THE STUDY OF VIRAL GENETICS VIA THE CONSTRUCTION OF RECOMBINANT MURINE CYTOMEGALOVIRUSES

A thesis submitted to the

College of Graduate Studies and Research

in partial fulfillment of the requirements

for the degree of

Master of Science

in the

Department of Biochemistry

University of Saskatchewan

By

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ABSTRACT

Cytomegaloviruses are highly host specific, and murine cytomegalovirus (MCMV) has been widely used as a model for studying human cytomegalovirus (HCMV) infections to overcome the difficulty of experimentation with HCMV *in vivo*. The ability to manipulate the viral genome and introduce specific mutations into viral genes should facilitate the investigation of mechanisms governing cytomegalovirus host specificity. Our laboratory has utilized a modified MCMV genome lacking a *Swa*I site to construct recombinant MCMVs through homologous recombination. However, it was not known whether this modification of the MCMV genome by removing the *Swa*I site would affect the biological properties of MCMV, or whether the *Swa*I mutation could be reversed should the need arose. To this end, two recombinant bacterial artificial chromosomes, pMCMV_ETwt and pMCMV_ETSwa$^-$*, were constructed and characterized by restriction endonuclease analysis, demonstrating that only the expected genome modifications were present. Recombinant viruses were then reconstituted in tissue culture and their biological properties were compared to the Smith strain of MCMV as well as the parental MCMV_EGFP virus. Viral DNA isolated from infected cells showed the expected restriction patterns for MCMV-ETwt and MCMV-ETSwa$^-$. The expression of MCMV proteins M112-113, ppM44 and gB, representative of those expressed in the early, delayed early, and late phases of infection, respectively, did not differ significantly between the recombinant viruses or the Smith strain of MCMV. In addition, virus growth in permissive Balb/3T3 cells at both low and high multiplicities of infection, and semi-permissive COS-1 cells at a high multiplicity of infection showed growth kinetics or patterns of infection that were similar to the Smith strain of MCMV. Results from these preliminary experiments suggested that the modification of the MCMV genome by removal of the *Swa*I site did not appear to affect the biological properties of MCMV *in vitro*. Furthermore, analysis of MCMV_ETwt demonstrated that we could reconstitute the *Swa*I site in the MCMV genome if necessary, resulting in a virus that should be identical to the Smith strain of MCMV. Therefore, these results suggest that the *Swa*I$^-$ MCMV genome will enable easier construction of recombinant MCMVs with desired alterations in any region of the viral genome.

ACKNOWLEDGEMENTS

I would first of all like to express my deepest thanks towards my supervisor, Dr. L. C. Loh. He has given me countless guidance, encouragement and support during my graduate studies program, and I could not have done this without his help. I feel that he has given me the tools to be successful in my future career and helped me realize where my strengths are. For this I cannot thank him enough.

I would like to thank the members of my Graduate Advisory Committee, Drs. W. Roesler, R. Khandelwal, H. Wang, R. Warrington, V. Misra, and S. Tikoo for their time and suggestions about my project.

I appreciate and acknowledge the College of Medicine graduate scholarships and NSERC post-graduate scholarships that provided financial support during my graduate studies program.

I would like to thank present and past members of the laboratories of Drs. Loh and Laferté, including Brian, Andrea, Suzanne, Tricia, Vicki, Rebecca, and Nikki for their friendship and assistance in my project. I would also like to thank my friends, in particular Erin, Candice, Carolyn, Jean, Amanda, Katie and Jeanette for helping make graduate studies fun. Ryan, thank you so much for being there, for your encouragement and love, and for always believing in me.

Finally, I thank my family, namely my parents, my brother, Michael, my sister in law, Jana, and Didu for their understanding, support, and love during my years as a graduate student. I would not have been as successful without their unwavering belief in my abilities and constant encouragement.

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LIST OF ABBREVIATIONS

BAC	Bacterial artificial chromosome
Bak	BCl-2 homologous antagonist/killer
Bax	BCl-2 associated X protein
Bcl-2	B cell lymphoma/leukemia 2
Bid	BCl-2 interacting protein
Bim	BCl-2 interacting mediator of cell death
CMV	Cytomegalovirus
CTLs	Cytotoxic-T lymphocytes
CPE	Cytopathic effect
DAXX	death-associated protein 6
DE	Delayed-early
DMEM	Dulbecco's minimal essentials media
E	Early
E. coli	*Escherichia coli*
FCS	Fetal calf serum
FLIP	FLICE-like inhibitory protein
gc	glycoprotein complex
HCMV	Human cytomegalovirus
HDAC	histone deacetylase
HSV	Herpes-simplex virus
IE	Immediate-early
IE1	Immediate-early 1
IE2	Immediate-early 2
IFN	Interferon
IR	Internal repeat
IR_L	Internal repeat long
IR_S	Internal repeat short
L-Broth	Luria Broth
LTR	Left terminal repeat

MCMV	Murine cytomegalovirus
MHC-I	Major histocompatibility class I
MIE	Major immediate-early
MIEP	Major immediate-early promoter/enhancer
MOI	Multiplicity of infection
NK	Natural killer
ND10	Nuclear domain 10
*ori*Lyt	origin of lytic DNA replication
PFU	plaque-forming unit
PML	Promyelocytic leukemia protein
PODs	Promyelocytic oncogenic domains
RCMV	Rat cytomegalovirus
RTR	Right terminal repeat
SCMV	Simian cytomegalovirus
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
STAT	Signal transducers and activators of transcription
SUMO-1	Small ubiquitin-like modifier-1
TK	Thymidine kinase
TNF	Tumor necrosis factor
TR	Terminal repeat
TR_L	Terminal repeat long
TR_S	Terminal repeat short
U_L	Unique long
U_S	Unique short
vICA	viral inhibitor of caspase 8 activation
vMIA	viral mitochondrial-localized inhibitor of apoptosis
YAC	Yeast artificial chromosome

1.0 INTRODUCTION

1.1 Herpesviruses

The Herpesvirus family is very large and consists of viruses that are able to infect most animal species. Although the herpesvirus family consists of three subfamilies that differ in terms of their replication and host range, all herpesviruses share similar virion structure and the characteristic of being able to reside in a latent state in their host.

1.1.1 Classification

Classification of Herpesviruses into genera is currently based on factors such as sequence comparisons (for both genome organization and DNA sequences) and similarities between viral proteins. However, the family members of *Herpesviridae* were originally classified based on their biological properties, resulting in the three subfamilies of Alphaherpesvirinae, Betaherpesvirinae, and Gammaherpesvirinae (Pellett and Roizman, 2007). Each subfamily has distinctive characteristics related to viral replication and the host range that can be infected.

The alpha-herpesviruses have a variable host range while the beta- and gamma-herpesviruses have host ranges limited to their natural hosts. The three subfamilies also differ in terms of their reproductive cycle. Alpha-herpesviruses spread rapidly in cell culture due to their short replication cycles whereas beta-herpesviruses spread slowly due to their longer replication cycles. Cells infected with alpha-herpesviruses are damaged within a short period of time post-infection, whereas cells infected with beta-herpesviruses experience cytomegalia, or enlargement. Although some members of the gamma-herpesviruses are able to cause productive infections in epitheliod and fibroblast cells, viruses in this subfamily are usually specific for either T or B lymphocytes. Latent infections are established in sensory ganglia for the alpha-herpesviruses, and in secretory glands, lymphoreticular cells, kidneys and other tissues for the beta-herpesviruses (Pellett and Roizman, 2007).

Alpha-herpesviruses include the mammalian Simplexvirus (e.g. herpes simplex virus type 1, HSV-1), *Varicellovirus* (e.g. varicella zoster virus, VZV), the avian *Mardivirus* (e.g.

Gallid herpesvirus 2), and *Itovirus* (e.g. Gallid herpesvirus 1). Although this family also includes the reptilian herpesviruses, they have not yet been placed into specific genera (Pellet and Roizman, 2007). The beta-herpesviruses encompass the genera *Cytomegalovirus* (Human Cytomegalovirus, HCMV), *Muromegalovirus* (Murine Cytomegalovirus, MCMV), *Roseolovirus* (Human Herpesvirus-6), and putatively, *Proboscivirus* (Elephant Herpesvirus 1). The gamma-herpesviruses contain the genera *Lymphocryptovirus* (Epstein Barr Virus) and *Rhadinovirus* (Human Herpes Virus-8) although it is proposed that the latter include *Macavirus* and *Percavirus*.

1.1.2 Structure and genome organization

All members of the *Herpesviridae* family have similar virion structures, which were initially used as the basis for classifying a virus as a family member. The virions range in size from 120-260 nm depending on the size of the tegument layer and the morphology of the envelope. Virions include a large number of viral tegument, capsid, and core proteins, as well as some host proteins although it is not yet known why this is the case. The viral core contains linear double stranded viral DNA, which ranges in size from 120-230 kb depending on the virus, and is arranged around the protein core in a toroidal shape. The core is located inside an icosahedral nucleocapsid that is 100 nm in size and contains 162 capsomeres. Surrounding the capsid is a tegument layer that varies in thickness, and contrary to its appearance, is an ordered structure that contains several proteins. The tegument proteins are critical in helping the virus establish productive infection in a new cell by interfering with host functions and activating viral gene expression (Pellet and Roizman, 2007). The final component of the herpesvirus virion is the envelope, which contains several viral glycoproteins, and its lipid bilayer membrane is derived from the host.

In contrast to the virion structure, the genome organizations of *herpesviridae* members vary significantly. In very general terms, herpesvirus genomes consist of one or two unique sequences flanked by repeated sequences. However, several herpesviruses contain both unique long (U_L) and unique short (U_S) regions, flanked with direct and/or inverted terminal and internal repeats. In fact, the sizes of the same strain of virus can vary by as much as 10 kb based on the number of repeat segments present in the genome (Weir, 1998). Rearrangement of the viral genome is possible when either the U_S or U_L regions (or both) are flanked by

inverted repeats. However, if the genome arrangement consists of U_S and U_L regions flanked by direct repeats, no rearrangement of the genome is possible.

1.1.3 Replication

Herpesvirus replication follows the general stages outlined here. The first stage involves the attachment of the virion to specific receptors present on the host cell and penetration into the cell mediated by virus surface glycoproteins. Next, the viral capsid is transported to the nucleus where viral DNA is released. This is where viral DNA replication, transcription and assembly of new capsids take place. A common feature of herpesvirus infections is the ordered expression of viral genes, ensuring productive infection will occur under the right conditions. Immediate-early (IE) genes are the first to be expressed, and their gene products activate the expression of early (E) genes, which generally encode proteins important for DNA replication. Following viral DNA replication, the late (L) genes are expressed, which typically encode structural components of the virion (Greaves and Mocarski, 1998). Subsequently, viral DNA is assembled into a new capsid, which acquires its envelope from the inner nuclear membrane. The enveloped virus is then released into the cytoplasm where it undergoes de-envelopment, further modifications to the tegument, and re-envelopment prior to being released from the cell.

1.1.4 Latency

Infection of cells can either result in lytic infection, leading to cell death, or latency, a common feature of herpesviruses. Latent viral genomes are not actively replicating and only express a subset of viral genes. However they can undergo productive infection upon reactivation. As mentioned in a previous section, specific cell types can harbor herpesviruses during latency. There is some evidence that reactivation from latency is dependent on regulation by host cells (Pellet and Roizman, 2007).

1.2 Cytomegaloviruses

Cytomegalovirus (CMV) is a beta-herpesvirus that contains one of the largest viral genomes of any mammalian DNA virus. Cytomegalovirus infection *in vivo* results in cytomegaly, which is the enlargement of the infected cell, as well as the presence of inclusions in the nucleus. The viral life cycle can take as long as 72 hours to complete in HCMV. The CMV genome codes for more than 200 proteins, most of whose functions are unknown. Many

of these proteins are dispensable for virus growth in cell culture, but they may play an important role during infection of the natural host. Complex interactions between the virus and host often determine the outcome of CMV infections.

There are several types of CMVs, including HCMV, MCMV, Rat CMV (RCMV), Simian CMV (SCMV), Guinea Pig CMV, and Equine CMV. Human CMV is a major human pathogen, and infects a large proportion of the adult population asymptomatically although it can cause severe disease in newborns and immunocompromised individuals.

1.2.1 Characteristics that make MCMV a good model for HCMV

Due to the strict species specificity of HCMV, a model of HCMV infection is required in order to perform the *in vivo* testing necessary for the development of vaccines and anti-viral drugs. Therefore, a related CMV needs to be used as a model. HCMV is very closely related to Chimpanzee CMV, which has HCMV homologs in all open reading frames (Mocarski *et al.,* 2007). However, due to the impracticalities involved in using primates in most experiments, either MCMV or RCMV are commonly used as models for HCMV infection *in vivo*. In particular, MCMV is well suited to being a model since the mouse genome has been completely sequenced, and the mouse is the most well characterized laboratory animal in use (Rawlinson *et al.,* 1996). Both HCMV and MCMV have characteristics shared by most herpesviruses, including their virion structures and the orderly expression of viral genes. Furthermore, both viral genomes have been sequenced (Chee *et al.,* 1990; Rawlinson *et al.,* 1996), and the central regions of the 2 genomes are collinear, with 78 of the predicted open reading frames showing sequence similarity between the two viruses (Mocarski and Kemble, 1996). Human CMV and MCMV have similar mechanisms of pathogenesis in the infected host, thereby facilitating the use of MCMV to investigate the biological properties of CMV infections (Krmpotić *et. al*., 2003). Nevertheless, under certain conditions, MCMV has a less restricted host range compared to HCMV and presents an interesting perspective to the study of host specificity.

