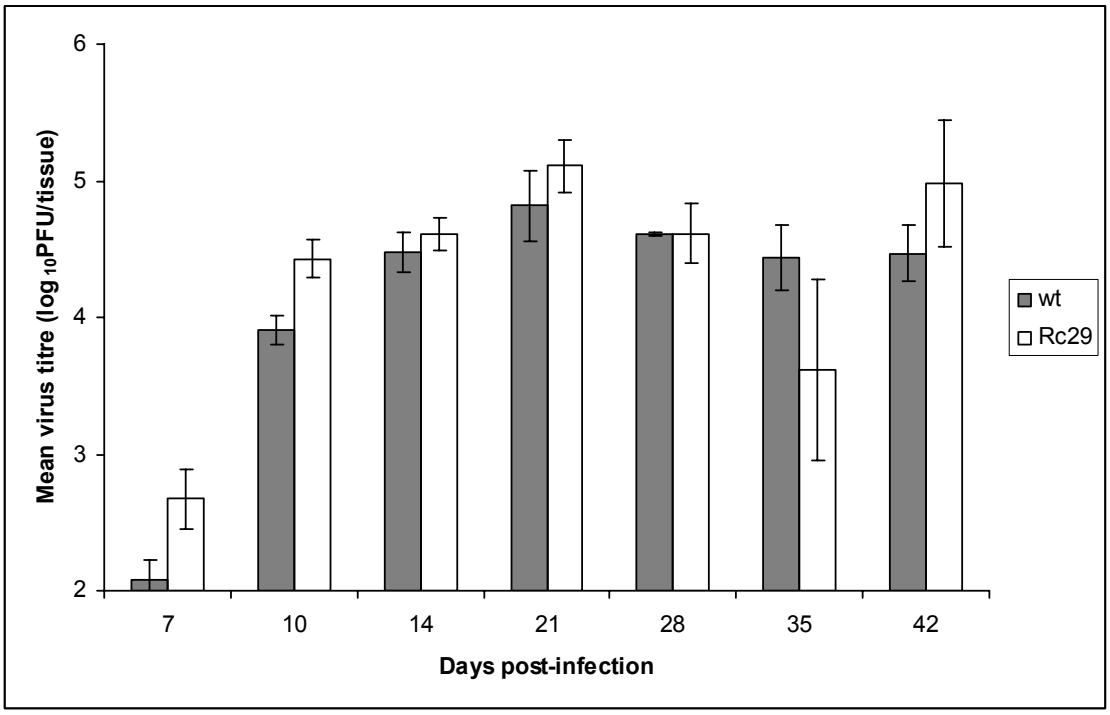


Figure 3.29 Virus titres in salivary glands of wt and Rc29 virus infected BALB/c mice. BALB/c mice were inoculated intraperitoneally with 10^4 PFU of virus. At 7, 10, 14, 21, 28, 35 and 42 days post-infection, animals were sacrificed, the salivary glands collected, homogenised and titrated for virus. The limit of detection was 100 PFU/tissue. The viral titres represent the mean of four animals ± standard deviation. Error bars that are not evident indicate that the standard deviation was less than or equal to the height of the symbols.

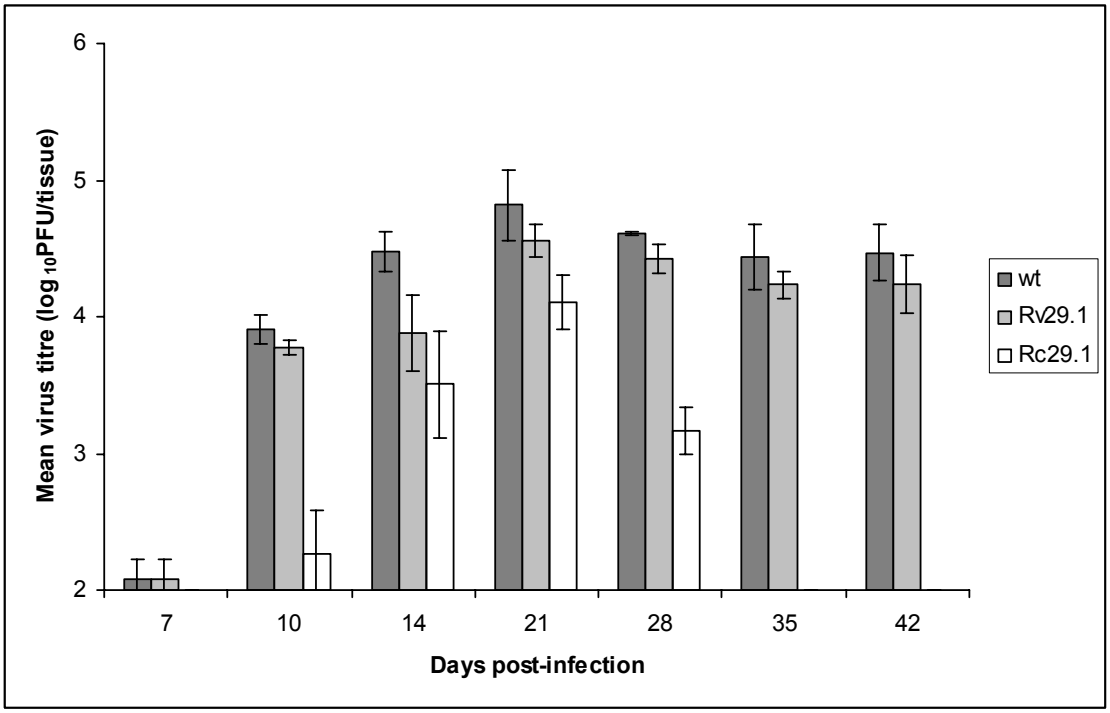


Figure 3.30 Virus titres in salivary glands of Rc29.1, Rv29.1 and wt virus infected BALB/c mice. BALB/c mice were inoculated intraperitoneally with 10^4 PFU of virus. At 7, 10, 14, 21, 28, 35 and 42 days post-infection, animals were sacrificed; the salivary glands collected, homogenised and titrated for the virus. The limit of detection was 100 PFU/tissue. The viral titres represent the mean of four animals ± standard deviation. Error bars that are not evident indicate that the standard deviation was less than or equal to the height of the symbols.

3.12 Replication of mutant viruses in immunodeficient animals

Immunodeficient animals, e.g. CB17 SCID mice, are extremely sensitive to MCMV infection (53, 115, 124, 133). To study the pathogenesis of mutant virus in these immunodeficient animals, the replication of Rc29.1 in different organs of the animals was examined at different times post-infection. CB17 SCID mice were injected intraperitoneally with 10^4 PFU of Rc29.1 and wt viruses. At 3, 7, 10, 14, 21, 23, 28 and 31 days post-infection, four mice from each virus group were sacrificed and the salivary glands, livers, hearts, lungs, kidneys and spleens were harvested. Mice injected with wt and Rc29.1 viruses became sick at 23 and 31 days post-infection respectively. Animals were monitored regularly for body weight, mobility, clinical illness etc and when animals exhibited substantial symptoms, defined according to the FELASA Working Group on Pain and Distress (44) and thus would probably die, Home Office regulations required the animals to be humanely euthanised (Figure 3.31). Virus was detected in all organs of mice infected with wt or Rc29.1 viruses. In wt infected mice, virus was first detected at days 3 (spleen and kidney), 7 (salivary glands and lung), 21 (liver) or 23 (heart) post-infection and yields in all organs were maximal at the time of death (Figure 3.32 & 3.33). In contrast, in Rc29.1 virus infected mice, virus was first detected at 10 (salivary glands, spleen, lung and kidney) or 28 (liver and heart) days post-infection and, in all organs, peak titres were again observed 31 days post-infection at the time of death (Figure 3.32 & 3.33). Virus yields were significantly lower in salivary glands at day 10, in spleen at days 14 & 21 and in kidneys at day 21 post-infection ($p<0.05$) (Figure 3.32) when compared to wt virus. In all organs, the

Rc29.1 virus showed delayed replication compared to wt virus (Figure 3.32 & 3.33). Thus, these results suggest that the delayed replication of Rc29.1 in these organs is due to disruption of m29.1 protein expression and that m29.1 ORF is important for optimal viral growth in immunodeficient hosts.

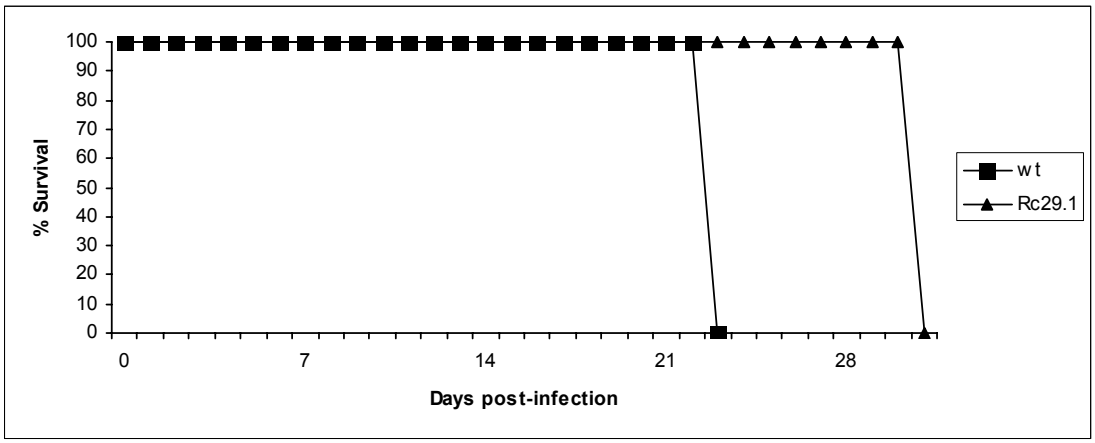


Figure 3.31 Survival rates of SCID mice inoculated with wt and Rc29.1 viruses. CB17 SCID (32 animals per group) mice were inoculated intraperitoneally with 10^4 PFU of each virus. The sickness of the animals was monitored every day for five weeks.