1.2.2 Genome Organization

The genome organization of HCMV includes U_L and U_S regions that are flanked by their own unique inverted terminal repeat (TR_L and TR_S) and internal repeat (IR_L and IR_S) sequences (Figure 1.1, Panel B). This allows both U_L and U_S to be inverted independently to

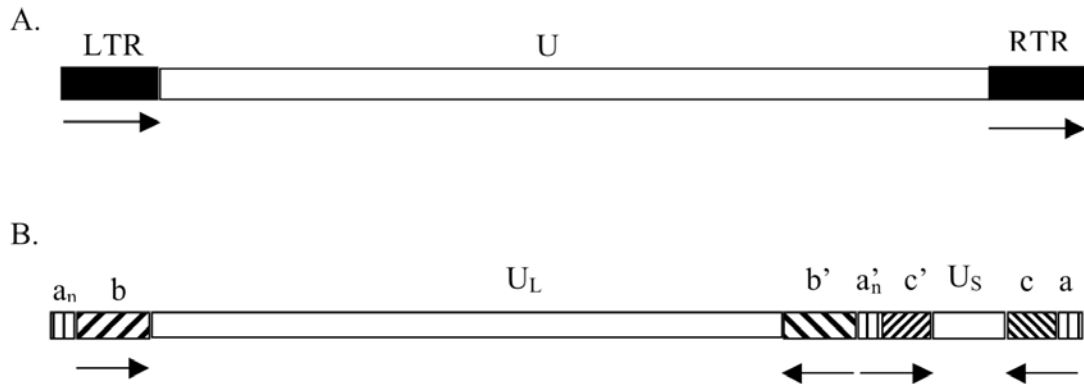

Figure 1.1: The prototypical genome arrangement of HCMV and MCMV

A. The MCMV genome consists of a single unique sequence (U) flanked by the direct repeats LTR (left terminal repeat) and RTR (right terminal repeat). **B.** The HCMV genome consists of two unique sequences (U_L and U_S) flanked by inverted repeats (b/b’ and c/c’, designated by boxes with diagonal stripes). The direct repeat a/a’ is present with a variable number of copies at the left terminus, and a single copy at the right terminus.

This figure is modified from Pellet and Roizman, 2007, and Weir, 1998.

generate 4 possible isomers of the virus (Weir, 1998; Mocarski *et al.,* 2007). In contrast, other beta-herpesviruses lack internal repeats (Pellet and Roizman, 2007). For example, the MCMV genome has a long unique sequence with short direct repeats at each terminus (Rawlinson *et al.,* 1996; Figure 1.1, Panel A).

The HCMV genome has around 140 genes that are conserved in all known strains and about 80 of these represent genes essential for viral replication *in vitro*. The MCMV genome has around 170 genes, and 78 of these (located in the central 180 kb of the MCMV sequence) share significant amino acid homology with HCMV (Rawlinson *et al.*, 1996). In contrast, the terminal 24 kb and 27 kb regions of MCMV contain unique genes which are not found in HCMV. Both viruses possess a single origin of replication.

Human CMV genes are labelled from left to right on the prototype form of the viral genome according to the region (U_L, U_S, TR_L, IR_L, TR_S or IR_S) where the gene is located. The numbering system for MCMV genes follows that of HCMV genes for the U_L region but MCMV homologs to genes in the U_S, TR_L, IR_L, TR_S or IR_S regions do not share the same numbering system (Rawlinson *et al.,* 1996). MCMV genes that have HCMV homologs are prefixed by an uppercase M. For example, *M36* is homologous to HCMV *UL36*. Those genes that are unique to MCMV are prefixed by a lowercase m (e.g. *m38.5*). In addition, in situations where there are additional MCMV genes located between genes homologous to HCMV, numbered suffixes are used so that the numbering system can be maintained (e.g. *M73.5*).

All herpesviruses contain a set of core genes that tend to be clustered in specific areas of the genome. Seven functionally conserved gene blocks are found at similar locations on the HCMV and MCMV genomes (Chee *et al.*, 1990; Rawlinson *et al.,* 1996). These include genes that encode proteins essential for viral replication: the DNA polymerase (*UL54/M54*), the DNA polymerase accessory factor (*UL44*/*M44*), the single-stranded DNA binding protein (*UL57*/*M57*), and the helicase-primase complex (*UL70*/*M70*, *UL102*/*M102*, and *UL105*/*M105*) genes (Rawlinson *et al.,* 1996). Although HSV-1 *UL9* is required for replication, there are no homologs to this gene in either MCMV or HCMV (Chee *et al.,* 1990; Rawlinson *et al.,* 1996). However, all of the additional genes required for HCMV replication, which mainly have regulatory roles, are present in MCMV, including the early genes *UL112-113*/*M112-113*, *UL84*/*M84*, and genes located in the major immediate-early (MIE) region (*UL122-123*/*M122-m123*, also known as *ie1-ie2*/*mie1-mie3*), the *TRS1-IRS1* region (the MCMV *US22* family

homolog *m143*), and the *UL36-38* region (*M36*, *m38.5*) (Rawlinson *et al.*, 1996). The important regulatory gene (*UL69*) of HCMV also has an MCMV homolog (*M69*), and is involved in the transactivation of early genes (Rawlinson *et al*., 1996).

Conserved herpesvirus structural genes are also found in both HCMV and MCMV, including genes encoding the major capsid protein (*UL86/M86)*, large tegument protein (*UL48/M48*), and the minor capsid protein (*UL46/M46*) (Rawlinson *et al.,* 1996). Both HCMV and MCMV contain homologous genes encoding structural proteins found only in beta-herpesviruses. These include genes encoding the upper and lower matrix proteins, *UL82*/*M82* and *UL84*/*M84*, respectively, phosphoproteins (*UL32/M32 and UL99/M99*), and proteins involved in virion assembly (*UL80 /M80* and *UL56*/*M56)* (Rawlinson *et al.,* 1996).

Genes encoding proteins responsible for nucleotide metabolism, replication and repair are also present in HCMV and MCMV. Examples include the uracil-DNA glycosylase *UL114*/*M114*, ribonucleotide reductase *UL45*/*M45*, dUTPase *UL72*/*M72*, phosphotransferase *UL97*/*M97*, and pyruvoyl decarboxylase *UL77*/*M77* (Chee *et al.*, 1990; Rawlinson *et al.,* 1996). Several conserved genes encoding glycoproteins in herpesviruses have homologs in HCMV and MCMV, including gB (*UL55/M55*), gH (*UL75/M75*), gM (*UL100/M100*), and gL (*UL115/M115*) (Rawlinson *et al*., 1996).

MCMV contains 6 gene families, 4 of which are homologous to HCMV gene families and 2 of which are unique to MCMV (Rawlinson *et al.,* 1996). There are the *m02 (m02-m16* inclusive) and the *m145* (*m145*, *m146*, *m150-m155*, *m157*, *m158*, *m17*) gene families, both of which encode membrane glycoproteins (Rawlinson *et al.,* 1996). The *US22* gene family has members in both HCMV and MCMV (*M23, M24, m25.1, m25.2, M36, M43, m128/ie2,* and *m139-m143*) as well as other herpesviruses (Rawlinson *et al.,* 1996). MCMV also contains members of the HCMV *UL25* and *UL82* families (*M25*/*M35* and *M82*/*M83,* respectively) (Rawlinson *et al.,* 1996). The last gene family present in both MCMV and HCMV contains homologs to G-protein-coupled receptors (*UL33*/*M33*, *US27*, *US28* and *UL78/M78*) (Rawlinson *et al.,* 1996; Chee *et al.,* 1990). Homologs to the cellular gene encoding the major histocompatibility class I (MHC-1) protein, are present in both HCMV (*UL18)* and MCMV (*m144)* although the 2 viral genes do not share sequence homology with each other (Rawlinson *et al.,* 1996).

1.2.3 Viral Life Cycle

The first stage in the viral life cycle is the attachment and penetration of the virus into host cells. This is followed by the uncoating of the viral capsid in the cytoplasm, and the translocation of the viral genome to the nucleus where DNA replication takes place. Subsequently, viral capsids are assembled, and an envelope is acquired as the encapsidated virus is transported across the nuclear membrane. De-envelopment occurs in the cytoplasm, where additional tegument proteins are incorporated, and the final envelopment of the virion takes place. The mature virion is released from the host cell and proceeds to infect other susceptible cells.

Research in both HCMV and MCMV has contributed to the pool of knowledge about the CMV life cycle, and many of the important genes involved in the virus life cycle have HCMV and MCMV homologs. Even though the descriptions in the following sections are mostly derived from research on HCMV, we have listed the MCMV homologs along with the relevant HCMV genes, with the assumption that the MCMV genes may have similar functions.

1.2.3.1 Viral Entry

In order for viral replication to occur, the virus first needs to be able to attach to and enter a susceptible host cell. In herpesviruses, several conserved glycoproteins (gB, gH, gL, gM and gN) are known to be involved in this process. Homologs for all of these proteins have been identified in HCMV, and are part of three glycoprotein complexes known as gcI (gpUL55/gB), gcII (gpUL100/gM and gpUL73/gN) and gcIII (gpUL75/gH-gpUL115/gL-gpUL74/gO) (Gretch *et al.,* 1988a; Gretch *et al.,* 1988b; Kari and Gehrz, 1992; Kaye *et al.,* 1992; Kari *et al.,* 1994; Huber and Compton, 1998; Mach *et al.,* 2000). In addition, homologs have been identified and characterized in MCMV (Loh *et al*., 1988; Loh and Qualtiere, 1988; Loh, 1991; Rapp *et al*., 1992; Xu *et al*., 1992; Xu *et al*., 1994; Li *et al*., 1995; Scalzo *et al*., 1995a; Scalzo *et al*., 2004).

The first step in viral entry is attachment of the virus to receptors on the host cell, mediated by glycoproteins in the viral envelope. For HCMV, heparan sulfate, integrins, and epidermal growth factor receptor have been identified as cell surface receptors (Kari and Gehrz, 1992; Compton *et al*., 1993; Wang *et al*., 1995; Wang *et al*., 2003; Feire *et al*., 2004). The binding of HCMV gcII to heparan sulfate proteoglycans on the cell surface is a critical step of viral infection (Kari and Gehrz, 1992). The glycoproteins in this complex, gM and gN,

interact both covalently, through disulfide bonds, as well as non-covalently (Mach *et al*., 2000; Mach *et al*., 2005). The covalent interaction is not required for viral replication or the transport of the complex out of the endoplasmic reticulum (Mach *et al*., 2005). Glycoprotein N undergoes significant modifications that increase its size from 18 kDa to 50 or 60 kDa in the presence of gM (Mach *et al*., 2000). The MCMV gM homolog has a 47% amino acid identity with HCMV gM (Li *et al*., 1995; Scalzo *et al*., 1995a), and the MCMV gN homolog (*M73*) has recently been identified (Scalzo *et al*., 2004). Human CMV is unable to bind to cells lacking cell surface heparin sulfate or during competition with soluble heparin (Compton *et al*., 1993). While gB is able to bind to heparin through a site in the carboxy terminal region, it is able to remain associated with the surfaces of cells deficient in heparan sulfate proteoglycans (Compton *et al*., 1993; Carlson *et al*., 1997; Boyle and Compton, 1998). It was also determined that pre-treatment of cells with gB significantly affected the ability of HCMV to bind to these cells, perhaps due to the lack of availability of appropriate receptors (Boyle and Compton, 1998). It appears that the binding of HCMV to heparin sulfate proteoglycans is only required at the initial stages of viral attachment to cells, and other receptors are required at later steps of virus entry (Boyle and Compton, 1998). One potential candidate for this receptor may be cellular integrins (Feire *et al*., 2004; Wang *et al*., 2005).

In both HCMV and MCMV, the gB glycoprotein forms disulfide-linked complexes, is expressed late in infection, and is a major constituent of the viral envelope (Loh *et al*., 1988; Loh, 1991; Rapp *et al*., 1992; Navarro *et al*., 1993). Although gB genes from all herpesviruses share a great deal of conservation, those from MCMV and HCMV are the most similar. The gB glycoproteins from both viruses retain the conserved tertiary structures found in all herpesviruses and share 45% of their amino acid sequences (Rapp *et al*., 1992). In addition, the beta-herpesviruses share 86.5% amino acid identity in the disintegrin-like domain of gB (Feire *et al*., 2004). Murine CMV has been shown to interact with β1 integrins, whereas HCMV can interact with β1 and β3 integrins (Feire *et al.,* 2004). In particular, gH can interact with integrin αvβ3 (Wang *et al*., 2005).

The epidermal growth factor receptor has also been implicated as an additional cellular receptor for HCMV gB (Wang *et al*., 2003), and has been shown to interact with integrin αvβ3 during virus entry and signalling (Wang *et al*., 2005). However, recent data indicated that the epidermal growth factor receptor might not play an important role in HCMV entry or

downstream signalling (Isaacson *et al*., 2007). It remains unknown whether there is an additional receptor capable of interacting with integrins during HCMV entry into cells.

Following attachment of virus to host cells through specific receptors, fusion with the cell membrane occurs, a process that depends on the gB and gH/gL glycoproteins in other herpesviruses (Kinzler and Compton, 2005). A 92.5 kDa protein on the cell surface interacts with the gH glycoprotein of HCMV, and may play a role in fusion. However, the identity of this receptor is not yet known (Keay *et al*., 1989; Keay and Baldwin, 1991). Furthermore, early studies indicated that gB and gH/gL were required for cell-cell fusion rather than initial attachment of CMV to cells (Keay and Baldwin, 1991; Navarro *et al*., 1993). The MCMV homologs to gH and gL have been identified and show great similarity to their HCMV counterparts as well as other beta- and gamma-herpesviruses (Loh and Qualtiere, 1988; Xu *et al*., 1992; Rapp *et al.,* 1994; Xu *et al*., 1994).

In HCMV, the gH/gL complex also includes gO, resulting in a heterotrimeric complex (Huber and Compton, 1998), or pUL131-128 (Ryckman *et al*., 2008). If the gO gene is deleted, viral growth is attenuated but not prevented as in the case of gH/gL deletion (Hobum *et al*., 2000). The heterotrimeric complex is formed in two steps. The first step involves the association of gH and gL (Huber and Compton, 1999) and the second step involves the association of gO with the gH-gL complex to form the precursor gCIII complex in the endoplasmic reticulum. This complex is then processed to the mature gCIII complex in a post-endoplasmic reticulum compartment (Huber and Compton, 1999).

As expected, if the gB, gH, gL and gO glycoproteins of HCMV were expressed individually in cells, cell fusion did not occur. However, co-expression of gH and gL enabled cell fusion in certain cell lines and the process was inhibited by neutralizing antibodies against these glycoproteins (Kinzler and Compton, 2005). Expression of gB and/or gO along with gH/gL did not increase the extent of cell fusion (Kinzler and Compton, 2005). Interestingly, if gH/gL was co-transfected into cells infected with a strain of HCMV that caused fusion regardless of viral infection, cell fusion was enhanced (Milne *et al*., 1998).