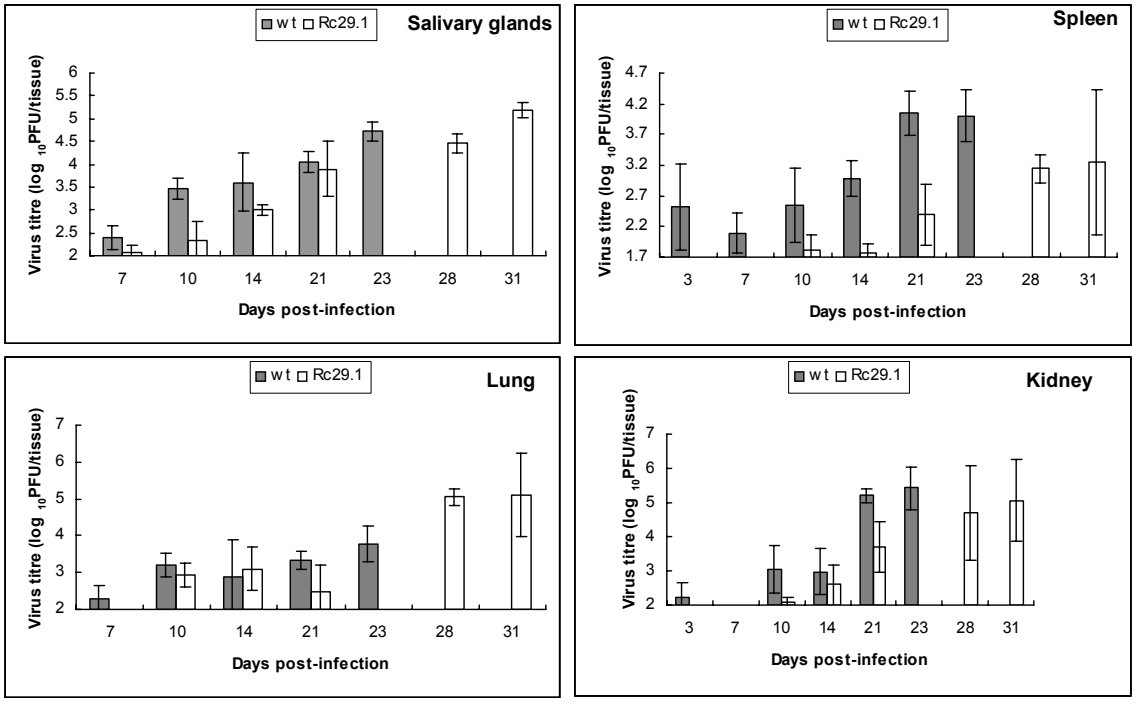


Figure 3.32 Virus titres in salivary glands, spleen, lung and kidneys of CB17 SCID mice infected with wt or Rc29.1 viruses. Mice were inoculated intraperitoneally with 10^4 PFU of virus. At different time points, animals were sacrificed. Their salivary glands, spleen, lung and kidneys collected, homogenised and titrated for virus. The limit of detection was 100 PFU/tissue for salivary glands, lung and kidneys; 50 PFU/tissue for spleen only. The viral titres represent the means of four animals ± standard deviation.

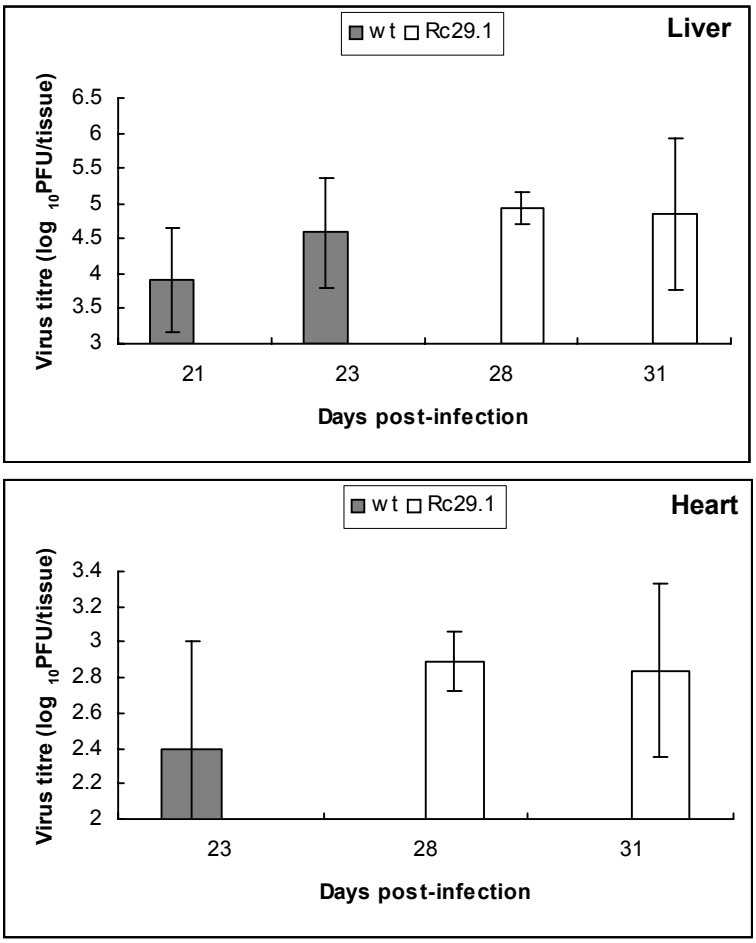


Figure 3.33 Virus titres in liver and heart of CB17 SCID mice infected with wt or Rc29.1 viruses. Mice were infected intraperitoneally with 10^4 PFU of virus. At different time points, animals were sacrificed. Their salivary glands, spleen, lung and kidneys collected, homogenised and titrated for virus. The limit of detection was 100 PFU/tissue. The viral titres represent the means of four animals ± standard deviation.

3.13 Point mutation is stable during viral growth in mice

MCMV mutants with point mutations could revert to wild type. Indeed, a point mutation identified in the M98 region (165) of mutant tsm5 (136) has been shown to revert to wild type after several passages in tissue-cultured cells (Timoshenko and Sweet, unpublished). Although the *in vitro* and *in vivo* phenotype of Rc29 and Rc29.1 viruses did not suggest that this had occurred it was important to confirm that virus replicating in mice was still mutant. Thus, salivary glands of Rc29 and Rc29.1 inoculated BALB/c mice were harvested 21 days post-infection, homogenised and inoculated onto NIH 3T3 cells. Viral DNA was purified from the infected cells and sequenced over m29 and m29.1 region. A point mutation in the m29 gene of Rc29 virus and in the m29.1 gene of Rc29.1 virus was confirmed to be at nt positions 35,896 and 36,484 respectively (data not shown). Thus, the point mutation in Rc29 and Rc29.1 appeared to be stable.

4. DISCUSSION

4.1 Sequence discrepancy to the published genome

The MCMV genome was predicted to encode more than 170 ORFs, 78 of which have extensive homology to those of HCMV (128). Brocchieri and colleagues (20) re-annotated the whole MCMV genome sequence based on the previously published MCMV sequence data (128) and predicted a total of 126 ORFs which were not noticeable in prior annotation efforts. However, few of these were examined to see if they are expressed. They also suggested that CMV genomes are likely to encode a greater number of overlapping genes than previously appreciated. A newly annotated ORF, m28.2 (20) overlapped with the published m29 ORF (128). Another re-analysis of the MCMV sequence identified 14 additional ORFs (170), some of which were shown to be expressed.

A sequence discrepancy to the published MCMV genome (128) was identified in the m29 and m29.1 ORFs region in this study. An extra nucleotide, a guanine residue, at nucleotide position 36,198 was found, altering the predicted ORFs of m29 and m29.1. To examine whether this mutation is distinctive of MCMV strains, this region in other MCMV isolates was sequenced. This difference was also found in MCMV strain K181 and Smith (Birmingham) (136) and natural MCMV wild type isolates (N1, K17A and G4) (89). Insertion of this G shortened the C terminus of m29 from position 35,747

to 36,471 and extended that of m29.1 from position 36,660 to 36,032. A further cytosine insertion at nt position 37263 was identified in our Smith strain MCMV BAC in the m30 ORF and confirmed through sequencing the MCMV Smith strain (Birmingham) and natural wild type isolates (N1, K17A and G4). Insertion of this cytosine residue alters the ORF such that the stop codon in m30 no longer exists and the predicted protein continues into the published M31 ORF. It would be useful to sequence the M31 ORF to determine the stop codon for m30. This is because both ORFs are coded on same strand and M31 overlaps m30. Further sequencing studies were not followed-up due to time constraints. Such types of sequence discrepancies were also reported by several other groups. Indeed, an incorrect G insertion into the m20 ORF at position 20,958 of the MCMV genome was reported by Kattenhorn and colleagues (70). Removal of this G extended the C terminus of m20 from position 20,805 to position 20,579. Therefore, further annotation efforts should be carried out to resolve the anomalies in these studies.

4.2 Organization of m29 and m29.1 ORFs and location of mutation

The functional analysis of a gene needs manipulation at the DNA level. Insertional or deletion mutations are routinely used to characterize the function of the MCMV encoded genes (169, 170, 191). Genetic analysis of DNA viruses is difficult due to the compactness of the genomes, which results in overlapping ORFs. The m29 and m29.1 ORFs are overlapping and coded on opposite strands of the viral double stranded DNA (128). The newly predicted ORF m28.2 also overlaps m29 and is coded on the same strand. The ORF m29.2

overlaps m30 but is encoded on the complementary strand just upstream of the m29.1 ORF (Figure 4.1). Thus, to generate mutants in this region care was needed not to affect neighbouring genes. In this study, point mutations that introduced premature stop codons into the m29 and m29.1 ORFs near to 5'-end of each ORF were constructed using BAC mutagenesis.

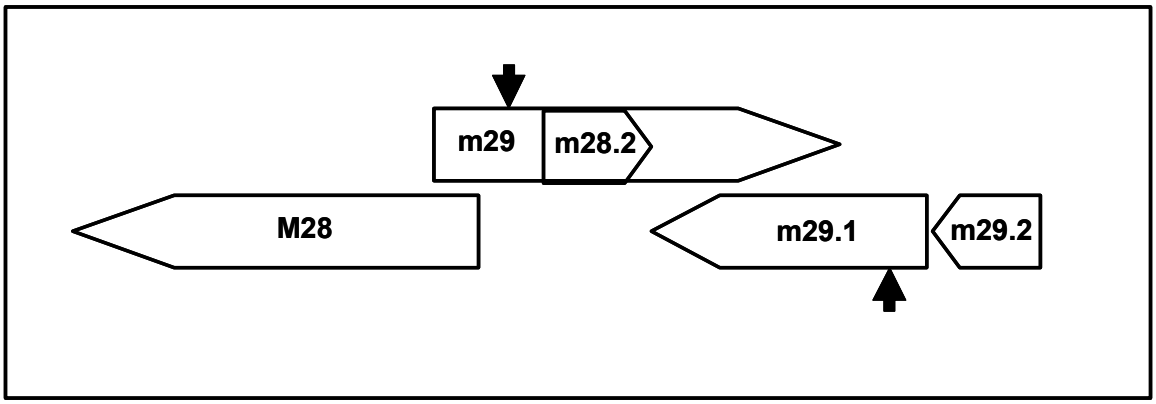


Figure 4.1 A probable map of m29 and m29.1 ORFs with neighbouring ORFs. The position of the point mutations in m29 and m29.1 is indicated by the arrow heads. The direction of transcription is indicated by the sharp end to the boxes, m29 and m28.2 being encoded on the top DNA strand, the others on the bottom strand.