It appeared that HCMV might use the gH/gL/pUL128-131 complex to enter epithelial and endothelial cells by endocytosis and fusion (Wang and Shenk, 2005; Ryckman *et al*., 2006) whereas entry into fibroblasts involved the gH/gL/gO complex that mediated fusion with the plasma membrane (Wang and Shenk, 2005; Ryckman *et al*., 2008). Each of the

pUL128-pUL131 proteins was able to bind to the gH/gL complex on their own, and in so doing either enhanced or inhibited further binding by the other proteins (Ryckman *et al*., 2008). Export of gH/gL from the endoplasmic reticulum into the golgi apparatus was greatly enhanced with pUL128-pUL131 regardless of the presence of gO (Ryckman *et al*., 2008). In addition, there is evidence to suggest that the formation of a gB/gH complex during viral entry is dependent on pUL131-pUL128. Thus these proteins are important mediators of CMV fusion (Patrone *et al*., 2007).

1.2.3.2 Pre-replication events

Following fusion of the viral envelope with the cellular membrane, the viral and its associated tegument proteins are released into the cytoplasm. The virion is uncoated and viral DNA is transported into the nucleus where viral DNA replication will take place. Although not a lot of detail is known about this stage of the viral life cycle in CMVs, tegument proteins encoded by *UL48*/*M48*, *UL47*/*M47*, *UL49*/*M49* as well as the major capsid protein encoded by *UL86*/*M86* formed a complex that might play a role (Rawlinson *et al.*, 1996; Bechtel and Shenk, 2002). *UL48*/*M48* is highly conserved in herpesviruses, and is homologous to HSV-1 *UL36*, which encodes a virion structural protein that plays a role in the release of viral DNA from capsids (Batterson *et al.*, 1983; Bradshaw *et al.*, 1994). In addition, *UL47* was shown by Bechtel and Shenk (2002) to have a role in the viral life cycle between viral entry and immediate-early gene expression, which was delayed in *UL47* mutants.

Following delivery of the viral genome to the nucleus and before viral DNA replication can occur, the virus must suppress the apoptotic response initiated by the host cell. A number of viral genes are involved in the prevention of apoptosis at very early times after CMV infection, facilitating viral DNA replication by minimizing the effect of host anti-viral defences. This is discussed in more detail in section 1.2.4.1.

During HCMV infection of permissive cells, the host cell cycle is blocked at various stages, and particularly during G_1-S, and G_2-M transitions (Wiebusch and Hagemeier, 1999). There is some controversy over when the cell cycle is arrested during virus infection. However, it is known that cell cycle arrest mediated by immediate-early 2 (*ie2)* is crucial for efficient viral replication (Petrik *et al*., 2006). One study demonstrated that *ie2* induced cell cycle arrest at the G_1 stage within 24 hours after infection (Wiebusch and Hagemeier, 1999). However, a subsequent study indicated that *ie2* induced a shift into the S phase of the cell

cycle during viral infection, and blocked cell cycle progression at this point (Murphy *et al.*, 2000). The ability of *ie2* to activate cyclin E was necessary for shuttling cells into the S phase (Wiebusch *et al.*, 2003). In cells lacking p53, immediate-early 1 (*ie1)* blocked the cell cycle at the G_1/S transition, whereas *ie2* blocked the cell cycle at the G_2/M checkpoint, allowing cellular DNA synthesis but preventing cell division (Castillo *et al.*, 2000; Song and Stinski, 2005).

The host cell also has intrinsic defences in place that help protect against CMV infection. Cellular proteins involved in these processes include the promyelocytic leukemia protein (PML), death-associated protein 6 (Daxx) and histone deacetylases (HDACs) (Tang and Maul, 2003; Cantrell and Bresnahan, 2006; Preston and Nicholl, 2006; Saffert and Kalejta, 2006; Tavalai *et al*., 2006). These proteins have been shown to act independently and have an additive effect on the repression of immediate early gene expression (Tavalai *et al.,* 2008). The HCMV tegument protein pp71, encoded by *UL82*, interacted with and mediated the degradation of hDaxx, preventing it from repressing major immediate-early promoter/enhancer (MIEP) activity and viral replication (Cantrell and Bresnahan, 2006; Preston and Nicholl, 2006; Saffert and Kalejta, 2006). In this way, pp71 has the crucial role of activating the MIEP to allow efficient viral replication to occur (Liu and Stinski, 1992). Murine homologs of *UL82* have been identified as *M82* and *M83* (a *UL82*/*UL83* homolog) (Cranmer *et al.,* 1996). The mIE1, HCMV IE1 and IE2 proteins were all able to deacetylate HDACs, thus inhibiting their repressive activities (Tang and Maul, 2003; Nevels *et al*., 2004b; Park *et al*., 2007).

The promyelocytic leukemia protein, hDaxx, Sp100, the Bloom (*BLM*) helicase, and SUMO-1 (small ubiquitin-like modifier-1) are constituents of the promyelocytic oncogenic domains (PODs), also known as nuclear domain 10 (ND10). Many viruses, including papovaviruses, polyomaviruses, and members of all subfamilies of *herpesviridae* have proteins that co-localize with PML nuclear bodies (Everett, 2006). During HCMV infection, pp71 localized to ND10, where it interacted with hDaxx, and facilitated efficient viral DNA replication (Ishov *et al*., 2002). Available evidence suggests that MCMV mIE1 is initially targeted to ND10 by either PML or Daxx, and subsequently helps to recruit HDAC-2 (Tang and Maul, 2003). Ahn and Hayward (1997) observed that both IE1 and IE2 were present in ND10 less than 2 hours post-infection, where the IE1 protein was visualized by immunoflourescence as a mixture of diffuse and punctate nuclear patterns. By 4-6 hours post-

infection the IE1 pattern became exclusively diffuse, indicating that IE1 was able to disperse PML, thereby allowing the virus to initiate productive infection more efficiently (Ahn and Hayward, 2000). In contrast, the IE2 protein remained distributed in a punctate pattern even up to 12 hours post-infection (Ahn and Hayward, 1997).

The modification of PML by SUMO-1 is required for recruitment of Daxx into PODs and its own punctate nuclear localization pattern (Ishov *et al.*, 1999). During HCMV infection, IE1 was able to desumoylate PML, preventing it from performing its organizational role within ND10, resulting in the dispersion of ND10 proteins (Ahn *et al.,* 1998; Muller and Dejean, 1999; Lee *et al.,* 2004). This in turn prevented PML from functioning as a transcriptional repressor (Xu *et al.*, 2001). Thus it is not surprising that a MCMV *mie1* deletion mutant had decreased ability to disrupt PML bodies in mouse fibroblasts early after infection (Ghazal *et al.,* 2005). It is generally thought that during an infection at high MOI (multiplicity of infection), other immediate-early or viral proteins are able to compensate for the IE1 defect, modifying PML bodies to some extent so that productive HCMV infection can occur (Gawn and Greaves, 2002). When HDAC-2 activity was inhibited with Trichostatin A, there was an increase in the amount of mIE1 and M112-113 (also known as E1) proteins produced, with 50% more cells expressing E1 (Tang and Maul, 2003). The authors suggested that two different processes might occur depending on the MOI of the infection. At low MOI, the cell might be able to segregate mIE1 into ND10, resulting in decreased viral activity. With a high MOI, there is abundant production of mIE1, resulting in the sequestering of repressive cellular proteins via interactions with mIE1 (Tang and Maul, 2003).

HCMV IE1 and IE2 proteins were shown to interact with SUMO-1 and undergo sumoylation (Ahn *et al.*, 2001; Spengler *et al.*, 2002), a process that increased viral replication as well as expression of the IE2 protein, but was not required for interaction with PML (Xu *et al.*, 2001; Nevels *et al.,* 2004a). During HCMV infection, the expression of PML enhanced the rate of IE1 sumoylation (Xu *et al.,* 2001). There are differing reports regarding whether sumoylation of IE1 is required for its intracellular localization although it appeared that SUMO-1 did not target IE1 to ND10 (Xu *et al.*, 2001; Spengler *et al.,* 2002, Sadanari *et al.*, 2005). In a similar fashion, SUMO-1 modification was not required for the targeting of IE2 to ND10. However, it enhanced IE2 transactivation (Ahn *et al.*, 2001).

Once CMV is able to circumvent the cellular defences against viral infection, the HCMV IE1 and IE2 proteins and their MCMV homologs, mIE1 and mIE3, have crucial roles as transcriptional regulators. The HCMV *ie2*, and its homologous MCMV *mie3* genes are essential for viral replication and the initiation of productive infection (Angulo *et al.*, 2000; Marchini *et al.,* 2002). The IE2 protein is known as the major transactivator of gene expression and is able to activate a wide range of viral and cellular promoters (Pizzorno *et al.*, 1988; Stenberg *et al.*, 1990). In contrast, IE1/mIE1 is only required for viral replication in human/mouse cells at low MOI, but not at high MOI (Mocarski *et al.*, 1996; Messerle *et al.,* 1997; Greaves and Mocarski, 1998). Although another *mie1* mutant described by Ghazal *et al*. (2005) did not show any impairment in either early gene expression or ability to replicate in different cell lines at either high or low MOI, the discrepancy might be attributed to differences in the construction of the mutant viruses.

HCMV IE1 appears to have a role in transactivating delayed early (DE) genes (Gawn and Greaves, 2002). For instance, when IE1 was deleted, the accumulation of ppUL44 (the DNA polymerase processivity factor, a DE protein) during low MOI infection was delayed, and DNA replication compartments were not established (Greaves and Mocarski, 1998). In contrast, there is normal accumulation of IE2 and ppUL112-113 proteins in cells infected with *ie1* deletion mutants at low MOI, indicating that these proteins are not as strongly dependent on IE1 expression as ppUL44 (Gawn and Greaves, 2002). These experiments have also been performed in semi-permissive cells with similar results, where the expression of certain late proteins such as ppUL99 might even be delayed at high MOI (Ahn and Hayward, 2000). The IE2 protein may also be able to direct DE gene transcription as the amount of IE2 present at PML bodies increased during infection with IE1-negative viruses (Ahn and Hayward, 2000). These growth properties are similar to those observed during MCMV infection of non-murine cell lines (unpublished results in our laboratory).

The IE1 protein was shown to positively autoregulate the transcription of the HCMV MIEP through an NFκB site in the enhancer (Stenberg and Stinski, 1985; Cherrington and Mocarski, 1989). In this way, IE1 is able to augment IE2 activation of early and late viral genes (Malone *et al.*, 1990; Stenberg *et al.*, 1990). In addition, the IE2 protein was able to repress the MIEP by directly binding to the *cis*-repression signal (Stenberg *et al.*, 1990; Lang and Stamminger, 1993). In addition to IE1 and IE2, viral accessory transactivators such as

TRS1/IRS1, UL36-UL38, and UL112-UL113 were also necessary for activation of DE promoters (Stasiak and Mocarski, 1992; Pari and Anders, 1993; Iskenderian *et al.*, 1996). In addition, when co-transfected with genes encoding IE1 and IE2, IRS1 and TRS1 enhanced the activity of a late promoter (Stasiak and Mocarski, 1992). Further studies showed that these proteins were able to up-regulate the activity of promoters only in the presence of IE1 and IE2 (Romanowski and Shenk, 1997). These proteins also up-regulated the activity of immediate-early promoters with the help of pUL69, a tegument protein present in virions (Romanowski *et al.,* 1997). Similarly, IE2 interacted with components of the basal transcription machinery, such as the TATA binding protein, resulting in the activation of several promoters (Caswell *et al.*, 1993). In addition, both HCMV IE1 and IE2 were able to interact with specific transcription factors and nuclear proteins such as c-Jun, SP-1, E2F1, Cardiotrophin 1, the $hTAF_{II}130$ component of TFIID, p107, and PML (Lukac *et al.*, 1994; Hayhurst *et al.*, 1995; Margolis *et al.*, 1995; Scully *et al.,* 1995; Poma *et al.*, 1996; Lukac *et al.,* 1997; Ahn *et al.*, 1998).

Initiation of the formation of HCMV DNA replication compartments occurred in structures that bud from PODs and then progressed into larger nuclear compartments that were clearly visible in infected cells (Ahn *et al.,* 1999). The HCMV *UL112/113* or MCMV *M112/113* genes encode four phosphoproteins (pp34, pp43, pp50 and pp84 in HCMV) that co-localize with viral DNA prior to viral DNA replication and play a crucial role in the initial steps of replication compartment formation (Iwayama *et al.*, 1994; Yamamoto *et al.*, 1998; Ciocco-Schmitt *et al.*, 2002). When all 4 proteins were expressed, they localized to PODs, and the pp84 protein in particular was required for the targeting of the other 3 proteins (Park *et al.*, 2006). In addition, pUL84, which contained a nuclear localization signal (Xu *et al.*, 2002), was essential for the formation of replication compartments (Sarisky and Hayward, 1996). The pre-replication compartments contained several other viral proteins, including IE2 (but not IE1) and the essential replication proteins ppUL44 and pUL57 (Penfold and Mocarski, 1997; Ahn *et al.*, 1999; Xu *et al.*, 2002). In particular, the localization of ppUL44 to these compartments required the presence of all four ppUL112/113 proteins as well as pUL84 (Xu *et al.*, 2004; Park *et al.*, 2006). However, the localization of IE2 to replication compartments appeared to be more complex. Both pUL84 and an interaction between IE2 and viral DNA have been suggested to be responsible for its recruitment into ND10 (Xu *et al.,* 2004; Sourvinos *et al.*,

2007). In addition, MCMV ppM112-113 proteins have been demonstrated to interact with mIE3 (the MCMV homolog of HCMV IE2) in a complex with PML, thereby sequestering mIE3 into replication compartments and preventing the negative regulation of the major immediate early promoter (Tang *et al.,* 2005).

1.2.3.3 Viral DNA Replication

A set of 11 viral genes is required for the initiation of DNA replication from *ori*Lyt, the origin of DNA replication during lytic infection in CMV. This included 6 core herpesvirus genes which are homologous to the essential HSV-1 replication genes: a DNA polymerase (*UL54*/*M54*), a polymerase processivity factor (*UL44*/*M44*), a single stranded DNA-binding factor (*UL57*/*M57*), and the helicase-primase complex consisting of a primase-helicase (*UL70*/*M70*), a helicase (*UL105*/*M105*) and a primase-associated factor (*UL102*/*M102*) (Pari and Anders, 1993; Pari *et al.*, 1993; Loh *et al.*, 1994; Rawlinson *et al.*, 1996; McMahon and Anders, 2002). In addition, 5 other genes that show no obvious homology to genes in alpha- or gamma-herpesviruses are required to complement *ori*Lyt-dependent DNA replication: *UL84*/*M84*, *UL112-UL113*/*M112-M113*, and the regulatory genes *UL36-38* (*M36*, *m38.5*), *ie1-ie2* (*mie1-mie*3), and *TRS1*/*IRS1* (*m143*) (Pari and Anders, 1993; Pari *et al.*, 1993; Rawlinson *et al.*, 1996).