4.3 m29, and m29.1 ORFs are detectable in NIH 3T3 cells

RT-PCR is generally used to detect MCMV gene expression in infected cells, this has an increased sensitivity over that of microarray assay (170). Transcription from the m29 and m29.1 region was first detected in NIH 3T3 cells at 3 and 2 hours post-infection, respectively using RT-PCR and expression was detected at all time points up to 24 hours. These observations were consistent with those of Tang and colleagues (170) who showed expression of the m29 and m29.1 ORFs in MCMV infected NIH 3T3 cells 24

hours post-infection using microarray assays. Attempts were made to characterise the number and size of the m29 and m29.1 transcripts in infected cells using northern blotting. Unfortunately, no RNA species were detected from either ORF in infected cells using probes derived from either randomly primed DNA or single stranded PCR products. An antisense RNA probe from the m29 or m29.1 ORFs would need to be synthesised by *in vitro* transcription as it has increased sensitivity over DNA or PCR probes. Due to time constraints no further northern blotting experiments were performed.

The kinetic class expression pattern of IE, E and L genes was confirmed using protein and DNA synthesis inhibitors. Expression of the m123(ie1/3) ORF and lack of expression of M112/113(e1) in infected cells at IE times post-infection clearly showed that the dose of cycloheximide used was adequate to inhibit protein synthesis. The M75 gene has been classified to be an E-L gene. The intensity of RT-PCR products produced from the M75 transcript in the presence and absence of phosphonoacetic acid confirmed that inhibition of DNA synthesis occurred which agreed with the findings of Chambers and colleagues (28). In this study, it has been shown that m29 expression was inhibited in the presence of cycloheximide but not phosphonoacetic acid and thus belongs to the E gene family whereas m29.1 expression occurred in the presence of cycloheximide and thus was classed as a member of the IE gene family.

4.4 The MCMV genes m29 and m29.1 are dispensable

The majority of MCMV genes (60%) are found to be non-essential for virus replication in cell culture (24). Indeed, a mutant with a deletion of the entire M27 to M31 region has recently been reported to be viable in tissue culture (24). Previous work in our laboratory with Kn29 and Kn29.1 viruses, with an insertional mutation in the m29 and m29.1 ORFs, showed that m29 and m29.1 ORFs are dispensable (Kirby, personal communication). The Rc29 and Rc29.1 BAC DNAs with a point mutation in the m29 and m29.1 ORFs respectively were transfected into NIH 3T3 cells to reconstitute infectious viruses. Infectious virus was readily obtained upon transfection, further suggesting that m29 and m29.1 ORFs are dispensable for replication in fibroblast cultures.

4.5 Stop codon mutation should not affect transcription but should affect translation

Due to insertion of a premature stop codon mutation at the 5' end of the m29 and m29.1 ORFs in Rc29 and Rc29.1 viruses respectively, transcripts from the both ORFs region should be expressed in each mutant. RT-PCR results strongly showed that this was the case (Figure 3.20). Similarly, expression of overlapping ORFs m29.2 and M28 was unaffected (Figure 3.28). However, no functional m29 or m29.1 proteins should be produced. Western blot analysis of the m29.1 protein provided direct evidence to show that the m29.1 protein was not expressed in Rc29.1 virus infected cells but it was in Rc29 and Rv29.1 virus infected cells (Figure 3.23). On the other hand, no m29 protein product

was detected in wt, Rc29.1 or Rc29 virus infected cells. Antibody to the m29.1 protein was raised in rabbits to purified protein expressed in *E. coli* cells and thus was monospecific and polyclonal. However, several attempts to express m29 protein in bacterial cells were unsuccessful. Likely epitopes in this protein were identified from its sequence and one of these was synthesised and used to immunise rabbits. Unfortunately, this antibody failed to detect m29 protein in western blots possibly because this epitope is part of a conformational epitope not present on the denatured protein run on gels or indeed is not antigenic at all. Thus, the premature stop codon mutation in the m29.1 ORF of Rc29.1 has prevented the expression of functional protein.

4.6 Properties of Rc29 and Rc29.1 viruses

The Rc29 and Rc29.1 viruses were investigated for their growth phenotype *in vitro*. MEF cells were infected at low (0.05) and high (5.0) MOI with Rc29 and Rc29.1 viruses and their growth characteristics compared to wt virus. At high MOI, no significant growth difference was found between Rc29 and wt virus. At low MOI, Rc29 virus grew to slightly lower titres when compared to the wt virus, suggesting a defect in virus release. Therefore, in this study, it has been concluded that m29 ORF is likely to play a role in virus release during replication but it seems to have a minor effect.

On the other hand, at both high and low MOI, Rc29.1 showed a significantly slower growth than wt virus. Thus, although m29.1 is not an essential ORF its product augments virus replication and appears to be required for maximal

growth. Another interesting feature is that Rc29.1 virus formed smaller plaques than the parental virus in MEF cells (Figure 3.26). As m29.1 belongs to IE gene family and as the MCMV ie1 mutant virus also showed slower growth and formed smaller plaques than the parental virus in MEF cells (102) it is interesting to speculate that m29.1 may have a similar function to ie1.

To study the replication of Rc29 and Rc29.1 in immunocompetent and immunodeficient hosts, an *in vivo* experiment was carried out with adult BALB/c and SCID mice. In immunocompetent mice, e.g. BALB/c, innate and adaptive immunity are able to combat viral replication. The experiment with these mice was expected to show, whether or not, the mutation in m29 or m29.1 ORFs had a growth defect *in vivo*. Therefore, to study the viral replication *in vivo*, BALB/c mice are routinely injected intraperitoneally with 10^4 PFU and examined over a short period of time (82, 169, 191). In contrast, immunodeficient animals, e.g. CB17 SCID mice, are extremely sensitive to MCMV infection (53, 115, 124, 133). A low dose of 10 PFU is capable of killing half of the infected animals (124). The experiment with these mice was expected to show, whether or not, the Rc29 or Rc29.1 viruses were capable of replication or causing death as the mice have no adaptive immune response. Therefore, SCID mice are routinely injected intraperitoneally with 10^4 PFU and examined over a short period of time to study the viral replication, virulence and pathogenicity *in vivo* (82, 169, 191).

In BALB/c mice, no significant difference was observed between wt and Rc29 viruses in salivary glands. On the other hand, Rc29.1 was delayed in

replication in the salivary glands. For example, the wt virus was first detected with titres ~200 PFU/tissue at 7 days post-infection and was detectable with titres ~$4.0x10^4$ PFU/tissue at 42 days post-infection in salivary glands. But Rc29.1 virus was first detected with titres ~$2.2x10^2$ PFU/tissue at 10 days post-infection and was detectable with titres ~$1.5x10^3$ PFU/tissue at 28 days post-infection in the same organs (Figure 3.30). No viruses were recovered from the lung, spleen, kidneys, liver and heart of BALB/c mice that were intraperitoneally infected with either wt or Rc29 or Rc29.1 viruses. As Rc29 virus showed no growth difference in BALB/c mice, it was assumed that the same results would be observed in SCID mice. Therefore, Rc29 virus was not injected into SCID mice. In SCID mice, Rc29.1 was also delayed in replication in the salivary glands, lungs, spleens, kidneys, livers and hearts. For example, the Rc29.1 virus was first detected in spleen, lung and kidneys at 10 days post-infection with titres 75, $1.0x10^3$, $1.3x10^2$ PFU/tissue respectively, whereas wt virus was first detected in these organs at 3, 7 and 3 days post-infection respectively. In salivary glands, both viruses (wt and Rc29.1) were first detected at 7 days post-infection. Rc29.1 showed slightly reduced growth but the difference was not significant (Figure 3.32). In liver and heart, wt virus was first detectable at 21 and 23 days post-infection, respectively and in the same organs Rc19.1 was first detectable at 28 days post-infection (Figure 3.33). Moreover, all SCID mice infected with wt were found sick at 23 days post-infection, whereas those infected with Rc29.1 were not sick until 31 days post-infection (Figure 3.31). All sick mice were sacrificed due to Home Office license agreement. The Rc29.1 virus exhibited a delayed level of virulence in killing SCID mice as compared to that of the wt virus. From the above discussion, it

strongly suggests that m29 and m29.1 ORFs are not essential for viral replication *in vivo*, but m29.1 ORF improves the growth of MCMV significantly both in immunocompetent and immunodeficient hosts.

It is possible that the observed change in the level of growth of the Rc29.1 is due to other adventitious mutations introduced during the construction and growth of mutant virus in tissue culture cells. However, several lines of evidence suggest that this is unlikely. First, the revertant virus Rv29.1 (the mutation in m29.1 ORF was restored to wt sequence) grew similarly in infected cultured cells (Figure 3.25) and in salivary glands of the infected BALB/c mice (Figure 3.30) to wt virus. As Rv29.1 and wt viruses grew equally in BALB/c, it was assumed that the same phenotype would be observed in SCID mice. Thus, the revertant virus Rv29.1 was not injected into SCID mice. Second, restoration of the wt phenotype in Rv29.1 occurred together with the restoration of m29.1 protein expression (Figure 3.23). Third, Rc29.1 virus produced smaller plaques than wt virus on MEF cell monolayers. The Rv29.1 virus produced similar sized plaques to wt virus (Figure 3.26). Fourth, the mutation in the m29.1 ORFs were stable during replication in animals (Section 3.12). Thus, these results suggest that the observed change in the level of growth of the Rc29.1 is due to disruption of m29.1 protein expression as a consequence of a stop codon insertion at the 5' end.

4.7 RACE analysis of m29.1 transcript

The MCMV immediate early region produces two alternative spliced transcripts that encode the ie1 protein pp89 and the 88 kDa ie3 protein (101). Indeed, an MCMV ie1 mutant virus showed slower growth and formed smaller plaques than the parental virus in MEF cells (102). In this study, it has been concluded that the m29.1 gene belongs to the IE class of genes (Section 3.2.3) and the phenotypes observed for the Rc29.1 virus were similar to those of the MCMV ie1 mutant virus (102). Therefore, it was possible that the m29.1 ORF might produce a spliced transcript(s). To examine this, a 5' and 3' RACE analysis of mRNA extracted from wt virus infected cells was performed. RACE analysis has been routinely used to examine mRNA species in CMV infected cells (4, 90, 175). 5'-RACE revealed two transcription start sites at nt 36,752 and 36,754, 92bp and 94bp upstream from the previously predicted initiator methionine (128) and an appropriate TATA sequence at position 36,784. 3'-RACE analysis identified an AAUAAA polyadenylation signal at nt 34,359, followed by a poly(A) tag at nt 34,342 (Figure 3.27). Therefore, the transcript from the m29.1 ORF was about 2.4kb in length. However, two other interesting observations were made from analysis of the RACE results. First, analysis of the MCMV genomic sequence revealed that ORF M28 may be contained within the m29.1 mRNA because the first AAUAAA polyadenylation signal is located 3` to the M28 ORF. Second, a low abundance spliced transcript (1 out of 8 clones) was found to be expressed from the m29.1 ORF in which a 123bp intron had been removed. However, further northern blot analyses with more

suitable probes will need to be carried out to confirm the unspliced and spliced transcripts expressed from the m29.1 ORF.