Thus far only one *ori*Lyt has been identified in both MCMV and HCMV. It is located upstream (5') of the *UL57/M57* genes for both viruses (Hamzeh *et al.*, 1990; Anders and Punturieri, 1991; Masse *et al.,* 1997), and shows little sequence homology to the origins of replication in alpha-herpesviruses (Hamzeh *et al.*, 1990). The MCMV and HCMV *ori*Lyt contain several duplicated and inverted sequences, as well as core regions of 1.7 kb and 1.5 kb, respectively (Hamzeh *et al.*, 1990; Anders and Punturieri, 1991; Masse *et al.*, 1992; Masse *et al.,* 1997). In HCMV and MCMV, *ori*Lyt activity also requires sequences flanking the core region, and sequences in the left flanking region in particular (Anders *et al.*, 1992; Masse *et al.,* 1997; Borst and Messerle, 2005). These flanking regions contain binding sites for transcription factors which could function as promoter sequences (Hamzeh *et al.*, 1990; Anders *et al.*, 1992; Masse *et al.,* 1997; Borst and Messerle, 2005). One of these promoters lies between *UL57* and *ori*Lyt and was shown to be responsible for regulating *UL57* expression and activating *ori*Lyt (Kiehl *et al.*, 2003). The smallest replicator transcript (SRT) RNA, which might be involved in regulation of lytic DNA replication, was transcribed from HCMV *ori*Lyt

(Huang *et al.*, 1996), which also contained sites for two virus-associated RNAs (vRNA-1 and vRNA-2) that formed RNA-DNA hybrid structures during or after DNA replication (Prichard *et al.*, 1998).

The initiator of HCMV DNA replication, pUL84, bound to *ori*Lyt specifically through a stem-loop region within this RNA/DNA hybrid structure (Colleti *et al.,* 2007), and was absolutely required for viral DNA replication to occur, but not for the expression of viral genes (Xu *et al.*, 2004). It contains a nuclear targeting domain that includes two leucine-rich nuclear export signals, enabling the shuttling of proteins between the nucleus and the cytoplasm (Lischka *et al.*, 2006). This suggested that pUL84 might have an as yet undefined role in the cytoplasm (Lischka *et al.*, 2006). The pUL84 protein interacted with many cellular proteins, including casein kinase II, p32, ubiquitin-conjugating enzyme E2, and annexin A2 (Gao *et al.,* 2008). It could interact with other pUL84 molecules through an oligomerization domain, as well as viral proteins such as pp65, a component of the capsid, and IE2 (through a leucine zipper domain) (Colletti *et al.*, 2004; Gao *et al.*, 2008;). Both the pUL84-pUL84 and the pUL84-IE2 interactions appeared to be required for DNA replication (Colletti *et al.*, 2004). Interestingly, pUL84 was able to inhibit transactivation of viral gene expression by IE2, and enhanced the negative autoregulation of the MIEP by this gene (Gebert *et al.,* 1997). The pUL84 protein also interacted with ppUL44, implying a potential role for the polymerase accessory protein in the initiation of DNA synthesis (Gao *et al.*, 2008).

Other protein-protein interactions were shown to be important for viral DNA replication. For example, interaction between pUL54 and ppUL44 resulted in increased processivity of the DNA polymerase (Ertl and Powell, 1992; Weiland *et al.*, 1994). A region of pUL54 distinct from the ppUL44-binding domain contained two nuclear localization signals, although only one is required for nuclear localization (Alvisi *et al.*, 2006). The accessory proteins UL36-38, ppUL112-113, IRS1, and IE1/IE2 cooperated with each other to regulate the expression of the 6 core proteins required for lytic DNA replication (Stasiak and Mocarski, 1992; Iskenderian *et al.,* 1996). In addition, ppUL112-113 expression is regulated by IRS1 and UL36-38 (Iskenderian *et al.*, 1996). The MIE proteins had the strongest affect on core protein expression, followed by UL36-38 (Iskenderian *et al.*, 1996). The IR1 (inverted repeat-1) sequence in the *UL54* promoter was required for its regulation by MIE proteins and ppUL112-113 proteins at early times of infection (Kerry *et al.*, 1996). In particular, the IE2 protein

bound to IR1 in a complex with the cellular transcription factor SP1 (Luu and Flores, 1997; Wu *et al.*, 1998). However, at late times of infection, the *UL54* promoter was regulated by the activating transcription factor-1 (Kerry *et al.,* 1997).

1.2.3.4 Virus Assembly and Egress

Following viral DNA replication, CMV genomes were arranged in head-to-tail concatemers that needed to be cleaved into genome-size lengths prior to packaging into capsids. The large subunit of the terminase, encoded by *UL56/M56*, was responsible for binding to the *pac* sites on the viral genome (Bogner *et al.*, 1998) while cleavage at these sites was carried out by the small subunit of the terminase encoded by *UL89/M89* (Scheffczik *et al.*, 2002). The two terminase subunits interacted with each other and localized to viral replication centers, suggesting a potential association between the DNA replication and packaging processes (Hwang and Bogner, 2002; Thoma *et al.*, 2006). Once the viral DNA genome was cleaved, it was translocated into capsids through a portal formed by pUL104 via interactions with the large terminase subunit (Dittmer *et al.*, 2005). This process was also dependent on pUL56 ATPase activity (Hwang and Bogner, 2002). More recently, pUL52/pM52 was shown to play a role in both the cleavage and packaging of viral DNA (Borst *et al.*, 2007). The unique subnuclear location of pUL52, which appeared to enclose compartments where DNA replication and packaging took place, suggested that it might provide a link between the two processes (Borst *et al.*, 2007).

Once capsids were assembled, they underwent maturation and egress from the nucleus, processes believed to be mediated by highly conserved components of the nuclear egress complex (pUL53/pM53 and pUL50/pM50). In addition, pUL32/pM32 might also be required as either a stabilization factor during capsid assembly or during nuclear egress (Meyer *et al.,* 1997; AuCoin *et al.,* 2006). It is generally thought that CMV virions undergo a primary envelopment, followed by a de-envelopment and then a secondary envelopment. Prior to primary envelopment, viral structural proteins (pUL83, pUL69 tegument proteins, and pUL86 and pUL49/49 capsid proteins) accumulated in the nuclear matrix, which was tightly associated with the inner nuclear membrane (Sanchez *et al.*, 1998). The proteins of the nuclear egress complex played a major role in the primary envelopment of CMV capsids by designating the site on the nuclear lamina where viral capsid egress could occur (Muranyi *et al.*, 2002). The M50 protein was responsible for the penetration of capsids through the nuclear

envelope, a process that required the dissolution of the nuclear lamina through the action of protein kinase C (Muranyi *et al.*, 2002). A proline rich region in both pM50 and its HCMV homolog pUL50 was required for nuclear egress of capsids containing viral DNA (Rupp *et al.*, 2007).

Two of the most abundant tegument proteins, pUL99 and pUL32, as well as glycoprotein B (UL55) were not found to be associated with the nuclear matrix (Sanchez *et al.*, 1998). These proteins, along with pUL53, ppUL83 (pp65), gH and gp65 appeared to accumulate in a cytoplasmic assembly compartment where additional proteins might be incorporated into virus particles following their egress from the nucleus and de-envelopment in the cytoplasm (Sanchez *et al.*, 2000a; Dal Monte *et al.*, 2002; Silva *et al.,* 2003). When pUL99 was transfected alone into cells, it localized to the endoplasmic reticulum-golgi intermediate compartment, suggesting that other viral proteins were required for its translocation to the assembly compartments in the cytoplasm (Sanchez *et al.,* 2000a; Sanchez *et al.,* 2000b). The pUL26 tegument protein had a role in regulating the phosphorylation status of the pUL99 and pUL83 tegument proteins and maintaining their stability (Munger *et al.*, 2006). This stability is crucial, as pUL99/pM99 appeared to be involved in the final envelopment of CMV in the cytoplasm (Silva *et al.*, 2003). Mutations in this protein resulted in the accumulation of non-enveloped capsids in the cytoplasm in spite of normal expression of late proteins (Silva *et al.,* 2003).

The proteins present in both the MCMV and HCMV virions have been identified (Kattenhorn *et al.*, 2004; Varnum *et al.*, 2004). In HCMV, 71 virally encoded proteins and 70 cellular proteins were found to be associated with virions (Varnum *et al.*, 2004). The most prevalent viral glycoprotein and protein were gM and pUL83, respectively (Varnum *et al.,* 2004). In MCMV, 7 cellular proteins and 58 virally encoded proteins were found to be associated with virions, including capsid, tegument, regulatory, and replication proteins, as well as glycoproteins (Kattenhorn *et al.,* 2004). Annexin II represented a cellular protein that was associated with both HCMV and MCMV virions and was known to interact with gB (Pietropaolo and Compton, 1997; Kattenhorn *et al.,* 2004; Varnum *et al.*, 2004;). Since this interaction was non-essential for viral entry into cells, it had been suggested that it might be required for the acquisition of primary and final envelopes during virion production (Pietropaolo and Compton, 1999).

1.2.4 Viral-host interactions

The interactions between virus and host cell often dictate whether an infection will be successful. The nature of these interactions can be affected by the types of cells and tissues infected by the virus, or the species of the infected host. Since HCMV and other CMVs are able to enter cells and express a subset of viral genes in non-permissive hosts, the block to viral replication is likely at a point in the viral life cycle after entry (Mocarski *et al.,* 2007).

1.2.4.1 Host and tissue specificity

A characteristic trait of CMVs is their host species specificity, which means that they usually only undergo productive infection in their natural host. This is quite different from the alpha-herpesviruses, including HSV-1, which have a broad host range. However, even amongst CMVs there are differences in the degree of species specificity. For instance, HCMV is known to have very strict species specificity and is only able to undergo productive infection in human diploid fibroblasts (Lafemina and Hayward, 1988). In contrast, MCMV is noted to have the ability to productively infect many cell lines, including those derived from other species, albeit only at high MOI (Kim and Carp, 1971; unpublished data from our laboratory). Murine CMV was able to absorb to and cause cytopathic effects (CPE) in human diploid cells and complete a portion of the replicative cycle even though productive infection did not occur (Kim and Carp, 1972). The ability of MCMV to cause typical CPE in infected human cells was not dependent on viral DNA synthesis (Kim and Carp, 1972). Similar observations were made during HCMV infection of guinea pig cells (Fioretti *et al.,* 1973). Interestingly, while MCMV was able to infect rat cells, RCMV was unable to infect murine or human cells and productive infection was limited to rat embryo fibroblasts (Bruggeman *et al.,* 1982).

There are distinct differences in the permissiveness of human, monkey, and murine cells for HCMV, SCMV and MCMV (Lafemina and Hayward, 1988). Murine CMV and SCMV were shown to have a much broader host range than HCMV. Although infection of different types of diploid fibroblasts results in IE gene expression for all three viruses, only MCMV progresses to the viral DNA replication stage in each cell type (Lafemina and Hayward, 1988). Productive MCMV infection can occur in human cells if HCMV IE1 and tegument proteins are provided (Tang and Maul, 2006). These proteins have important functions required for effective cross-species infection, including interactions of the tegument protein pp71 with the Daxx repressor, repression of the interferon response by pp65 and

inhibition of Daxx and HDAC by IE1 (Tang and Maul, 2006). In contrast, SCMV DNA replication was observed in monkey and human cells, whereas HCMV DNA replication only occurred in human cells (Lafemina and Hayward, 1988). By comparison, SCMV was able to express IE genes in a wider range of cells, and HCMV was the most restricted (Lafemina and Hayward, 1988). Interestingly, HCMV is able to progress to the viral DNA replication stage of the life cycle in primary chimpanzee skin fibroblasts but not transformed chimpanzee fibroblasts, illustrating the close lineage between the human and chimpanzee species (Perot *et al.,* 1992).

A study by Jurak and Brune (2006) highlighted the importance of apoptosis during the infection of human cells by MCMV. Although MCMV was able to infect and replicate in human embryonic kidney cells, the release of virus was delayed. In addition, when these cells were infected with MCMV at low MOI, virus production and cell-cell spread was lower than in permissive cells (Jurak and Brune, 2006). It was determined that MCMV infection of non-permissive human cells caused apoptosis, a process that was likely prevented during permissive infection. As the authors noted, human cell lines permissive for MCMV infection contain the adenoviral genes *E1A*, which is pro-apoptotic, and *E1B*, which is anti-apoptotic (Jurak and Brune, 2006). Interestingly, when non-permissive human cells were transfected with vectors expressing the *E1B*-19k gene, apoptosis induced by MCMV was prevented, and productive infection occurred. Thus the barrier to cross-species infections appeared to be the induction of apoptosis in non-permissive cells (Jurak and Brune, 2006). However, even when the presence of an anti-apoptotic protein facilitated MCMV replication in human cells, the progress of infection was still delayed, suggesting that there were other factors involved.

The first HCMV genes identified as having a role in preventing apoptosis of infected cells were *ie1* and *ie2*, which blocked apoptosis induced by either tumor necrosis factor (TNF)-α, or superinfection by EIB-19k-deficient adenovirus, but they did not interact with either Bcl-2 (B cell lymphoma/leukemia 2) or Bax (Bcl-2-Associated X Protein) (Zhu *et al.,* 1995). Since these were immediate-early genes, their expression prevented the host cell from mounting an early antiviral response (Zhu *et al.,* 1995). The IE1 and IE2 proteins activated the phosphatidylinositide 3'-OH kinase, which in turn activated Akt (a cellular kinase), leading to the inhibition of apoptosis (Yu and Alwine, 2002). In addition, IE2 was able to interact with and repress the transcription of p53, a well-known apoptotic protein, thereby preventing it

from activating the apoptosis pathway (Tsai *et al.,* 1996). This interaction is likely critical to the success of CMV infection in permissive cells, especially since p53 is stabilized during HCMV infection (Muganda *et al.,* 1994). Human CMV was also able to sequester p53 into replication centers in infected cells, which might have the dual effect of repressing p53 apoptotic activity and enhancing HCMV replication at the same time (Fortunato and Spector, 1998; Casavant *et al.,* 2006).

Although other herpesviruses contain homologs of Bcl-2 or FLIP (FLICE-like inhibitory protein), which are anti-apoptotic proteins, no CMV homologs to these cellular proteins are known to exist (Goldmacher *et al.,* 1999). However, as previously mentioned, *UL37* encodes a viral mitochondrial-localized inhibitor of apoptosis (vMIA) which has a similar mechanism of action to Bcl-2. This protein was designated vMIA due to its localization to the mitochondria as well as its anti-apoptotic activity (Goldmacher *et al.*, 1999). Murine CMV *m38.5* is located in the same region of the viral genome as HCMV *UL37*, and appeared to function in the same way as vMIA, by localizing to the mitochondria and inhibiting apoptosis induced by proteasome inhibitors. However, it was unable to prevent Fas-mediated apoptosis (McCormick *et al.,* 2005), and did not share sequence homology with *UL37* (McCormick *et al.*, 2005).

Arnoult *et al.* (2004) demonstrated that vMIA could prevent apoptosis mediated by Bax but not Bak (Bcl-2 homologous antagonist/killer). The vMIA protein was able to inactivate Bax-mediated membrane permeabilization, whereas Bcl-2 inactivated truncated Bid (Bcl-2 interacting protein) mediated activities (Arnoult *et al.,* 2004). In addition, HCMV vMIA did not prevent Fas activation of caspase 8 or cleavage of Bid (Goldmacher *et al.,* 1999). If the cellular *bcl-2* or *bcl-XL* genes, or the HCMV *UL37* gene were over-expressed during MCMV infection of human cells, or if human cells were infected by recombinant MCMV containing HCMV *UL37*, apoptosis was prevented (Jurak and Brune, 2006). Interaction of vMIA with GADD45 (growth arrest and DNA damage 45) was required for its anti-apoptotic activity (Smith and Mocarski, 2005).

Another anti-apoptotic protein of CMV is vICA (viral inhibitor of caspase 8 activation), the gene product of *UL36*, which has a distinct mechanism compared to vMIA (Skaletskaya *et al.,* 2001). Although vICA has a similar function to FLIPs, it lacks any homology to them, much the same as the case for vMIA and Bcl-2 (Skaletskaya *et al.,* 2001).

Cells expressing UL36 were resistant to apoptosis mediated by Fas, TNF-α, TNF-related apoptosis-inducing ligand, doxorubicin, and infection with *E1B*-19k deficient adenovirus. The pUL36 protein was able to interact with the pro domain of caspase 8, preventing its activation, and thus functioned at an earlier point in the Fas-mediated apoptosis pathway than vMIA (Goldmacher *et al.,* 1999; Skaletskaya *et al.,* 2001). The M36 protein of MCMV is homologous to HCMV vICA and designated M-vICA. It is part of the *US22* family of genes, and requires motif IV for its activity, suggesting that one of the roles of the *US22* family is to mediate anti-apoptotic activities (McCormick *et al.,* 2003). *M36* was not required for virus growth on endothelial cells, but its anti-apoptotic activity increased the viability of macrophages (Ménard *et al.,* 2003). However, unlike HCMV vICA, M-vICA was essential for *in vivo* viral replication (Ménard *et al.,* 2003; Goldmacher, 2005).

It was initially thought that *UL36* represented a redundant viral function protecting against apoptosis since studies leading to this conclusion used a viral strain lacking a functional *UL36* (Reboredo *et al.,* 2004). The deletion of *UL37* in a viral strain that contained a functional *UL36* gene did not affect the replication of HCMV or induce caspase 3 dependent apoptosis (McCormick *et al.,* 2005). It was suggested that vICA initially prevented apoptosis of HCMV infected cells, and vMIA protected against apoptosis induced by proteasome inhibitors later on in infection (McCormick *et al.,* 2005).

The MCMV *M45*, a homolog of the ribonucleotide reductases, was another anti-apoptotic protein essential for the growth of MCMV on endothelial cells. However, this gene was not required to prevent apoptosis in fibroblasts (Brune *et al.,* 2001). In contrast, the gene product of HCMV *UL45* did not have an anti-apoptotic function (Hahn *et al.,* 2002). Murine CMV *m41*, which is part of the *m41*, *m40* and *m39* gene cluster, localized to the golgi apparatus and had an anti-apoptotic function at late stages of infection (Brune *et al.,* 2003). Although expression of *m41* prevented premature apoptosis in infected cells, it was not essential for viral growth (Brune *et al.,* 2003).

MCMV was able to protect virally infected dendritic cells from apoptosis (Andoniou *et al.,* 2004). During this process, Bim (Bcl-2 interacting mediator of cell death), a Bcl-2 family member, was upregulated whereas other Bcl-2 family members did not experience any change in expression. The pro-apoptotic protein Bim localized to the mitochondria as a result of induction of cellular apoptotic pathways. In addition, the expression of an anti-apoptotic

protein Bfl-1/A1 was upregulated during MCMV infection of dendritic cells. Bax remained targeted to and oligomerized at the mitochondria in these infected cells even when growth factors were not present, and the mitochondrial membrane potential did not change (Andoniou *et al.,* 2004). However, an amino terminal epitope of Bax was not exposed during MCMV infection of dendritic cells in the absence of growth factors. This anti-apoptotic function was not regulated by *M36*, *m38.5* or *M45* (Andoniou *et al.,* 2004).

In summary, the host range of CMVs appears to be regulated by several viral and cellular factors. Many of these factors are likely to be involved in evading the host defences. For instance, there appears to be a link between productive viral infection and the blocking of apoptosis in infected cells. Viral infection can cause cellular stress, cell cycle arrest, and DNA damage, leading to apoptosis. This host strategy allows infected cells to be eliminated before virions can be released to infect other cells in the host system. Elucidation of the factors involved should be beneficial to the development of HCMV vaccines and anti-viral drugs.

1.2.4.2 Host defences against viral infection

Upon viral infection, the host organism mounts innate, cell-mediated and humoral immune responses. Innate responses involve natural killer and dendritic cells, the action of interferons, and the activation of dsRNA activated protein kinases. Cell-mediated responses involve recognition of viral proteins by cytotoxic T-lymphocytes (CTLs). Humoral responses involve the recognition of viral antigens by the immune system and the production of antibodies against these antigens. The virus must be able to circumvent these host defences in order to initiate productive infection of the host organism.

1.2.4.2.1 Innate immune responses

Natural killer (NK) cells are able to recognize MHC class I molecules on the surface of virus infected cells and are critical in the control of MCMV infection. Cell surface receptors such as the Ly49H activation receptor encoded by the *Cmv1* resistance locus, a part of the NK cell gene complex, are expressed in NK cells (Brown *et al.,* 2001; Daniels *et al.,* 2001; Lee *et al.,* 2001a). During MCMV infection of resistant mouse strains (such as B6), the MHC class-I like m157 protein was expressed on the surface of infected cells and bound to the Ly49H receptor, causing the induction of NK cell mediated cell death, and secretion of interferon and chemokines (Arase *et al.,* 2002; Smith *et al.,* 2002). However, *Cmv1*-mediated resistance to MCMV infection is rare among inbred and outbred (wild) mouse strains (Scalzo *et al.,* 1995b;

Scalzo *et al.,* 2005). There are different mechanisms for resisting NK cell mediated cell death, depending on the strain of mouse infected with MCMV. For instance, resistance to MCMV infection in strains containing the *Cmv2* (the NZW strain), *Cmv3* (the MA/MY inbred strain), or *Cmv4* (PWK/Pas strain) loci did not involve interactions between Ly49H and m157. Instead, *Cmv3* resistance involves the NK cell receptor Ly49P, whereas receptors enabling *Cmv2* and *Cmv4* resistance had not yet been identified (Rodriguez *et al.,* 2004; Desrosiers *et al.,* 2005; Adam *et al.,* 2006).

Murine CMV and HCMV encode genes that are able to help counteract host immune responses. There is evidence that *m157* can act as a viral immune evasion gene by preventing the action of NK cells in susceptible mouse strains (Arase *et al.,* 2002). In addition, MCMV infected cells can avoid NK cell mediated cell death by the decoy action of *UL18*/*m144* genes which encoded MHC class-I like proteins (Beck *et al.,* 1988; Rawlinson *et al.,* 1996; Farrell *et al.,* 1997; Reyburn *et al.,* 1997). Human CMV *UL18* binds to the leukocyte immunoglobulin-like receptor on the cell surface and inhibits the activity of NK cells and other immune cells (Cosman *et al.,* 1997). In addition, MCMV *m145*, *m152*, and *m155* genes encode proteins that decrease the possibility that NK cell receptors would detect stress related molecules produced as a result of MCMV infection by down-regulating their expression (Hasan *et al.,* 2005; Krmpotic *et al.,* 2002; Lodoen *et al.,* 2003; Lodoen *et al.,* 2004; Krmpotic *et al.,* 2005). All these genes are highly conserved in MCMV strains isolated outside the laboratory (Smith *et al.,* 2006).

Another target for MCMV during viral infection are dendritic cells, which need to interact with NK cells to enable their activation (Andrews *et al.,* 2001). Initially, MCMV infection of dendritic cells caused the production of cytokines that in turn help to activate NK cells (Andrews *et al.,* 2001; Andoniou *et al.,* 2005). However, later on in infection, the infected dendritic cells became non-functional (Andrews *et al.,* 2001; Mathys *et al.,* 2003). Interestingly, in resistant mice containing the *Cmv1* gene locus, the propagation of NK cells expressing Ly49H on the cell surface depended on the presence of CD8α+ dendritic cells (Andrews *et al.,* 2003).

The IRS1 and TRS1 proteins also have a crucial role in the viral evasion of the host immune response by preventing the activation of a pathway that would block viral replication (Child *et al.,* 2004). These proteins were able to prevent the inhibition of protein synthesis by

the cell, a process involving the phosphorylation of the eukaryotic translation factor eIF-2α and the activation of RNase L, resulting in RNA degradation (Child *et al.,* 2004). IRS1 and TRS1 could also help the virus evade this dsRNA activated antiviral pathway by binding to dsRNA (Cassidy, 2005; Hakki and Geballe, 2005). TRS1 was able to retain protein kinase R in the nucleus, preventing its activation and the subsequent induction of the dsRNA antiviral pathway (Hakki *et al.,* 2006). TRS1, but not IRS1, is required for the production of infectious virus (Blankenship and Shenk, 2002).

Another important host defence mechanism that must be circumvented by viruses is the interferon (IFN) response. Interferons can be divided into two classes. Type I interferons are produced in response to viral infection, whereas type II interferons are released by NK cells and T cells when they are activated upon recognition of infected cells (Hengel *et al.,* 2005). Type I interferons use the IFN-α/β receptors whereas Type II interferons bind to IFN-γ receptors. Activation of the IFN-α/β receptor results in the induction of IFN-stimulated response elements whereas IFN-γ receptor signalling activates the gamma activated sequences (Hengel *et al.,* 2005). Both of these pathways represent different parts of the Jak-STAT (signal transducers and activators of transcription) pathway. Murine CMV was able to circumvent the host-mediated type I and type II interferon antiviral defences through *M27* (Hengel *et al.,* 1994; Lucin *et al.,* 1994; Heise *et al.,* 1998; Zimmermann *et al.,* 2005). The M27 protein bound to STAT2 and caused its degradation. Thus lack of this gene resulted in susceptibility to type I interferon, as well as type II interferon, which required functional type I interferon for its activity (Zimmermann *et al.,* 2005). In addition, IE2 could block the IFN-β response while IE1 counteracted the type I-IFN response during HCMV infection (Taylor and Bresnahan, 2005; Paulus *et al.,* 2006).

1.2.4.2.2 Cell-mediated responses

Cell-mediated immune responses represent an important host defence mechanism against CMV infections. Cytotoxic T-lymphocytes are able to kill virus-infected cells by recognizing viral peptides presented by the MHC class-I molecules on the surfaces of these cells. In MCMV, the major target of CTLs was mIE1 (Reddehase *et al.,* 1987). Although IE1 did represent a target, the major target for CTLs in HCMV-infected cells was pp65 (Riddell *et al.,* 1991; Wills *et al.,* 1996; Gyulai *et al.,* 2000; Khan *et al.,* 2002). Due to the vast sequence diversity in *mie1* in wild mice, CTLs specific for *mie1* from the K181 and Smith laboratory

strains of MCMV did not recognize these variants (Lyons *et al.,* 1996). In contrast, the CTL epitopes on pp65 were highly conserved in HCMV strains, and although HCMV IE1 also showed significant sequence variation, CTLs were still able to recognize these variants (Solache *et al.,* 1999; Prod'homme *et al.,* 2003). The IE1 protein of HCMV was phosphorylated by pp65, thus preventing CTLs from recognizing it as a viral target (Gilbert *et al.,* 1996).

Both self and foreign antigens (such as MCMV IE1 and HCMV pp65) are presented to CTLs on cell surfaces by MHC class-I molecules. Therefore, viruses target these molecules as a way to evade CTL responses to viral infection. The HCMV genes involved in immune system evasion through modulating antigen presentation by MHC class-I molecules are *US2, US3, US6* and *US11,* all of which encode glycoproteins. *US2* and *US11* were involved in facilitating the destruction of newly synthesized MHC class I molecules by the proteasome in the cytosol (Wiertz *et al.,* 1996a, 1996b). The US3 protein prevented the maturation of MHC class-I and its translocation from the endoplasmic reticulum during the immediate-early phase of infection, a function that might require oligomerization of US3 molecules (Jones *et al.,* 1996; Misaghi *et al.,* 2004). The US6 protein formed a complex with the transporter of antigen processing, thus preventing peptides from being recognized by the immune system by retaining MHC class-I molecules in the endoplasmic reticulum (Lehner *et al.,* 1997). Murine CMV genes *m06*, *m152* and *m04* encoded gp48, gp40, and gp34, respectively. Both *m06* and *m152* were highly conserved amongst MCMV strains, whereas *m04* was quite variable (Smith *et al.,* 2006). The *m152* gene encoded a glycoprotein that prevented CTL recognition of the *ie1* peptide by blocking MHC class-I presentation of this antigen on the cell surface (Del Val *et al.,* 1992; Ziegler *et al.,* 1997). In contrast, the *m04* gene product did not prevent MHC class-I surface expression but was able to regulate the CTL response (Kleijnen *et al.,* 1997; Kavanagh *et al.,* 2001). The *m06* gene product bound to MHC class I molecules and targeted them for degradation (Bubeck *et al.,* 2002).