Several possibilities may explain how the m29.1 and M28 ORFs are expressed from one mRNA. These include: leaky scanning, re-initiation, ribosomal frameshifting, suppression of termination, splicing and an internal ribosome entry site (IRES). Leaky scannning usually occurs when the ribosome binds at the next AUG codon and is unlikely to operate in this case. Suppression of termination occurs relatively frequently with viruses e.g. the alphavirus nsP4 (85) and retrovirus gag-Pol genes (180) but read-through of the m29.1 stop codon would produce an m29.1/M28 fusion protein. Western blotting showed only a single protein indicative of the size expected for the m29.1 product. Ribosomal frameshift is unlikely for the same reason.

Re-initiation is a possibility as this has been observed for the HCMV gpUL4 (gp48) gene (26). However, the upstream ORF (uORF2) is short, only 22 codons in length, and represses expression from the down stream ORF as ribosomes stall on the uORF2 termination codon (26). Clearly m29.1 is an independent ORF and this mechanism is unlikely to explain how the M28 protein is produced. The most likely explanation is that an IRES may be located in between the m29.1 and M28 gene producing a bi-cistronic mRNA. All eukaryotic mRNAs possess a cap structure at the 5' end that plays a central role to promote ribosome binding to the mRNA and to control the rate of translation initiation (49, 143). Ribosomes may also access eukaryotic mRNA by binding to an IRES. Thus, the m29.1 protein is synthesized by the cap-

dependent initiation approach. Translation initiation of the M28 protein could be directed by the IRES segment which is predicted to be located upstream from the M28 initiation codon. IRESs were first discovered in picornavirus RNA, which have no 5' cap structure (64, 119) and the presence of IRESs have also been reported in herpesvirus mRNA (10).

IRES sequences are not conserved. Thus, it was not possible to identify an IRES sequence by a DNA database search. In future, a bicistronic luciferase reporter system (10) could be utilised to investigate whether the M28 protein is expressed from the 2.4kb bicistronic message utilising an IRES sequence. In this system, a bicistronic luciferase reporter plasmid could be constructed (pdLUC) (Figure 4.2), in which the genes for *Renilla* and firefly luciferases (which utilize different substrates) has been cloned in tandem downstream from a T7 RNA polymerase promoter (10). In this plasmid, ribosomes would have inefficient access to the downstream cistron (firefly luciferase), but would express the upstream cistron (*Renilla* luciferase). However, if an IRES sequence is inserted before the downstream ORF (firefly luciferase), translation should be considerably enhanced (up to 100-fold). Thus, DNA fragments immediately upstream from the M28 start codon could be cloned into plasmid pdLUC. The ratio of firefly luciferase activity to *Renilla* luciferase activity, one directed by putative IRES sequences and the other by cap-dependant ribosome scanning, could be used to compare the activities of the different DNA fragments. The above system has been used to detect an IRES in Kaposi's sarcoma- associated herpes virus (KSHV) (10).

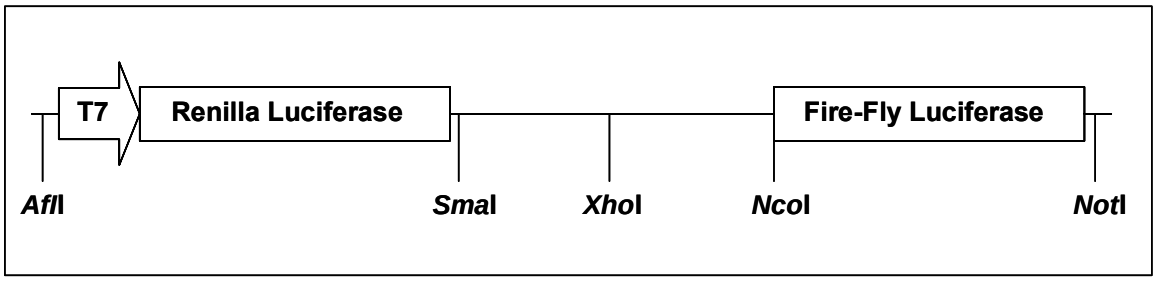


Figure 4.2 Map of the plasmid pdLUC. The plasmid has the coding sequence for the *Renilla* and firefly luciferase enzymes cloned downstream of a T7 RNA polymerase promoter. IRES sequences could be cloned between *Sma*I and *Nco*I restriction site.

4.8 The m29.1 ORF could be a transcription factor

CMV genes are transcribed in three sequential phases which are temporally regulated at IE, E or L times post infection. IE genes are the first viral genes which are transcribed upon infection without the need for prior viral protein synthesis. IE proteins have regulatory functions that are required for the expression of E and L genes. Three IE genes have been identified within the MCMV IE region. The most abundantly transcribed IE gene of MCMV, ie1, is an activator of transcription (75). The ie3 is a strong transcriptional activator of the MCMV e1 promoter and also shows an autoregulatory function by repression of the MCMV ei1/ie3 promoter (101). The ie2 is transcribed in the opposite direction to ie-1 and ie-3 and found to be non-essential for virus replication *in vitro* or *in vivo* (27). In this study, the m29.1 ORF has been classified as an IE gene. It is likely that the m29.1 ORF might have regulatory functions that are required for the expression of E and L genes. Indeed, the

Rc29.1 mutant virus showed a similar phenotype to that of the MCMV ie1 mutant virus. Thus, it is possible to speculate that the m29.1 ORF product could be an activator of transcription.

A global-DNA microarray has been developed to detect MCMV gene expression in infected cells (170). It would be useful to do a microarray experiment on mRNA extracted from Rc29.1 virus infected cells to observe whether the mutation in the m29.1 ORF down-regulates or up-regulates any other genes in the genome as the products of the MCMV ie-1(m123) and ie-3(M122) genes had been proved as transactivators and repressors respectively (75, 101). Similarly, the MCMV enhancer has five or more binding sites for cellular factors including NF-κB and AP-1 to regulate transcription. Thus, it was interesting to speculate that upstream of the m29.1 ORF there may be similar DNA binding motifs for cellular transcription factors. At least 1000bp nucleotide sequences upstream from the m29.1 ORF were checked but no DNA binding motifs were found.

4.9 Conclusion

The function of m29 and m29.1 ORF is unknown. Neither transcripts nor the protein products coded by these ORFs had been reported previously. This study indicates that m29 belongs to the early class gene family and is dispensable for virus replication both in tissue culture *in vitro* and in animals *in vivo*. However, it seems that m29 ORF has a minor effect on virus release during replication. In contrast, the m29.1 ORF is reported as an immediate

early gene in this study. It has been proposed that m29.1 ORF could be a transcription factor. However, apart from the kinetic experiments, no further evidence has been shown to support this assumption. A transcript of about 2.4kb is expressed from the m29.1 ORF which produces a protein product of about 28-30 kDa. The m29.1 ORF is also dispensable for virus replication both in tissue culture *in vitro* and in animals *in vivo*, but the virus grew to significantly lower yields and showed delayed replication due to lack of the m29.1 protein which suggests that m29.1 ORF is required for optimum viral growth.

5. REFERENCES

1. **Adler, S. P.** 1989. Cytomegalovirus and child day care. Evidence for an increased infection rate among day-care workers. N Engl J Med **321:**1290-6.

2. **Akel, H. M., and C. Sweet.** 1993. Isolation and preliminary characterisation of twenty-five temperature-sensitive mutants of mouse cytomegalovirus. FEMS Microbiol Lett **113:**253-60.

3. **Akira, S., and K. Takeda.** 2004. Toll-like receptor signalling. Nature Reviews Immunology **4:**499-511.

4. **Akter, P., C. Cunningham, B. P. McSharry, A. Dolan, C. Addison, D. J. Dargan, A. F. Hassan-Walker, V. C. Emery, P. D. Griffiths, G. W. G. Wilkinson, and A. J. Davison.** 2003. Two novel spliced genes in human cytomegalovirus. J Gen Virol **84:**1117-1122.

5. **Allan-Yorke, J., M. Record, C. de Preval, C. Davrinche, and J.-L. Davignon.** 1998. Distinct pathways for tumor necrosis factor alpha and ceramides in human cytomegalovirus infection. J. Virol. **72:**2316-2322.

6. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25:**3389-402.

7. **Anders, D. G., and L. A. McCue.** 1996. The human cytomegalovirus genes and proteins required for DNA synthesis. Intervirology **39:**378-88.

8. **Arase, H., E. S. Mocarski, A. E. Campbell, A. B. Hill, and L. L. Lanier.** 2002. Direct recognition of cytomegalovirus by activating and inhibitory NK Cell receptors. Science **296:**1323-1326.

9. **Berenguer, J., J. Gonzalez, F. Pulido, B. Padilla, J. L. Casado, R. Rubio, and J. R. Arribas.** 2002. Discontinuation of secondary prophylaxis in patients with cytomegalovirus retinitis who have responded to highly active antiretroviral therapy. Clin Infect Dis **34:**394-7.

10. **Bieleski, L., and S. J. Talbot.** 2001. Kaposi's sarcoma-associated herpesvirus vCyclin open reading frame contains an internal ribosome entry site. J. Virol. **75:**1864-1869.

11. **Biron, C. A., K. B. Nguyen, G. C. Pien, L. P. Cousens, and T. P. Salazar-Mather.** 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annual Review of Immunology **17:**189-220.

12. **Biron, K. K.** 2006. Antiviral drugs for cytomegalovirus diseases. Antiviral Research **71:**154-163.

13. **Biron, K. K., R. J. Harvey, S. C. Chamberlain, S. S. Good, A. A. Smith, III, M. G. Davis, C. L. Talarico, W. H. Miller, R. Ferris, R. E. Dornsife, S. C. Stanat, J. C. Drach, L. B. Townsend, and G. W. Koszalka.** 2002. Potent and selective inhibition of human cytomegalovirus replication by 1263W94, a Benzimidazole L-Riboside with a unique mode of action. Antimicrob. Agents Chemother. **46:**2365-2372.

14. **Boeckh, M., W. Leisenring, S. R. Riddell, R. A. Bowden, M. L. Huang, D. Myerson, T. Stevens-Ayers, M. E. Flowers, T. Cunningham, and L. Corey.** 2003. Late cytomegalovirus disease and mortality in recipients of allogeneic hematopoietic stem cell transplants: importance of viral load and T-cell immunity. Blood **101:**407-14.

15. **Boppana, S. B., and W. J. Britt.** 1996. Recognition of human cytomegalovirus gene products by HCMV-specific cytotoxic T cells. Virology **222:**293-296.

16. **Boppana, S. B., M. A. Polis, A. A. Kramer, W. J. Britt, and S. Koenig.** 1995. Virus-specific antibody responses to human cytomegalovirus (HCMV) in human immunodeficiency virus type 1-infected persons with HCMV retinitis. J Infect Dis **171:**182-5.