1.2.4.2.3 Humoral responses

Humoral immune responses produce antibodies against specific viral antigens that can neutralize virus infectivity and prevent cells from being infected. Humoral responses initially involve the production of IgM antibodies, followed by IgG antibodies, which persist in the host. During HCMV infection, gB represented the main target of the humoral response, and it

was the target of more than half of the neutralizing antibodies produced by the host (Britt *et al.*, 1990). In MCMV, gB represented a target for both neutralizing and non-neutralizing antibodies (Loh *et al.,* 1988; Rapp *et al.,* 1992). In addition, several neutralizing antibodies were directed against gH during HCMV infection (Urban *et al.,* 1994). Non-neutralizing antibodies specific for other HCMV proteins, such as pp150 (encoded by *UL32*), had also been identified (Jahn *et al.,* 1987).

Human CMV was able to evade humoral immune responses by up-regulating the expression of Fc receptors that could bind IgG molecules (MacCormac and Grundy, 1996). This could help to prevent virus specific antibodies from interacting with complement and inducing antiviral immune responses. Secondly, cytomegaloviruses encoded homologs of the Fc receptor, such as the TRL11/IRL11 and UL119/118 proteins in HCMV, and the m138 protein in MCMV (Thale *et al.,* 1994; Lilley *et al.,* 2001; Atalay *et al.,* 2002). Although the MCMV Fc receptor homolog was required for productive infection in normal or immunosuppressed mice, it was not required during infection of newborn mice (Crnković-Mertens *et al.,* 1998). A third way for cytomegaloviruses to evade the humoral immune response was through the regulation of complement activation. The infecting virus was able to control the expression of cellular regulators of complement activation in infected cells (Spiller *et al.,* 1996, 1997). Normally, during viral infection, complement could recognize and attack viral particles that had antibody bound to their envelope glycoproteins. Murine CMV infection resulted in the upregulation of CD46, which functioned to prevent the complement-mediated lysis of virally infected cells (Nomura *et al.,* 2002).

1.2.5 Pathogenesis

Cytomegaloviruses infect 40-60% of the population asymptomatically, often with the disease remaining undiagnosed. However, the virus can remain in the host in a latent state in specific cell types, and cycle between periods of latency and reactivation. In an asymptomatically infected host, the virus can still be transmitted upon reactivation. The ability of CMV to remain undetected and undisturbed in the host for significant periods of time highlights the importance of virus-host interactions to pathogenesis. However, in immuno-compromised hosts and newborns, primary CMV infections could cause severe disease (Singzer and Jahm, 1996). Interestingly, MCMV strains (e.g. Smith strain) that are adapted to tissue culture are unable to cause lytic or latent infections in immunocompetent or

immunocompromised hosts. In contrast, MCMV isolated from saliavary glands of mice infected with tissue-culture adapted virus was highly virulent, but the virulence was lost upon a single passage through tissue culture (Krmpotić *et al.,* 2003).

Human CMV could infect several organs and a wide variety of cell types *in vivo* and *in vitro* (Myerson *et al.,* 1984; Toorkey and Carrigan, 1989). In cell culture systems, HCMV was able to replicate in many different human cell lines, and could infect cells of non-human origin although viral DNA replication did not take place (Heieren *et al.,* 1988; Lafemina and Hayward, 1988; Tumilowicz, 1990; Ibanez *et al.,* 1991; Waldman *et al.,* 1991). Epithelial, endothelial, and fibroblast cells were the primary sites of HCMV infection *in vivo,* and these cells were widely distributed throughout the body (Sinzger *et al.,* 1995). Viral replication and antigen production had been observed in epithelial cells of the gastrointestinal tract, genitourinary tract, and respiratory tract (Escudero *et al.,* 1992; Sinzger *et al.,* 1995). It has been suggested that these epithelial cells may be sources of viral transmission as well as infection (Sinzger and Jahn, 1996). Human CMV is transmitted in the population by direct contact through bodily fluids such as saliva and urine (Mocarski *et al.,* 2007). In addition, transmission from mother to child could occur during birth, via breast milk, or through the placenta during pregnancy (Reynolds *et al.,* 1973; Stagno *et al.,* 1980; Dworsky *et al.,* 1983). While pathogenesis of MCMV and HCMV is very similar, there is one area in which the two viruses differ, as MCMV could not be transmitted across the placenta although the other methods of transmission from mother to child are still applicable (Johnson, 1969; Li and Tsutsui, 2000). Infection of neonatal mice involved a longer period of lytic infection prior to the establishment of latency when compared to adult mice (Reddehase *et al.,* 1994). In addition, MCMV infection in neonatal mice could cause damage to the liver, brain, lymphatic system, skin, and coronary arteries, as well as inflammation of the heart and skeletal muscles (Fitzgerald *et al.,* 1990; Krmpotić *et al.,* 2003).

Human CMV could infect endothelial cells of capillaries and venules in several organs throughout the body (Wu *et al.,* 1992; Sinzger *et al.,* 1993; Sinzger *et al.,* 1995). Endothelial cells had been observed to enter the bloodstream and in this way, could help to disseminate the virus (Grefte *et al.,* 1993; Perecivalle *et al.,* 1993). The third main cell type that is a target for HCMV infection is the fibroblast, which is located in connective tissues involved in the generation of the extracellular matrix. Due to the presence of connective tissues in several

organs, infection of fibroblasts could take place in the placenta, lung, and intestines (Sinzger *et al.,* 1993; Sinzger *et al.,* 1995).

Leukocytes or white blood cells such as macrophages, monocytes, granulocytes, and lymphocytes were also susceptible to HCMV infection (Taylor-Wiedeman *et al.,* 1991; Gerna *et al.,* 1992). In fact, HCMV infection caused by blood transfusions could be prevented by the removal of white blood cells from donors latently infected with the virus (Lang *et al.,* 1977; Winston *et al.,* 1980). However, evidence suggested that leukocytes were merely vehicles for viral transmission rather than sites of viral replication. Viral DNA could be detected in mononuclear cells such as monocytes and their differentiated cell types such as macrophages and lymphocytes, as well as polymorphonuclear phagocytes, such as neutrophils (Rinaldo *et al.,* 1977; Turtinen *et al.,* 1987; Saltzman *et al.,* 1988; Dankner *et al.,* 1990). However, some viral genes involved in replication were not expressed in these leukocytes (Turtinen *et al.,* 1987; Grundy *et al.,* 1988).

Human CMV could use the bloodstream to spread itself from the initial site of infection throughout the infected organism (Sinzger and Jahn, 1996). Although the way the virus is transmitted from the site of entry into the bloodstream is unknown, studies on MCMV indicated that the virus might be transported via phagocytes to other organs (Stoddart *et al.,* 1994). In addition, monocytes might be involved in the transmission of CMV since they became permissive for the virus once they differentiated into macrophages (Ibanez *et al.,* 1991).

Human CMV infection in organs could cause disease directly or indirectly. Evidence of direct viral involvement in disease was implicated by the presence of high levels of viral replication at the site of infection, as well as reversal of symptoms with antiviral therapy (Dieterich *et al.,* 1988; Sinzger *et al.,* 1995). Human CMV is believed to cause direct clinical consequences in the retina and the gastrointestinal tract, as well as pneumonitis, the inflammation of lung tissues (Vasudevan *et al.,* 1990; Heinemann, 1992; Sinzger *et al.,* 1995). In certain cases, such as pneumonitis in transplant recipients, the symptoms were not resolved through antiviral therapy, and there were no high levels of viral replication in lung tissues (Shepp *et al.,* 1985; Zaia *et al.,* 1986). Therefore it is uncertain at this time whether HCMV-induced host immune response led to pneumonitis. Human CMV infection is believed to

indirectly cause liver dysfunction during liver transplantation and alternative blood cell formation during hemotopoiesis (Arnold *et al.,* 1992; Theise *et al.,* 1993).

1.3 Studying viral genetics via the construction of recombinant viruses

Determining the roles of various viral genes has many important implications towards different areas of research in virology. Once the role of a gene is known, it is possible to apply this knowledge to study the pathogenesis of the virus, to improve gene therapy protocols, and to help develop viral vaccines and treatments for viral diseases (Adler *et al.,* 2003). Disrupting the function of a gene can help elucidate the importance of the gene during the viral life cycle. In the past, mutations in genes were either induced chemically, or isolated from naturally occurring mutants. The functions of these viral genes were often elucidated through *in vitro* experiments away from the context of viral infection. Although this approach will yield some useful information about the viral gene in question, oftentimes, viral infection is necessary in order to observe the full function of the gene. When genes are studied individually, they often behave differently than they do during viral infection. For instance, the products of genes may need to interact with each other, or be modified in some way by other viral factors in order to carry out their functions during *in vitro* or *in vivo* infections. As an example, the CMV helicase/primase complex, which is essential for viral DNA replication, is formed by interactions between three viral gene products (*UL70*/*M70*, *UL105*/*M105* and *UL102*/*M102*).

Therefore, the manipulation of viral genomes is an important tool that can be used to introduce mutations or alterations into the viral genome. The biological effects of the recombinant virus containing the desired genomic changes can then be observed, either through *in vitro* or *in vivo* studies. Small viral genomes such as those of Adenoviruses or Retroviruses can be easily manipulated using a plasmid-based approach. One of the classical methods used to generate recombinant adenoviral genomes was developed by Stow (1981). In this method, a plasmid containing mutated DNA sequences in the E1 region of adenovirus was ligated *in vitro* with a restriction fragment representing the rest of the adenovirus genome. The ligated DNA was then transfected into a cell line that was able to compensate for the missing E1 gene products, and recombinant virus was produced. Thus, the use of plasmid DNA enabled the easy manipulation of the adenoviral genome.

Adenoviral genomes can also be manipulated through homologous recombination *in vivo*. If a plasmid vector containing the adenoviral genome with an insert in the E1 region was

co-transfected with another plasmid containing a wild-type or altered E1 region, homologous recombination *in vivo* occurred between the two plasmids (McGrory *et al.,* 1988). Although adenoviruses could package sequences into virions that are 5% larger than the viral genome, the insert in the E1 region made the parental virus larger than the packaging limit (McGrory *et al.,* 1988). Therefore, recombinant adenoviruses containing the desired alterations in the E1 region were preferentially packaged and rescued. However, this method required more extensive plaque purification compared to other methods in order to separate the recombinant virus from wild-type (Stow, 1981; McGrory *et al.,* 1988).

Due to the low efficiency of recombination *in vivo* in a mammalian system, methods have been developed that enable the recombination to take place prior to transfection of viral DNA into mammalian cells (Ketner *et al*., 1994; Chartier *et al*., 1996; Crouzet *et al*., 1997). The first method involved the construction of a YAC (yeast artificial chromosome) containing the adenovirus genome in yeast spheroplasts (Ketner *et al.,* 1994). Homologous recombination could occur in yeast between the adenovirus YAC and linearized plasmid DNA containing mutated adenoviral genes, followed by the transfection of YAC DNA into cells to produce infectious virus (Ketner *et al*., 1994). Advantages of the YAC method over the *in vivo* homologous recombination method are that mutations can be placed anywhere in the genome regardless of restriction site availability, and homologous recombination in yeast is very efficient. Moreover, since the recombinant YAC can be characterized before transfection, the recovered recombinant virus does not need to be plaque-purified as extensively.

In a similar manner, recombinant adenoviral genomes could be constructed via homologous recombination in *E. coli* prior to transfection of mammalian cells with viral DNA to produce viable virus. Recombination occurred in a recombination-competent ($RecA^+$) bacterial strain between a plasmid vector containing the adenoviral genome and DNA containing the desired mutated sequence flanked by homologous viral sequences. The recombinant adenoviral DNA was then transformed into a $RecA^-$ bacterial strain in order to generate higher yields of DNA with increased stability. For example, the adenoviral genome on the plasmid used by Chartier *et al.* (1996) was flanked by *Pac*I sites (absent from the genome) so that it could be excised by digestion with *Pac*I prior to transfection into mammalian cells to produce recombinant virus. The advantages of homologous recombination in *E. coli* are that any region of the viral genome can be altered, and the frequency of isolating

correct recombinants is high. Thus, transfection of the resulting recombinant viral genome into mammalian cells would allow the rescue of the expected recombinant virus with high probability (Chartier *et al*., 1996). Although the above method was initially used to manipulate the E1 region of the adenoviral genome, a subsequent method described the mutagenesis of any region of the genome (Crouzet *et al*., 1997). However, since the latter method required a two-step cloning strategy and resolution of cointegrates containing both the adenoviral genome and a plasmid with the desired mutation, viral revertants would be produced, and a screening process was necessary to distinguish between wild-type and recombinant adenoviral genomes.

Another method for manipulating viral genomes involved the incorporation of viral DNA fragments into a P1 cloning vector that was packaged into bacteriophage P1 *in vitro* (Sternberg, 1990). Bacteriophages were then injected into *E. coli* and the DNA was circularized by the action of the P1 *loxP* recombination sites present on the vector and the P1 *Cre* recombinase present in bacterial cells (Sternberg, 1990). However, the bacteriophage P1 system was limited by the maximum insert size of 100 kbp dictated by the *in vitro* packaging system (Sternberg, 1990; Ioannou *et al.,* 1994). A cloning system based on both the BAC (bacterial artificial chromosome) and bacteriophage P1 methods called P1-derived artificial chromosomes could accommodate inserts between 100-300 kbp (Ioannou *et al*., 1994). In this system, a vector containing a SacB gene was used for selection of recombinant DNA clones derived from bacteriophage P1, and the DNA was transformed into *E. coli* by electroporation (Ioannou *et al*., 1994).

1.3.1 Methods for constructing recombinant herpesvirus genomes

Herpesviral genomes are large and cannot be manipulated using plasmids. There are two general strategies for introducing alterations into herpesviral genomes via homologous recombination. The first involved *in vivo* homologous recombination that could be accomplished either by insertional mutagenesis or co-transfection of overlapping cosmid clones. The second used a BAC-based system so that homologous recombination could take place in bacterial cells prior to the rescue of recombinant virus in cell culture. The BAC method has significant advantages over the *in vivo* methods as mutations can be introduced into any viral gene, including essential genes, since construction of the recombinant viral genome takes place *in vitro*, and is not dependent on the rescue of viable virus *in vivo*. Moreover, the viral genome can be characterized before time is spent isolating recombinant

virus (Messerle *et al.*, 1997). In addition, very large DNA fragments (over 300 kbp) can be incorporated into the BAC vector. Thus even the large MCMV or HCMV genomes could be, and have been, cloned as a BAC (Messerle *et al.*, 1997; Brune *et al.*, 2000).