17. **Borst, E. M., G. Hahn, U. H. Koszinowski, M. Messerle, I. Crnkovic, W. Hammerschmidt, and H. Ziegler.** 1999. Cloning of the human cytomegalovirus (HCMV) genome as an infectious bacterial artificial chromosome in Escherichia coli: a new approach for construction of HCMV mutants. J Virol **73:**8320-9.

18. **Brennan, D. C.** 2001. Cytomegalovirus in renal transplantation. J Am Soc Nephrol **12:**848-855.

19. **Britt, W.** 2006. Human cytomegalovirus infections and mechanisms of disease, p. 1-28. *In* M. J. Reddehase (ed.), Cytomegalovirus Molecular Biology and Immunology. Caister Academic Press, Norfolk, UK.

20. **Brocchieri, L., T. N. Kledal, S. Karlin, and E. S. Mocarski.** 2005. Predicting coding potential from genome sequence: application to betaherpesviruses infecting rats and mice. J Virol **79:**7570-96.

21. **Brown, M. G., A. O. Dokun, J. W. Heusel, H. R. C. Smith, D. L. Beckman, E. A. Blattenberger, C. E. Dubbelde, L. R. Stone, A. A. Scalzo, and W. M. Yokoyama.** 2001. Vital involvement of a natural killer cell activation receptor in resistance to viral infection. Science **292:**934-937.

22. **Browne, E. P., B. Wing, D. Coleman, and T. Shenk.** 2001. Altered cellular mRNA levels in human cytomegalovirus-infected fibroblasts: viral block to the accumulation of antiviral mRNAs. J. Virol. **75:**12319-12330.

23. **Brune, W., M. Messerle, and U. H. Koszinowski.** 2000. Forward with BACs: new tools for herpesvirus genomics. Trends Genet **16:**254-9.

24. **Brune, W., M. Wagner, and M. Messerle.** 2006. Manipulating cytomegalovirus genomes by BAC mutagenesis: Strategies and applications, p. 63-89. *In* M. J. Reddehase (ed.), Cytomegalovirus Molecular Biology and Immunology. Caister Academic Press, Norfolk, UK.

25. **Bubic, I., M. Wagner, A. Krmpotic, T. Saulig, S. Kim, W. M. Yokoyama, S. Jonjic, and U. H. Koszinowski.** 2004. Gain of virulence caused by loss of a gene in murine cytomegalovirus. J. Virol. **78:**7536-7544.

26. **Cao, J., and A. Geballe.** 1996. Coding sequence-dependent ribosomal arrest at termination of translation. Mol. Cell. Biol. **16:**603-608.

27. **Cardin, R. D., G. B. Abenes, C. A. Stoddart, and E. S. Mocarski.** 1995. Murine cytomegalovirus IE2, an activator of gene expression, is dispensable for growth and latency in mice. Virology **209:**236-41.

28. **Chambers, J., A. Angulo, D. Amaratunga, H. Guo, Y. Jiang, J. S. Wan, A. Bittner, K. Frueh, M. R. Jackson, P. A. Peterson, M. G. Erlander, and P. Ghazal.** 1999. DNA microarrays of the complex human cytomegalovirus genome: profiling kinetic class with drug sensitivity of viral gene expression. J. Virol. **73:**5757-5766.

29. **Chang, W. L. W., and P. A. Barry.** 2003. Cloning of the full-length rhesus cytomegalovirus genome as an infectious and self-excisable bacterial artificial chromosome for analysis of viral pathogenesis. J. Virol. **77:**5073-5083.

30. **Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, T. Horsnell, C. A. Hutchison, 3rd, T. Kouzarides, J. A. Martignetti, and et al.** 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr Top Microbiol Immunol **154:**125-69.

31. **Chomczynski, P.** 1992. One-hour downward alkaline capillary transfer for blotting of DNA and RNA. Analytical Biochemistry **201:**134-139.

32. **Colucci, F., J. P. Di Santo, and P. J. Leibson.** 2002. Natural killer cell activation in mice and men: different triggers for similar weapons? Nat Immunol **3:**807-13.

33. **Compton, T.** 2004. Receptors and immune sensors: the complex entry path of human cytomegalovirus. Trends in Cell Biology **14:**5-8.

34. **Compton, T., E. A. Kurt-Jones, K. W. Boehme, J. Belko, E. Latz, D. T. Golenbock, and R. W. Finberg.** 2003. Human cytomegalovirus activates inflammatory cytokine responses via CD14 and Toll-Like receptor 2. J. Virol. **77:**4588-4596.

35. **Compton, T., D. M. Nowlin, and N. R. Cooper.** 1993. Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate. Virology **193:**834-841.

36. **Daniels, K. A., G. Devora, W. C. Lai, C. L. O'Donnell, M. Bennett, and R. M. Welsh.** 2001. Murine cytomegalovirus is regulated by a discrete subset of natural killer cells reactive with monoclonal antibody to Ly49h. J. Exp. Med. **194:**29-44.

37. **Davison, A. J., A. Dolan, P. Akter, C. Addison, D. J. Dargan, D. J. Alcendor, D. J. McGeoch, and G. S. Hayward.** 2003. The human cytomegalovirus genome revisited: comparison with the chimpanzee cytomegalovirus genome. J Gen Virol **84:**17-28.

38. **De Clercq, E., and H. J. Field.** 2006. Antiviral prodrugs - the development of successful prodrug strategies for antiviral chemotherapy. Br J Pharmacol **147:**1-11.

39. **Deayton, J., A. Mocroft, P. Wilson, V. C. Emery, M. A. Johnson, and P. D. Griffiths.** 1999. Loss of cytomegalovirus (CMV) viraemia following highly active antiretroviral therapy in the absence of specific anti-CMV therapy. Aids **13:**1203-6.

40. **Depatie, C., E. Muise, P. Lepage, P. Gros, and S. M. Vidal.** 1997. High-resolution linkage map in the proximity of the host resistance locus Cmv1. Genomics **39:**154-163.

41. **Dolan, A., C. Cunningham, R. D. Hector, A. F. Hassan-Walker, L. Lee, C. Addison, D. J. Dargan, D. J. McGeoch, D. Gatherer, V. C. Emery, P. D. Griffiths, C. Sinzger, B. P. McSharry, G. W. G. Wilkinson, and A. J. Davison.** 2004. Genetic content of wild-type human cytomegalovirus. J Gen Virol **85:**1301-1312.

42. **Ehsani, M. E., T. W. Abraha, C. Netherland-Snell, N. Mueller, M. M. Taylor, and B. Holwerda.** 2000. Generation of mutant murine cytomegalovirus strains from overlapping cosmid and plasmid clones. J Virol **74:**8972-9.

43. **Farrell, H. E., and G. R. Shellam.** 1991. Protection against murine cytomegalovirus infection by passive transfer of neutralizing and non-neutralizing monoclonal antibodies. J Gen Virol **72:**149-156.

44. **FELASA.** 1994. Pain and distress in laboratory rodents and lagomorphs. Report of the Federation of European Laboratory Animal Science Associations (FELASA) Working Group on Pain and Distress accepted by the FELASA Board of Management November 1992.

45. **Fishman, J. A., and R. H. Rubin.** 1998. Infection in organ-transplant recipients. N Engl J Med **338:**1741-1751.

46. **Forbes, C. A., M. G. Brown, R. Cho, G. R. Shellam, W. M. Yokoyama, and A. A. Scalzo.** 1997. The cmv1 host resistance locus is closely linked to the Ly49 multigene family within the natural killer cell gene complex on mouse chromosome 6. Genomics **41:**406-413.

47. **Gerna, G., E. Percivalle, F. Baldanti, S. Sozzani, P. Lanzarini, E. Genini, D. Lilleri, and M. G. Revello.** 2000. Human cytomegalovirus replicates abortively in polymorphonuclear leukocytes after transfer from infected endothelial cells via transient microfusion events. J. Virol. **74:**5629-5638.

48. **Gerna, G., M. Zavattoni, F. Baldanti, M. Furione, L. Chezzi, M. G. Revello, and E. Percivalle.** 1998. Circulating cytomegalic endothelial cells are associated with high human cytomegalovirus (HCMV) load in AIDS patients with late-stage disseminated HCMV disease. Journal of Medical Virology **55:**64-74.

49. **Gingras, A. C., B. Raught, and N. Sonenberg.** 1999. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu Rev Biochem **68:**913-63.

50. **Gonczol, E., K. Berencsi, S. Pincus, V. Endresz, C. Meric, E. Paoletti, and S. A. Plotkin.** 1995. Preclinical evaluation of an ALVAC (canarypox)-human cytomegalovirus glycoprotein B vaccine candidate. Vaccine **13:**1080-1085.

51. **Greber, U. F., and M. Way.** 2006. A Superhighway to Virus Infection. Cell **124:**741-754.

52. **Griffiths, P. D., D. A. Clark, and V. C. Emery.** 2000. Betaherpesviruses in transplant recipients. J. Antimicrob. Chemother. **45:**29-34.

53. **Grundy, J., and C. Melief.** 1982. Effect of Nu/Nu gene on genetically determined resistance to murine cytomegalovirus. J Gen Virol **61:**133-136.

54. **Gunturi, A., R. E. Berg, and J. Forman.** 2004. The role of CD94/NKG2 in innate and adaptive immunity. Immunologic Research **30:**29-34.

55. **Hahn, G., R. Jores, and E. S. Mocarski.** 1998. Cytomegalovirus remains latent in a common precursor of dendritic and myeloid cells. Proc Natl Acad Sci U S A **95:**3937-42.

56. **Hahn, G., H. Khan, F. Baldanti, U. H. Koszinowski, M. G. Revello, and G. Gerna.** 2002. The human cytomegalovirus ribonucleotide reductase homolog UL45 is dispensable for growth in endothelial cells, as determined by a BAC-cloned clinical isolate of human cytomegalovirus with preserved wild-type characteristics. J. Virol. **76:**9551-9555.

57. **Hahn, G., D. Rose, M. Wagner, S. Rhiel, and M. A. McVoy.** 2003. Cloning of the genomes of human cytomegalovirus strains Toledo, TownevarRIT3, and Townelong as BACs and site-directed mutagenesis using a PCR-based technique. Virology **307:**164-177.

58. **Hasan, M., A. Krmpotic, Z. Ruzsics, I. Bubic, T. Lenac, A. Halenius, A. Loewendorf, M. Messerle, H. Hengel, S. Jonjic, and U. H. Koszinowski.** 2005. Selective down-regulation of the NKG2D ligand H60 by mouse cytomegalovirus m155 glycoprotein. J. Virol. **79:**2920-2930.

59. **Hendrix, M. G., M. Daemen, and C. A. Bruggeman.** 1991. Cytomegalovirus nucleic acid distribution within the human vascular tree. Am J Pathol **138:**563-7.

60. **Hirsch, M. S.** 1994. Cytomegalovirus infection, p. 794-797, Harrison's Principles of Internal Medicine, 13th ed. McGraw Hill Inc., St. Louis.

61. **Hobom, U., W. Brune, M. Messerle, G. Hahn, and U. H. Koszinowski.** 2000. Fast screening procedures for random transposon libraries of cloned herpesvirus genomes: mutational analysis of human cytomegalovirus envelope glycoprotein genes. J Virol **74:**7720-9.

62. **Holtappels, R., J. Podlech, G. Geginat, H.-P. Steffens, D. Thomas, and M. J. Reddehase.** 1998. Control of murine cytomegalovirus in the lungs: relative but not absolute immunodominance of the immediate-early 1 nonapeptide during the antiviral cytolytic T-lymphocyte response in pulmonary infiltrates. J. Virol. **72:**7201-7212.

63. **Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdorfer, T. Giese, S. Endres, and G. Hartmann.** 2002. Quantitative expression of Toll-like receptor 1-10 mrna in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol **168:**4531-4537.

64. **Jang, S. K., H. G. Krausslich, M. J. Nicklin, G. M. Duke, A. C. Palmenberg, and E. Wimmer.** 1988. A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J. Virol. **62:**2636-2643.

65. **Jarvis, M. A., and J. A. Nelson.** 2002. Human cytomegalovirus persistence and latency in endothelial cells and macrophages. Current Opinion in Microbiology **5:**403-407.

66. **Johnson, D. C., and P. G. Spear.** 1982. Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. J. Virol. **43:**1102-1112.

67. **Jonjic, S., I. Bubic, and A. Krmpotic.** 2006. Innate immunity to cytomegaloviruses, p. 285-319. *In* M. J. Reddehase (ed.), Cytomegalovirus Molecular Biology and Immunology. Caister Academic Press, Norfolk, UK.

68. **Jonjic, S., I. Pavic, B. Polic, I. Crnkovic, P. Lucin, and U. Koszinowski.** 1994. Antibodies are not essential for the resolution of primary cytomegalovirus infection but limit dissemination of recurrent virus. J. Exp. Med. **179:**1713-1717.

69. **Karavellas, M. P., S. P. Azen, J. C. MacDonald, C. L. Shufelt, C. Y. Lowder, D. J. Plummer, B. Glasgow, F. J. Torriani, and W. R. Freeman.** 2001. Immune recovery vitritis and uveitis in AIDS: clinical predictors, sequelae, and treatment outcomes. Retina **21:**1-9.

70. **Kattenhorn, L. M., R. Mills, M. Wagner, A. Lomsadze, V. Makeev, M. Borodovsky, H. L. Ploegh, and B. M. Kessler.** 2004. Identification of Proteins Associated with Murine Cytomegalovirus Virions. J. Virol. **78:**11187-11197.

71. **Kemble, G., G. Duke, R. Winter, and R. Spaete.** 1996. Defined large-scale alterations of the human cytomegalovirus genome constructed by cotransfection of overlapping cosmids. J Virol **70:**2044-8.

72. **Kemp, L. M., and D. S. Latchman.** 1989. Regulated transcription of herpes simplex virus immediate-early genes in neuroblastoma cells. Virology **171:**607-10.

73. **Kern, F., I. P. Surel, N. Faulhaber, C. Frommel, J. Schneider-Mergener, C. Schonemann, P. Reinke, and H.-D. Volk.** 1999. Target structures of the CD8+-T-cell response to human cytomegalovirus: the 72-kilodalton major immediate-early protein revisited. J. Virol. **73:**8179-8184.

74. **Khanna, R., and D. J. Diamond.** 2006. Human cytomegalovirus vaccine: time to look for alternative options. Trends in Molecular Medicine **12:**26-33.

75. **Koszinowski, U. H., G. M. Keil, H. Volkmer, M. R. Fibi, A. Ebeling-Keil, and K. Munch.** 1986. The 89,000-Mr murine cytomegalovirus immediate-early protein activates gene transcription. J. Virol. **58:**59-66.

76. **Kothari, S., J. Baillie, J. G. Sissons, and J. H. Sinclair.** 1991. The 21bp repeat element of the human cytomegalovirus major immediate early enhancer is a negative regulator of gene expression in undifferentiated cells. Nucleic Acids Res **19:**1767-71.

77. **Krmpotic, A., M. Hasan, A. Loewendorf, T. Saulig, A. Halenius, T. Lenac, B. Polic, I. Bubic, A. Kriegeskorte, E. Pernjak-Pugel, M. Messerle, H. Hengel, D. H. Busch, U. H. Koszinowski, and S. Jonjic.** 2005. NK cell activation through the NKG2D ligand MULT-1 is selectively prevented by the glycoprotein encoded by mouse cytomegalovirus gene m145. J. Exp. Med. **201:**211-220.

78. **Krosky, P. M., M.-C. Baek, and D. M. Coen.** 2003. The human cytomegalovirus UL97 protein kinase, an antiviral drug target, is required at the stage of nuclear egress. Journal Of Virology **77:**905-914.

79. **Krug, A., A. R. French, W. Barchet, J. A. A. Fischer, A. Dzionek, J. T. Pingel, M. M. Orihuela, S. Akira, W. M. Yokoyama, and M. Colonna.** 2004. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. Immunity **21:**107-119.

80. **Kubota, A., S. Kubota, H. E. Farrell, N. Davis-Poynter, and F. Takei.** 1999. Inhibition of NK Cells by murine CMV-encoded Class I MHC homologue m144. Cellular Immunology **191:**145-151.

81. **Lanier, L. L.** 2003. Natural killer cell receptor signaling. Current Opinion in Immunology **15:**308-314.

82. **Lee, M., J. Xiao, E. Haghjoo, X. Zhan, G. Abenes, T. Tuong, W. Dunn, and F. Liu.** 2000. Murine cytomegalovirus containing a mutation at open reading frame M37 is severely attenuated in growth and virulence in vivo. J Virol **74:**11099-107.

83. **Lee, S. H., S. Girard, D. Macina, M. Busa, A. Zafer, A. Belouchi, P. Gros, and S. M. Vidal.** 2001. Susceptibility to mouse cytomegalovirus is associated with deletion of an activating natural killer cell receptor of the C-type lectin superfamily. Nat Genet **28:**42-5.

84. **Li, C. R., P. D. Greenberg, M. J. Gilbert, J. M. Goodrich, and S. R. Riddell.** 1994. Recovery of HLA-restricted cytomegalovirus (CMV)-specific T-cell responses after allogeneic bone marrow transplant: correlation with CMV disease and effect of ganciclovir prophylaxis. Blood **83:**1971-9.

85. **Li, G. P., and C. M. Rice.** 1989. Mutagenesis of the in-frame opal termination codon preceding nsP4 of Sindbis virus: studies of translational readthrough and its effect on virus replication. J. Virol. **63:**1326-1337.

86. **Liu, R., J. Baillie, J. G. Sissons, and J. H. Sinclair.** 1994. The transcription factor YY1 binds to negative regulatory elements in the human cytomegalovirus major immediate early enhancer/promoter and mediates repression in non-permissive cells. Nucleic Acids Res **22:**2453-9.

87. **Lodoen, M., K. Ogasawara, J. A. Hamerman, H. Arase, J. P. Houchins, E. S. Mocarski, and L. L. Lanier.** 2003. NKG2D-mediated natural killer cell protection against cytomegalovirus is impaired by viral gp40 modulation of retinoic acid early inducible 1 gene molecules. J. Exp. Med. **197:**1245-1253.

88. **Loser, P., G. S. Jennings, M. Strauss, and V. Sandig.** 1998. Reactivation of the previously silenced cytomegalovirus major immediate-early promoter in the mouse liver: involvement of NFkappaB. J Virol **72:**180-90.

89. **Lyons, P. A., J. E. Allan, C. Carrello, G. R. Shellam, and A. A. Scalzo.** 1996. Effect of natural sequence variation at the H-2Ld-restricted CD8+ T cell epitope of the murine cytomegalovirus ie1-encoded pp89 on T cell recognition. J Gen Virol **77 (Pt 10):**2615-23.

90. **MacDonald, M. R., M. W. Burney, S. B. Resnick, and H. W. Virgin, IV.** 1999. Spliced mRNA encoding the murine cytomegalovirus chemokine homolog predicts a beta chemokine of novel structure. J. Virol. **73:**3682-3691.

91. **Mach, M.** 2006. Antobody-mediated neutralization of infectivity, p. 265-283. *In* M. J. Reddehase (ed.), Cytomegalovirus Molecular Biology and Immunology. Caister Academic Press, Norfolk, UK.

92. **Marchini, A., H. Liu, and H. Zhu.** 2001. Human cytomegalovirus with IE-2 (UL122) deleted fails to express early lytic genes. J. Virol. **75:**1870-1878.

93. **McGregor, A., and M. R. Schleiss.** 2001. Recent advances in herpesvirus genetics using bacterial artificial chromosomes. Molecular Genetics and Metabolism **72:**8-14.

94. **McLaughlin-Taylor, E., H. Pande, S. J. Forman, B. Tanamachi, C. R. Li, J. A. Zaia, F. D. Greenberg, and S. R. Riddell.** 1994. Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for CD8+ virus-specific cytotoxic T lymphocytes. Journal of Medical Virology **43:**103-110.

95. **McVoy, M. A., D. E. Nixon, J. K. Hur, and S. P. Adler.** 2000. The Ends on herpesvirus dna replicative concatemers contain pac2 cis cleavage/packaging elements and their formation is controlled by terminal cis sequences. J. Virol. **74:**1587-1592.

96. **Medzhitov, R., P. Preston-Hurlburt, and C. A. Janeway.** 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature **388:**394-397.

97. **Medzhitov, R., P. Preston-Hurlburt, E. Kopp, A. Stadlen, C. Chen, S. Ghosh, and C. A. Janeway.** 1998. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Molecular Cell **2:**253-258.

98. **Meier, J. L., and M. F. Stinski.** 2006. Major immediately-early enhancer and its gene products, p. 152-166. *In* M. J. Reddehase (ed.), Cytomegalovirus Molecular Biology and Immunology. Caister Academic Press, Norfolk, UK.

99. **Meier, J. L., and M. F. Stinski.** 1996. Regulation of human cytomegalovirus immediate-early gene expression. Intervirology **39:**331-42.

100. **Mendelson, M., S. Monard, P. Sissons, and J. Sinclair.** 1996. Detection of endogenous human cytomegalovirus in CD34+ bone marrow progenitors. J Gen Virol **77 (Pt 12):**3099-102.

101. **Messerle, M., B. Buhler, G. M. Keil, and U. H. Koszinowski.** 1992. Structural organization, expression, and functional characterization of the murine cytomegalovirus immediate-early gene 3. J. Virol. **66:**27-36.

102. **Messerle, M., I. Crnkovic, W. Hammerschmidt, H. Ziegler, and U. H. Koszinowski.** 1997. Cloning and mutagenesis of a herpesvirus genome as an infectious bacterial artificial chromosome. Proc Natl Acad Sci U S A **94:**14759-63.

103. **Messerle, M., G. M. Keil, and U. H. Koszinowski.** 1991. Structure and expression of murine cytomegalovirus immediate-early gene 2. J. Virol. **65:**1638-1643.