As mentioned earlier, YACs provided an alternative method of constructing recombinant viral genomes. Although a YAC cloning vector could incorporate much larger DNA sequences (up to 2 Mbp) than BACs, there are several reasons the BAC system is preferable over YACs (Ramsay, 1994; Brune *et al.*, 2000). It is commonly known that more than one YAC could be transformed into a single yeast cell, perhaps because of the high DNA concentrations used to circumvent the low transformation efficiency of yeast (Schalkywk *et al.*, 1995). On the other hand, BACs could be transformed into *E.coli* at high efficiencies by electroporation at a copy number of 1-2 per cell (Shizuya *et al.*, 1992). Therefore, a significant percentage of isolated YAC clones contained chimeras produced by recombination between the multiple YACs transformed into the same cell, whereas very few BAC clones were produced as chimeric molecules (Shizuya *et al.*, 1992). Whereas YACs were maintained as linear molecules, BACs were maintained as circular plasmids that facilitated the handling of the DNA, and decreased the possibility of shearing the large DNA molecules (Shizuya *et al.*, 1992). Bacterial artificial chromosomes characterized by restriction analysis were found to be much more stable than YACs, which often suffered deletions within the inserted sequences (Shizuya *et al.*, 1992; Monaco and Larin, 1994). The use of recombination-defective strains could decrease the possibility of unexpected genome alterations. However, this also resulted in decreased transformation efficiency (Schalkwyk, 1995). In addition, there was great difficulty in purifying YAC DNA away from yeast chromosomal DNA, which was not the case for BACs (Schalkwyk *et al.*, 1995).

1.3.1.1 *In vivo* complementation

Since homologous recombination is a rare event in mammalian cells, strategies to select for recombinant viruses need to be developed. Often the region of the viral genome one is interested in investigating contains no readily available selectable markers (Post and Roizman, 1981). Therefore, markers such as beta-galactosidase or beta-glucuronidase could be inserted into the genome at specific sites as a way to either inactivate viral genes or isolate viral recombinants containing the desired alterations (Jones *et al.*, 1991; Takekoshi *et al.*, 1991). However, the selection of recombinants using these 2 markers is carried out after virus

is produced, in contrast to the use of *neo*, thymidine kinase (TK) or *E. coli gpt* genes as selection markers, which conferred growth advantages to the desired recombinant viruses and increased the probability of success (Post and Roizman, 1981; Wolff *et al.*, 1993; Greaves *et al.*, 1995). Both TK and *gpt* could also be selected against, thereby providing the investigator with the option to select for the absence of these markers in recombinant viruses. To completely eliminate the necessity of incorporating foreign sequences such as marker genes into the viral genome, a method was developed where recombinant viruses could be produced through co-transfection of cloned overlapping DNA fragments encompassing the entire viral genome into susceptible cells (van Zijl *et al.*, 1988). Homologous recombination *in vivo* resulted in the reconstitution of full-length viral genomes containing the desired alterations and the rescue of viable recombinant viruses.

1.3.1.1.1 Insertional mutagenesis

Insertional mutagenesis involves the insertion of a sequence into a gene to render it nonfunctional. This is often accomplished by co-transfection of viral DNA with a plasmid containing a selection marker flanked by homologous viral sequences to target a region of interest on the viral genome. Homologous recombination *in vivo* results in the replacement of the wild-type gene with a nonfunctional mutant.

The insertion of beta-galactosidase into a viral gene enables the identification of viral recombinants containing the interrupted gene through a colorimetric assay. Briefly, cells infected with virus recovered from the transfection were overlaid with agarose containing X-gal (5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside), and recombinants that had incorporated the beta-galactosidase marker into their genomes could be isolated from blue plaques (Takekoshi *et al.*, 1991). This method has been used to identify several viral genes that were not required for growth in cell culture, including HCMV *UL18*, *UL16*, and MCMV *ie2* (Manning and Mocarski, 1988; Browne *et al.*, 1992; Kaye *et al.*, 1992). Recombinant viruses containing genes disrupted by beta-galactosidase insertion were stable, with a low rate of reversion. However, the disadvantage of this method is that when an essential gene has been disrupted this way, viable virus cannot be isolated (Manning and Mocarski, 1988).

A similar technique was developed using the beta-glucuronidase gene and resulted in the production of stable recombinants (Jones *et al.*, 1991). Initial concerns that beta-glucuronidase could not be used as a marker gene due to the presence of high levels of

endogenous beta-glucuronidase were unfounded, and this method was used to demonstrate that the HCMV *US10* and *US11* genes were not required for *in vitro* replication (Jones *et al*., 1991). However, the frequency of recombinant production was only 5%, compared to a frequency of 10% when beta-galactosidase was used (Jones *et al*., 1991; Takekoshi *et al*., 1991). It was suggested that the smaller size of the beta-glucuronidase gene (1.9 kb) compared to beta-galactosidase (3.1 kb) might decrease the probability of unexpected deletions in the recombinant viral genome if the packaging limits was exceeded (Jones *et al*., 1991).

Although the expression of beta-galactosidase or beta-glucuronidase allowed for the identification of viral recombinants, there was no selection pressure when virus recovered after transfection was allowed to spread. Since propagation of wild-type virus alongside the desired recombinants could not be prevented during the isolation process, several cycles of plaque purification were required to minimize the contamination of recombinant virus stocks with wild-type virus. In addition, it is possible that even following several cycles of plaque purification and selection, reversion to a wild-type phenotype may occur, as no selection for the recombinant phenotype is applied during the propagation of the recombinant virus (Wolff *et al*., 1993).

A method of creating recombinant HCMV by insertional mutagenesis which does involve selection during viral growth utilizes the bacterial *neo* gene. This gene encodes a neomycin/kanamycin phosphotransferase and could be inserted into the viral genome via homologous recombination (Wolff *et al*., 1993). After transfection into mammalian cells, the progeny virus was used to infect cells grown in the presence of G418, which selected for the presence of the *neo* gene (Wolff *et al*., 1993). The virus was further purified through additional passages in media containing G418. This method has been used to create HCMV mutants, and it was observed that following the initial selection, 20% of the virus pool represented recombinant virus (Wolff *et al*., 1993). After 3 passages in the presence of G418, all viruses isolated through plaque purification had incorporated the *neo* gene into their genomes. The *neo* selection method is relatively quick compared to the colorimetric methods, and selects for stable recombinants during virus propagation (Wolff *et al*., 1993). However, selection for *neo* resulted in the death of uninfected cells (Greaves *et al*., 1995).

Insertional mutagenesis using TK as a selectable marker has one important advantage over the *neo* gene, as the investigator can choose to select for, or against TK (Post and

Roizman, 1981). To introduce specific mutations into the viral genome, two selection steps were required. The first step involved the insertion of TK into the target gene through homologous recombination between the viral genome and a plasmid containing the TK gene flanked by viral sequences homologous to the target gene. The TK-expressing virus was selected from the pool of recovered viruses by plating these viruses on TK^- cells in media containing hypoxanthine, thymidine and methotrexate. In the second step, cells were co-transfected with DNA from the TK-expressing virus produced in the first step and a plasmid containing the mutated or wild-type target gene. Selection against TK was applied by propagating the virus in the presence of thymidine arabinoside, which was toxic to cells expressing thymidine kinase. Thus recombinant virus incorporating the desired mutation via homologous recombination, but lacking TK would be recovered preferentially (Post and Roizman, 1981).

Although this is a useful method for the construction of recombinant viruses, there are disadvantages to this procedure. Most notably, insertional mutagenesis using the TK gene requires the availability of mutant cell lines deficient in TK (Mulligan and Berg, 1981). In fact, the limited host range of HCMV and the lack of permissive TK^- human cell lines is the reason the TK selection procedure has not been adapted for CMV mutagenesis, and the development of alternative selection schemes are required (Greaves *et al.*, 1995). In addition, insertional mutagenesis using the TK gene can only be applied to viral genomes that lack the TK gene, further limiting the utility of this approach.

The *E. coli gpt* gene, which encodes a xanthine-guanine phosphoribosyltransferase, is another selectable marker that allows for both positive and negative selection. During positive selection, cells were infected with wild-type virus and then transfected with a plasmid containing the *gpt* gene flanked by sequences homologous to the target gene, resulting in the insertion of the *gpt* gene into the viral genome via homologous recombination. Selection for recombinants expressing *gpt* was carried out by propagating the virus in media containing mycophenolic acid (an inhibitor of *de novo* purine synthesis), xanthine, and adenine (Mulligan and Berg, 1981). If specific alterations of the target gene were desired, an additional step involving selection against *gpt* was performed. Cells deficient in mammalian hypoxanthine phosphoribosyltransferase were transfected with a plasmid containing the target gene with the desired deletion or mutation, and infected with recombinant virus containing the *gpt* insertion.

Recovered virus was propagated in media containing a toxic guanine analog (6-thioguanine), which allowed selection against viruses expressing *gpt*. Therefore, only viruses that had undergone homologous recombination and incorporated the desired alterations would be preferentially recovered.

The *gpt* gene can be used as a selection marker in many different cell lines and has widespread utility as a way to construct recombinant viruses. Selection utilizing *E. coli gpt* is efficient enough that cells can be infected by virus, and then transfected with plasmid DNA containing the marker genes flanked by homologous viral sequences, or vice versa (Vieira *et al*., 1994). Cell death did not occur as a result of *gpt* selection and thus, in contrast to selection by G418 resistance, recombinant virus could be isolated by plaque purification (Greaves *et al*., 1995).

As an example, Greaves *et al*. (1995) demonstrated that the *E. coli gpt* selection technique could be used to modify genes either through insertion of the *gpt* gene, rendering the viral gene non-functional, or by deletion of the gene. The *gpt* gene was inserted into HCMV *US*3, and deletion mutants lacking regions from the *IRS*1 and *US3-US5* genes were generated (Greaves *et al*., 1995). Some resistance towards selection with 6-thioguanine was observed, resulting in the growth of *gpt* containing viruses. The authors determined that these revertant viruses had acquired mutations or deletions in the *gpt* gene, thereby allowing their propagation in the presence of the toxic compound (Greaves *et al*., 1995). However, they were able to solve this problem by hybridization with a *gpt* probe to screen for recombinant viruses that did not contain the *gpt* gene.

Although the techniques of insertional mutagenesis utilizing marker genes are useful in studying functions of various herpesvirus genes, particularly HSV-1 genes, there are several key limitations to this approach. First, virus generated through homologous recombination *in vivo* required selection against wild-type virus (Wagner *et al*., 2002), and the selection of virus mutants with a growth disadvantage against their wild-type parents could be inefficient (McGregor and Schleiss, 2001). Second, insertional mutagenesis could not be used for constructing recombinant viruses containing lethal mutations in an essential gene. Lastly, it has been noted that the presence of the marker gene in the viral genome may in fact have an impact on the phenotype of the recombinant virus and the ability to recover the virus mutant (Kemble *et al*., 1996; Ehsani *et al*., 2000).

1.3.1.1.2 Construction of recombinant virus from cloned overlapping fragments

There are two methods of producing recombinant virus through cloned overlapping viral DNA fragments. One is a purely cosmid-based system, while the other uses both cosmids and plasmids. The cosmid-based system involved the generation of a set of cosmids containing overlapping subgenomic fragments that span the entire viral genome. When these cosmids were co-transfected into cells, viable virus could be recovered via homologous recombination *in vivo* between overlapping regions of the viral genome. The second system worked essentially in the same way, except that one of the cosmid clones was replaced with a set of 2 plasmids, which overlapped with each other and contained the desired mutation. The use of plasmids made it easier to manipulate viral DNA fragments and introduce point mutations into large herpesvirus genomes.

The cosmid co-transfection approach did not involve the insertion of marker genes into the viral genome (McGregor and Schleiss, 2001). Unlike insertional mutagenesis, which usually allowed one gene to be altered at a time, the cosmid-based system could be used to introduce multiple alterations to the viral genome in one experiment (van Zijl *et al.*, 1988). In this system, the viral genome was re-constituted solely via homologous recombination *in vivo* between the available cosmids, so any virus produced should contain all the desired mutations, thereby minimizing the number of steps required for recombinant virus isolation (Cohen and Seidel, 1993).

However, this protocol still has several disadvantages. Finding unique restriction sites in the large cosmids to use for genetic manipulation is difficult, and reconstitution of the viral genome in cell culture required several recombination events that could lead to unexpected genome alterations (Brune *et al.*, 2000; McGregor and Schleiss, 2001). Furthermore, maintaining stable cosmid clones with large viral DNA inserts containing multiple repeat elements might be problematic, since these sequences were known to be susceptible to rearrangements or deletions (Kim *et al.*, 1992). In addition, construction of revertant viruses to ascertain that the phenotype observed in the recombinant virus is due to the expected mutation is laborious (Brune *et al.*, 2000). The determination of whether the correct recombinant had been created could only be performed after the virus was isolated and grown up in cell culture (Messerle *et al.*, 1997). Several examples of the utility of this approach to herpesvirus mutagenesis are described below.

Overlapping cosmid and plasmid clones were used to generate recombinant HSV-1 containing point mutations in *UL26* (Register and Shafer, 1996). These point mutations could be reverted back to wild-type sequences, and the revertant viruses were shown to be identical to wild-type HSV-1. This demonstrated that no unexpected second-site mutations occurred, and the observed phenotype of the recombinant HSV-1 was indeed due to the point mutation introduced (Register and Shafer, 1996). Furthermore, work done by Ehsani *et al*. (2000) demonstrated that the *in vitro* and *in vivo* growth characteristics of MCMV reconstituted from a set of 7 cosmids were comparable to those of wild-type MCMV. There was no incorporation of cosmid vector sequences or the presence of unwanted secondary mutations or genome rearrangements in the reconstituted MCMV genome (Ehsani *et al*., 2000).

A set of 8 overlapping cosmid clones was used to regenerate the 230 kbp HCMV genome (Kemble *et al*., 1996). Furthermore, this method was used to construct a chimeric virus in which a 13 kbp DNA fragment from a virulent strain of HCMV (Toledo) replaced the corresponding region of the avirulent Towne viral genome. A change in plaque morphology was observed as a result of this genome alteration (Kemble *et al*., 1996). Cihlar *et al*. (1998) used a set of 7 cosmid clones and 2 overlapping plasmids to generate recombinant HCMV, and observed that this approach was just as efficient as the use of 8 cosmid clones even though the regions of overlap were shorter. A recombinant virus containing more than one mutation in *UL54* was constructed and the usefulness of the cosmid co-transfection technique was demonstrated (Cihlar *et al*., 1998).