104. **Minagawa, H., S. Tanaka, Y. Toh, and R. Mori.** 1994. Detection of herpes simplex virus type 1-encoded RNA by polymerase chain reaction: different pattern of viral RNA detection in latently infected murine trigeminal ganglia following in vitro or in vivo reactivation. J Gen Virol **75:**647-650.

105. **Minton, E. J., C. Tysoe, J. H. Sinclair, and J. G. Sissons.** 1994. Human cytomegalovirus infection of the monocyte/macrophage lineage in bone marrow. J Virol **68:**4017-21.

106. **Mocarski, E. S., and C. T. Courcelle.** 2001. Cytomegalovirus and their replication, p. 2629-2673. *In* D. M. Knipe and P. M. Howley (ed.), Fields Virology, 4th edn, vol. 2. Philadelphia, Lippincott Williams and Wilkins.

107. **Murphy, E., D. Yu, J. Grimwood, J. Schmutz, M. Dickson, M. A. Jarvis, G. Hahn, J. A. Nelson, R. M. Myers, and T. E. Shenk.** 2003. Coding potential of laboratory and clinical strains of human cytomegalovirus. Proc Natl Acad Sci U S A **100:**14976-81.

108. **Muyrers, J. P., Y. Zhang, V. Benes, G. Testa, W. Ansorge, and A. F. Stewart.** 2000. Point mutation of bacterial artificial chromosomes by ET recombination. EMBO Rep **1:**239-43.

109. **Muyrers, J. P., Y. Zhang, F. Buchholz, and A. F. Stewart.** 2000. RecE/RecT and Redalpha/Redbeta initiate double-stranded break repair by specifically interacting with their respective partners. Genes Dev **14:**1971-82.

110. **Muyrers, J. P., Y. Zhang, G. Testa, and A. F. Stewart.** 1999. Rapid modification of bacterial artificial chromosomes by ET-recombination. Nucleic Acids Res **27:**1555-7.

111. **Muyrers, J. P. P., Y. Zhang, and A. F. Stewart.** 2001. Techniques: Recombinogenic engineering-new options for cloning and manipulating DNA. Trends in Biochemical Sciences **26:**325-331.

112. **Navarro, L., K. Mowen, S. Rodems, B. Weaver, N. Reich, D. Spector, and M. David.** 1998. Cytomegalovirus activates interferon immediate-early response gene expression and an interferon regulatory factor 3-containing interferon-stimulated response element-binding complex. Mol. Cell. Biol. **18:**3796-3802.

113. **Newcomb, W. W., R. M. Juhas, D. R. Thomsen, F. L. Homa, A. D. Burch, S. K. Weller, and J. C. Brown.** 2001. The UL6 gene product forms the portal for entry of DNA into the herpes simplex virus capsid. J. Virol. **75:**10923-10932.

114. **Nowlin, D. M., N. R. Cooper, and T. Compton.** 1991. Expression of a human cytomegalovirus receptor correlates with infectibility of cells. J Virol **65:**3114-21.

115. **Okada, M., and Y. Minamishima.** 1987. The efficacy of biological response modifiers against murine cytomegalovirus infection in normal and immunodeficient mice. Microbiol Immunol **31:**45-57.

116. **Pande, H., K. Campo, B. Tanamachi, S. J. Forman, and J. A. Zaia.** 1995. Direct DNA immunization of mice with plasmid DNA encoding the tegument protein pp65 (ppUL83) of human cytomegalovirus induces high levels of circulating antibody to the encoded protein. Scand J Infect Dis Suppl **99:**117-20.

117. **Pass, R. F.** 2001. Cytomegalovirus, p. 2675-2705. *In* D. M. Knipe and P. M. Howley (ed.), Fields Virology, 4th ed, vol. 2. Philadelphia, Lippincott Williams and Wilkins.

118. **Pass, R. F.** 1985. Epidemiology and transmission of cytomegalovirus. J. Infect. Dis. **152:**10–16.

119. **Pelletier, J., and N. Sonenberg.** 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature **334:**320-325.

120. **Plachter, B., C. Sinzger, and G. Jahn.** 1996. Cell types involved in replication and distribution of human cytomegalovirus. Adv Virus Res **46:**195-261.

121. **Plotkin, S. A.** 2001. Vaccination against cytomegalovirus. Archives Of Virology. Supplementum**:**121-134.

122. **Plotkin, S. A., S. E. Starr, H. M. Friedman, K. Brayman, S. Harris, S. Jackson, N. B. Tustin, R. Grossman, D. Dafoe, and A. Barker et.** 1991. Effect of towne live virus vaccine on cytomegalovirus disease after renal transplant. a controlled trial. Annals Of Internal Medicine **114:**525-531.

123. **Podlech, J., R. Holtappels, M.-F. Pahl-Seibert, H.-P. Steffens, and M. J. Reddehase.** 2000. Murine model of interstitial cytomegalovirus pneumonia in syngeneic bone marrow transplantation: persistence of protective pulmonary cd8-t-cell infiltrates after clearance of acute infection. J. Virol. **74:**7496-7507.

124. **Pollock, J., and H. Virgin.** 1995. Latency, without persistence, of murine cytomegalovirus in the spleen and kidney. J. Virol. **69:**1762-1768.

125. **Preston, C. M., A. N. Harman, and M. J. Nicholl.** 2001. Activation of interferon response factor-3 in human cells infected with herpes simplex virus type 1 or human cytomegalovirus. J. Virol. **75:**8909-8916.

126. **Prichard, M., G. Duke, and E. Mocarski.** 1996. Human cytomegalovirus uracil DNA glycosylase is required for the normal temporal regulation of both DNA synthesis and viral replication. J. Virol. **70:**3018-3025.

127. **Raulet, D. H.** 2003. Natural killer cells, p. 365-391. *In* W. E. Paul (ed.), Fundamental Immunology. Philadelphia Lippincott Williams & Wilikns.

128. **Rawlinson, W. D., H. E. Farrell, and B. G. Barrell.** 1996. Analysis of the complete DNA sequence of murine cytomegalovirus. J Virol **70:**8833-49.

129. **Razonable, R. R., V. C. Emery, and F. 11th Annual Meeting of the IHMF (International Herpes Management.** 2004. Management of CMV infection and disease in transplant patients. 27-29 February 2004. Herpes: The Journal Of The IHMF **11:**77-86.

130. **Reddehase, M. J., W. Mutter, K. Munch, H. J. Buhring, and U. H. Koszinowski.** 1987. CD8-positive T lymphocytes specific for murine cytomegalovirus immediate-early antigens mediate protective immunity. J. Virol. **61:**3102-3108.

131. **Redwood, A. J., M. Messerle, N. L. Harvey, C. M. Hardy, U. H. Koszinowski, M. A. Lawson, and G. R. Shellam.** 2005. Use of a murine cytomegalovirus K181-derived bacterial artificial chromosome as a vaccine vector for Immunocontraception. Journal of Virology **79:**2998-3008.

132. **Revello, M. G., M. Zavattoni, A. Sarasini, E. Percivalle, L. Simoncini, and G. Gerna.** 1998. Human cytomegalovirus in blood of immunocompetent persons during primary infection: prognostic implications for pregnancy. J Infect Dis **177:**1170-5.

133. **Reynolds, R. P., R. J. Rahija, D. I. Schenkman, and C. B. Richter.** 1993. Experimental murine cytomegalovirus infection in severe combined immunodeficient mice. Lab Anim Sci **43:**291-5.

134. **Robain, M., N. Carre, E. Dussaix, D. Salmon-Ceron, and L. Meyer.** 1998. Incidence and sexual risk factors of cytomegalovirus seroconversion in HIV-infected subjects. The SEROCO Study Group. Sex Transm Dis **25:**476-80.

135. **Roizman, B., and P. E. Pellet.** 2001. The family of Herpeviridae: A brief introduction, p. 2381-2397. *In* D. M. Knipe and P. M. Howley (ed.), Fields Virology, vol. 2. Philadelphia, Lippincott Williams and Wilkins.

136. **Sammons, C. C., and C. Sweet.** 1989. Isolation and preliminary characterization of temperature-sensitive mutants of mouse cytomegalovirus of differing virulence for 1-week-old mice. J Gen Virol **70 (Pt 9):**2373-81.

137. **Sarisky, R., and G. Hayward.** 1996. Evidence that the UL84 gene product of human cytomegalovirus is essential for promoting oriLyt-dependent DNA replication and formation of replication compartments in cotransfection assays. J. Virol. **70:**7398-7413.

138. **Scalzo, A., N. Fitzgerald, A. Simmons, A. La Vista, and G. Shellam.** 1990. Cmv-1, a genetic locus that controls murine cytomegalovirus replication in the spleen. J. Exp. Med. **171:**1469-1483.

139. **Scalzo, A., N. Fitzgerald, C. Wallace, A. Gibbons, Y. Smart, R. Burton, and G. Shellam.** 1992. The effect of the Cmv-1 resistance gene, which is linked to the natural killer cell gene complex, is mediated by natural killer cells. J Immunol **149:**581-589.

140. **Scalzo, A. A., P. A. Lyons, N. A. Fitzgerald, C. A. Forbes, W. M. Yokoyama, and G. R. Shellam.** 1995. Genetic mapping of Cmv1 in the region of mouse chromosome 6 encoding the NK gene complex-associated loci Ly49 and musNKR-P1. Genomics **27:**435-441.

141. **Schaffer, P. A.** 1975. Temperature-sensitive mutants of herpesviruses. Current Topics in Microbiology and Immunology **70:**51-100.

142. **Shanley, J. D., M. C. Jordan, and J. G. Stevens.** 1981. Modification by adoptive humoral immunity of murine cytomegalovirus infection. J Infect Dis **143:**231-7.

143. **Shatkin, A. J.** 1976. Capping of eucaryotic mRNAs. Cell **9:**645-53.

144. **Simmen, K. A., J. Singh, B. G. M. Luukkonen, M. Lopper, A. Bittner, N. E. Miller, M. R. Jackson, T. Compton, and K. Fruh.** 2001. Global modulation of cellular transcription by human cytomegalovirus is initiated by viral glycoprotein B. PNAS **98:**7140-7145.

145. **Simon, C. O., C. K. Seckert, N. K. A. Grzimek, and M. J. Reddehase.** 2006. Murine model of cytomegalovirus latency and reactivation: the silencing/desilencing and immune sensing hypothesis, p. 483-500. *In* M. J. Reddehase (ed.), Cytomegalovirus Molecular Biology and Immunology. Caister Academic Press, Norfolk, UK.

146. **Sinclair, J., and P. Sissons.** 2006. Latency and reactivation of human cytomegalovirus. J Gen Virol **87:**1763-1779.

147. **Sinclair, J. H., J. Baillie, L. A. Bryant, J. A. Taylor-Wiedeman, and J. G. Sissons.** 1992. Repression of human cytomegalovirus major immediate early gene expression in a monocytic cell line. J Gen Virol **73 (Pt 2):**433-5.

148. **Singh, N.** 2006. Cytomegalovirus infection in solid organ transplant recipients: new challenges and their implications for preventive strategies. J Clin Virol **35:**474-7.

149. **Sissons, J. G. P., and A. J. Carmichael.** 2002. Clinical aspects and management of cytomegalovirus infection. Journal of Infection **44:**78-83.

150. **Sjolin, H., E. Tomasello, M. Mousavi-Jazi, A. Bartolazzi, K. Karre, E. Vivier, and C. Cerboni.** 2002. Pivotal role of KARAP/DAP12 adaptor molecule in the natural killer cell-mediated resistance to murine cytomegalovirus infection. J. Exp. Med. **195:**825-834.

151. **Smith, H. R. C., H. H. Chuang, L. L. Wang, M. Salcedo, J. W. Heusel, and W. M. Yokoyama.** 2000. Nonstochastic coexpression of activation receptors on murine natural killer cells. J. Exp. Med. **191:**1341-1354.

152. **Smith, H. R. C., J. W. Heusel, I. K. Mehta, S. Kim, B. G. Dorner, O. V. Naidenko, K. Iizuka, H. Furukawa, D. L. Beckman, J. T. Pingel, A. A. Scalzo, D. H. Fremont, and W. M. Yokoyama.** 2002. Recognition of a virus-encoded ligand by a natural killer cell activation receptor. PNAS **99:**8826-8831.

153. **Smyth, R. L., J. Sinclair, J. P. Scott, J. J. Gray, T. W. Higenbottam, T. G. Wreghitt, J. Wallwork, and L. K. Borysiewicz.** 1991. Infection and reactivation with cytomegalovirus strains in lung transplant recipients. Transplantation **52:**480-2.

154. **Spector, S. A., K. Hsia, M. Crager, M. Pilcher, S. Cabral, and M. J. Stempien.** 1999. Cytomegalovirus (CMV) DNA load is an independent predictor of CMV disease and survival in advanced AIDS. J. Virol. **73:**7027-7030.

155. **Stagno, S., R. F. Pass, G. Cloud, W. J. Britt, R. E. Henderson, P. D. Walton, D. A. Veren, F. Page, and C. A. Alford.** 1986. Primary cytomegalovirus infection in pregnancy. Incidence, transmission to fetus, and clinical outcome. JAMA **256:**1904-1908.

156. **Stagno, S., D. W. Reynolds, R. F. Pass, and C. A. Alford.** 1980. Breast milk and the risk of cytomegalovirus infection. N Engl J Med **302:**1073-6.

157. **Stark, G. R., I. M. Kerr, B. R. G. Williams, R. H. Silverman, and R. D. Schreiber.** 1998. How cells respond to interferons. Annual Review of Biochemistry **67:**227-264.

158. **Stein, J., H. D. Volk, C. Liebenthal, D. H. Kruger, and S. Prosch.** 1993. Tumour necrosis factor alpha stimulates the activity of the human cytomegalovirus major immediate early enhancer/promoter in immature monocytic cells. J Gen Virol **74 (Pt 11):**2333-8.

159. **Stenberg, R. M.** 1996. The human cytomegalovirus major immediate-early gene. Intervirology **39:**343-9.

160. **Stenberg, R. M., and J. A. Kerry.** 1995. Cytomegalovirus genes: their structure and function. Scand J Infect Dis Suppl **99:**3-6.

161. **Stoddart, C. A., R. D. Cardin, J. M. Boname, W. C. Manning, G. B. Abenes, and E. S. Mocarski.** 1994. Peripheral blood mononuclear phagocytes mediate dissemination of murine cytomegalovirus. J. Virol. **68:**6243-6253.

162. **Streblow, D. N., S. M. Varnum, and R. D. Smith.** 2006. A proteomics analysis of human cytomegalovirus particles, p. 91-110. *In* M. J. Reddehase (ed.), Cytomegalovirus Molecular Biology and Immunology. Caister Academic Press, Norfolk, UK.

163. **Sullivan, V., C. L. Talarico, S. C. Stanat, M. Davis, D. M. Coen, and K. K. Biron.** 1992. A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells. Nature **358:**162-4.

164. **Sweet, C.** 1999. The pathogenicity of cytomegalovirus. FEMS Microbiol Rev **23:**457-82.

165. **Sweet, C., K. Ball, P. J. Morley, K. Guilfoyle, and M. Kirby.** 2007. Mutations in the temperature-sensitive murine cytomegalovirus (MCMV) mutants tsm5 and tsm30: a study of genes involved in immune evasion, DNA packaging and processing, and DNA replication. J Med Virol **79:**285-99.

166. **Tabeta, K., P. Georgel, E. Janssen, X. Du, K. Hoebe, K. Crozat, S. Mudd, L. Shamel, S. Sovath, J. Goode, L. Alexopoulou, R. A. Flavell, and B. Beutler.** 2004. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. PNAS **101:**3516-3521.

167. **Takeda, K., and S. Akira.** 2003. Toll receptors and pathogen resistance. Cellular Microbiology **5:**143-153.

168. **Takeuchi, O., and S. Akira.** 2001. Toll-like receptors; their physiological role and signal transduction system. International Immunopharmacology **1:**625-635.

169. **Tam, A., J. Zhu, R. Hai, E. Haghjoo, T. Tong, X. Zhan, S. Lu, and F. Liu.** 2003. Murine cytomegalovirus with a transposon insertional mutation at open reading frame M35 is defective in growth in vivo. J. Virol. **77:**7746-7755.

170. **Tang, Q., E. A. Murphy, and G. G. Maul.** 2006. Experimental confirmation of global murine cytomegalovirus open reading frames by transcriptional detection and partial characterization of newly described gene products. J. Virol. **80:**6873-6882.

171. **Taniguchi, T., K. Ogasawara, A. Takaoka, and N. Tanaka.** 2001. IRF family of transcription factors as regulators of host defense. Annu Rev Immunol **19:**623-55.

172. **Taniguchi, T., and A. Takaoka.** 2002. The interferon-[alpha]/[beta] system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors. Current Opinion in Immunology **14:**111-116.

173. **Thale, R., P. Lucin, K. Schneider, M. Eggers, and U. H. Koszinowski.** 1994. Identification and expression of a murine cytomegalovirus early gene coding for an Fc receptor. J. Virol. **68:**7757-7765.

174. **Vilches, C., and P. Parham.** 2002. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. Annual Review of Immunology **20:**217-251.

175. **Voigt, S., G. R. Sandford, G. S. Hayward, and W. H. Burns.** 2005. The English strain of rat cytomegalovirus (CMV) contains a novel captured CD200 (vOX2) gene and a spliced CC chemokine upstream from the major immediate-early region: further evidence for a separate evolutionary lineage from that of rat CMV Maastricht. J Gen Virol **86:**263-274.

176. **Wagner, M., U. H. Koszinowski, and M. Messerle.** 1999. Systematic excision of vector sequences from the BAC-Cloned Herpesvirus Genome during Virus Reconstitution. J. Virol. **73:**7056-7060.

177. **Walter, E. A., P. D. Greenberg, M. J. Gilbert, R. J. Finch, K. S. Watanabe, E. D. Thomas, and S. R. Riddell.** 1995. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N Engl J Med **333:**1038-1044.

178. **Wang, X., S. M. Huong, M. L. Chiu, N. Raab-Traub, and E. S. Huang.** 2003. Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus. Nature **424:**456-61.

179. **Wills, M. R., A. J. Carmichael, and J. G. Sissons.** 2006. Adaptive cellular immunity to human cytomegalovirus, p. 341-365. *In* M. J. Reddehase (ed.), Cytomegalovirus Molecular Biology and Immunology. Caister Academic Press, Norfolk, UK.

180. **Wills, N. M., R. F. Gesteland, and J. F. Atkins.** 1994. Pseudoknot-dependent read-through of retroviral gag termination codons: importance of sequences in the spacer and loop 2. Embo J **13:**4137-44.

181. **Yu, D., H. M. Ellis, E. C. Lee, N. A. Jenkins, N. G. Copeland, and D. L. Court.** 2000. An efficient recombination system for chromosome engineering in Escherichia coli. Proc Natl Acad Sci U S A **97:**5978-83.

182. **Yu, D., G. A. Smith, L. W. Enquist, and T. Shenk.** 2002. Construction of a self-excisable bacterial artificial chromosome containing the human cytomegalovirus genome and mutagenesis of the diploid TRL/IRL13 gene. J Virol **76:**2316-28.

183. **Yurochko, A., E. Hwang, L. Rasmussen, S. Keay, L. Pereira, and E. Huang.** 1997. The human cytomegalovirus UL55 (gB) and UL75 (gH) glycoprotein ligands initiate the rapid activation of Sp1 and NF-kappaB during infection. J. Virol. **71:**5051-5059.

184. **Yurochko, A., T. Kowalik, S. Huong, and E. Huang.** 1995. Human cytomegalovirus upregulates NF-kappa B activity by transactivating the NF-kappa B p105/p50 and p65 promoters. J. Virol. **69:**5391-5400.

185. **Yurochko, A. D., and E.-S. Huang.** 1999. Human cytomegalovirus binding to human monocytes induces immunoregulatory gene expression. J Immunol **162:**4806-4816.

186. **Zacny, V. L., E. Gershburg, M. G. Davis, K. K. Biron, and J. S. Pagano.** 1999. Inhibition of Epstein-Barr virus replication by a benzimidazole L-riboside: novel antiviral mechanism of 5, 6-dichloro-2-(isopropylamino)-1-beta-L-ribofuranosyl-1H-benzimidazole. J Virol **73:**7271-7.

187. **Zhang, Y., F. Buchholz, J. P. Muyrers, and A. F. Stewart.** 1998. A new logic for DNA engineering using recombination in Escherichia coli. Nat Genet **20:**123-8.

188. **Zhang, Y., J. P. Muyrers, J. Rientjes, and A. F. Stewart.** 2003. Phage annealing proteins promote oligonucleotide-directed mutagenesis in Escherichia coli and mouse ES cells. BMC Mol Biol **4:**1.

189. **Zhu, H., J.-P. Cong, G. Mamtora, T. Gingeras, and T. Shenk.** 1998. Cellular gene expression altered by human cytomegalovirus: Global monitoring with oligonucleotide arrays. PNAS **95:**14470-14475.

190. **Zhu, H., J.-P. Cong, and T. Shenk.** 1997. Use of differential display analysis to assess the effect of human cytomegalovirus infection on the accumulation of cellular RNAs: Induction of interferon-responsive RNAs. PNAS **94:**13985-13990.

191. **Zhu, J., J. Chen, R. Hai, T. Tong, J. Xiao, X. Zhan, S. Lu, and F. Liu.** 2003. In Vitro and In Vivo Characterization of a Murine Cytomegalovirus with a Mutation at Open Reading Frame m166. J. Virol. **77:**2882-2891.

6. PUBLICATION LIST

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