1.3.3.2 Construction of recombinant viral genomes using bacterial artificial chromosomes

The BAC cloning vector developed by Shizuya *et al* (1992) is based on the F (fertility) factor plasmid from *E. coli*. This vector contains the F factor *oriS* and *repE* genes, which are involved in monitoring replication, and *par*A and *par*B genes, which have the function of ensuring a low copy number of 1-2 per cell (Shizuya *et al*., 1992). In addition, the BAC vector contains a chloramphenicol resistance gene to allow for antibiotic selection (Shizuya *et al*., 1992).

Three strategies for constructing recombinants with BAC vectors are described below: the shuttle plasmid method, the recE/recT method, and a method involving a defective lambda prophage. All of these methods take advantage of the efficiency of homologous recombination

in bacteria, which is much higher than that of similar reactions in mammalian cells *in vivo*. The general steps in constructing recombinant herpesviruses via BAC vectors include the insertion of BAC vector sequences into the viral genome by *in vivo* homologous recombination, mutagenesis of the BAC through homologous recombination in *E. coli*, and reconstitution of viable virus in mammalian cells (Messerle *et al.,* 1997). The DH10B strain of *E. coli* is generally regarded as the preferred bacterial strain for stable maintenance of BACs. This bacterial strain has high transformation efficiency and a *recA* mutation that renders it deficient for recombination. In addition, it contains mutations that allow methylated DNA sequences and large plasmids to be stably maintained (Ioannou *et al*., 1994).

Homologous recombination with a shuttle plasmid is a two-step process where a cointegrate was initially formed between a plasmid containing the mutated gene and a BAC containing the viral genome. The shuttle plasmid contained a temperature sensitive origin of replication, and its replication could be inhibited by increasing the temperature from 30°C to 42°C (Wagner *et al.,* 2002). Subsequently, the cointegrate was resolved, producing either wild-type virus or a mutated viral genome (Wagner *et al*., 2002). The recombination reaction took place in an *E. coli* strain containing a temperature-sensitive *recA* gene, thereby allowing the reaction to be tightly controlled (Brune *et al*., 2000). The first herpesvirus genome to be cloned as an infectious BAC was the 230 kbp MCMV genome (Messerle *et al*., 1997). The authors constructed a *mie1* mutant and demonstrated that the BACs remained stable after several weeks of propagation in bacteria (Messerle *et al*., 1997).

Although Messerle *et al*. (1997) demonstrated that BAC vectors could be successfully used for the construction of MCMV mutants, these viruses contained BAC vector sequences as well as a deletion in a region of the genome nonessential for viral replication *in vitro*. Both alterations to the viral genome could affect the *in vivo* replication of MCMV. In fact, Wagner *et al*. (1999) investigated the *in vivo* growth properties of wild-type MCMV and the recombinant MCMV mutant produced by Messerle *et al*. (1997), and they found that the 2 viruses showed significant differences during viral infection and the MCMV mutant was much less virulent (Wagner *et al*., 1999).

Wagner *et al* (1999) used the strategy illustrated in Figure 1.2 to re-insert the deleted MCMV sequences into the viral genome already present in the BAC vector via homologous recombination, resulting in a new MCMV BAC where the BAC vector sequences were

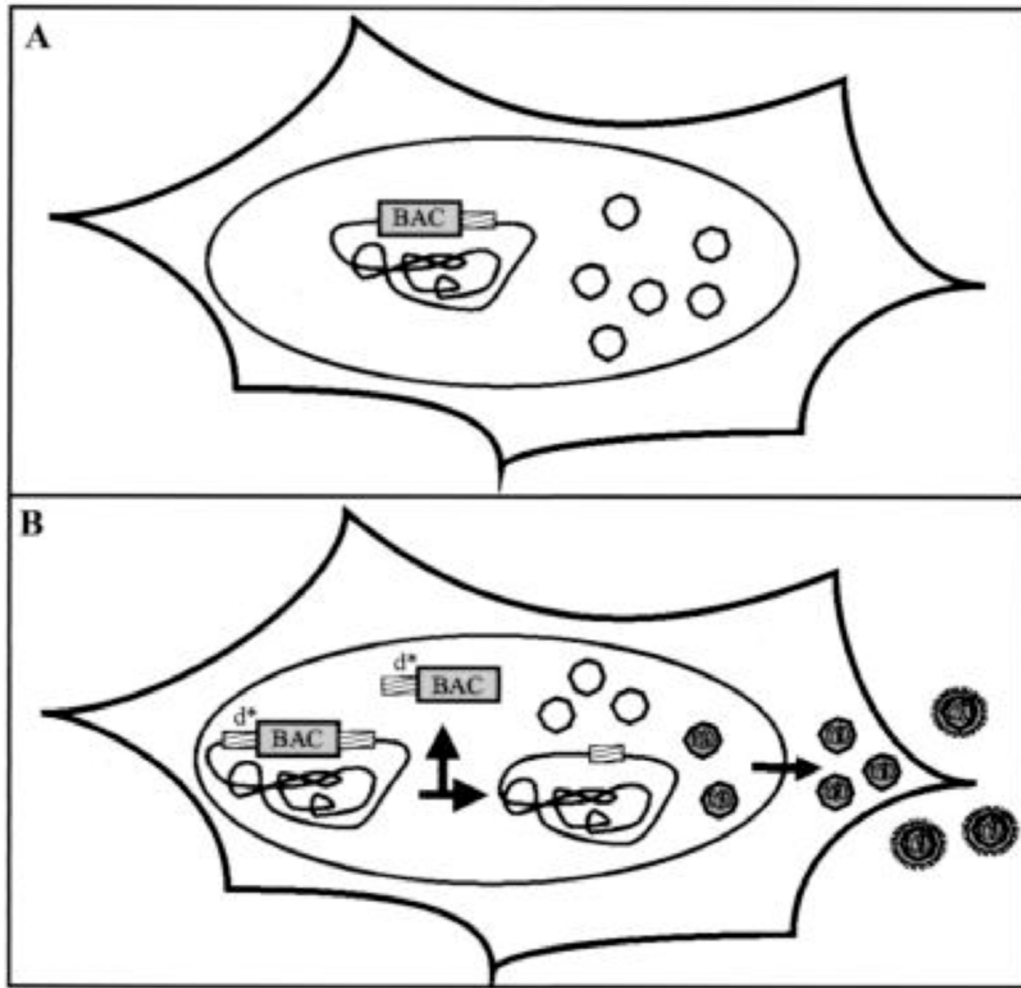

Figure 1.2: Excision of the BAC vector from the viral genome in eukaryotic cells.

A: The MCMV genome containing the BAC vector sequences is too large to be efficiently packaged into virions. **B:** A sequence (d*) is inserted along with the BAC vector into the MCMV genome, resulting in the BAC vector sequence being flanked with duplicate viral sequences (represented with Hatched boxes). Homologous recombination can then occur as a result of the duplicated sequence, resulting in the removal of the BAC vector from the MCMV genome. The MCMV genomes lacking the BAC vector will have the correct length for efficient packaging into virions, resulting in the production of wild-type virus. (This figure is borrowed from Wagner *et al*., 1999)

flanked by duplicate viral sequences. The presence of these flanking sequences enabled the removal of vector sequences from the MCMV genome by homologous recombination *in vivo*. The smaller viral genomes resulting from the loss of vector sequences were preferentially packaged over several passages in tissue culture, and wild-type MCMV was recovered (Wagner *et al*., 1999). The authors were able to distinguish between wild-type MCMV (Smith strain) and the reconstituted virus because the re-inserted sequence was derived from the K181 strain of MCMV, and contained a point mutation not present in the Smith strain of MCMV. Wagner *et al*. (1999) then compared the growth properties of wild-type and recombinant MCMV viruses in mice, and demonstrated that removal of the BAC vector sequences coupled with the re-insertion of the missing MCMV sequences resulted in disease progression comparable to that of wild-type MCMV (Wagner *et al*., 1999).

An additional example highlighted the importance of carefully selecting the site of BAC vector insertion into the viral genome. During recombinant virus construction, F plasmid vector sequences were inserted into the gG locus of the pseudorabies virus genome, a region believed to be nonessential for both *in vitro* and *in vivo* infection. Although the behavior of wild-type and recombinant pseudorabies viruses were similar during *in vivo* infection, the F plasmid sequences were spontaneously deleted during the DNA transfection process. Interestingly, insertion of F plasmid sequences at another locus did not result in spontaneous deletion, demonstrating that the stability of the recombinant viral genome might depend on the site of insertion of vector sequences (Smith and Enquist, 1999).

Brune *et al*. (1999) developed an alternative approach to targeted mutagenesis by mutagenizing the MCMV genome via random transposon insertion. This was accomplished by growing a *recA*$^-$ strain of *E. coli* harboring a MCMV BAC and a temperature-sensitive transposon donor plasmid at 30°C under antibiotic selection for both components. Bacteria were spread on plates containing antibiotics that selected for BACs with transposon insertions and grown at 42°C so that replication of the donor plasmid was inhibited (Brune *et al*., 1999). The BAC DNA isolated from the resultant bacterial clones was analyzed by restriction enzyme digestion and direct sequencing. It was observed that 90% of the clones contained transposon insertions. The viability of these transposon mutants was evaluated in cell culture. If infectious virus was recovered after transfection, the insertion should be in a gene nonessential for MCMV replication in cell culture. In contrast, if an essential gene were inactivated by

transposon insertion, viable virus would be recovered only by co-transfection with DNA containing a functional copy of the disrupted gene. In addition, revertant viruses produced by homologous recombination with wild-type MCMV sequences in bacteria restored the parental phenotype of these mutants. The genomes of these transposon mutants were stable, and this procedure provided a useful way for quickly identifying essential and non-essential genes in MCMV (Brune *et al*., 1999).

Another way of manipulating viral genomes in BACs utilized linear DNA fragments instead of plasmids to introduce a specific mutation into the viral genome via homologous recombination. The recombination functions were mediated either by the *E. coli recE* and *recT* genes, the lambda *red*α and *red*β genes (Zhang *et al*., 1998; Muyrers *et al*., 1999), or a defective lambda prophage (Yu *et al*., 2000; Lee *et al*., 2001b). The *recE*/*recT* or *red*α/*red*β recombination genes were located on a plasmid that was transferred into a *recA*$^-$ *recBC*$^+$ strain of *E. coli.* The bacteria also harbored the target DNA sequence on a BAC. Linear DNA containing the mutated gene of interest and an antibiotic resistance gene was then transformed into *E. coli* cells, where a successful homologous recombination reaction would result in the insertion of the desired mutation into the viral genome. Typically, homology arms of 1-3 kilobases were required on either side of the gene of interest for the homologous recombination reaction to occur. However, the recombination system described here required only short homology regions of 25-50 nucleotides at both ends of the linear DNA for the reaction to proceed efficiently (Zhang *et al*., 1998; Muyrers *et al*., 1999; Wagner *et al*., 2002).

In order for *recE*/*recT* or *red*α/*red*β mediated recombination to occur in *recBC*$^+$ strains of *E. coli*, the RecBCD exonuclease must be inhibited by Redγ (*gam*) to prevent premature degradation of the linear DNA fragment carrying the mutated gene. Therefore, plasmids harboring these genes also contained *gam* as well as an arabinose inducible promoter for *recE* or *red*α. In this way, the recombination reaction could be initiated by the addition of a chemical inducer (Zhang *et al.,* 1998; Muyrers *et al*., 1999).

A major advantage of this method is that the recombination reaction can be carried out in the *recA*$^-$ bacterial strains used to maintain the BACs (Messerle *et al*., 1997; Muyrers *et al*., 1999), since the recombination reaction is independent of *recA* function. In addition, the *recE*/*recT* or *red*α/*red*β recombination system did not require the presence of convenient restriction sites to facilitate the cloning or excision of the target DNA sequence. Instead, linear

DNA fragments used to introduce mutated sequences into the viral genome could be generated conveniently by PCR (polymerase chain reaction) (Zhang *et al.*, 1998; Muyrers *et al.*, 1999; Wagner *et al.*, 2002; Adler *et al.*, 2003).

One of the disadvantages of the *recE*/*recT* or *red*α/*red*β mediated recombination system is the necessity of removing the antibiotic resistance gene that was inserted into the viral genome via an unavoidable double crossover event. Removal of these sequences by site-specific recombinases could be facilitated by flanking the antibiotic resistance gene with FRT (FLP target recognition) or *loxP* sites (Zhang *et al.*, 1998). However, this process inevitably left a "scar" (short foreign DNA sequences) at the site of recombination that might affect the biological properties of the recombinant virus. Another potential problem concerned the stability of the BACs that are normally maintained in *recA*$^-$ bacteria. However, plasmid expression systems such as the ones described here are often leaky. In addition, herpesvirus genomes are known to contain multiple direct repeat sequences. Thus, even the presence of low levels of these powerful recombination genes in bacteria has the potential to cause BAC instability (Brune *et al.*, 2000; Lee *et al.*, 2001b).

As an alternative to maintaining a plasmid with the required recombination genes in bacteria, an *E. coli* strain that harbored a lambda prophage was constructed so that expression of the recombination genes *gam*, *exo*, and *bet* could be controlled by a temperature-sensitive cI-repressor (Yu *et al.*, 2000; Lee *et al.*, 2001b). In this system, expression of the recombination genes was briefly induced at 42°C, and the temperature shifted back down to 32°C. This was sufficient to mediate efficient homologous recombination between the target and BAC DNAs, requiring only short homology arms of ~40 bases at both ends of the target DNA fragment. To create the DY380 strain of *E. coli* for this purpose, Lee *et al.* (2001b) transferred a lambda prophage containing a tetracycline resistance marker (*tet*^{R}) into DH10B bacteria and demonstrated that this prophage-based system functioned with the same or better efficiency as the plasmid-based *recE*/*recT* system. Furthermore, the DY380 bacteria could be modified to facilitate the removal of selectable markers from BAC constructs via site-specific recombination at *FRT* or *lox*P sites. This was accomplished by replacing the *tet*^{R} gene on the prophage with the *araC* regulatory gene, thereby allowing precise control over the expression of arabinose-inducible genes *flpe* and *cre*.

1.4 Previous work performed in our laboratory

Our laboratory was involved in studying murine cytomegalovirus as an animal model for cytomegalovirus infections since its establishment in 1983. Our goal is to understand the molecular mechanism underlying the host specificity of cytomegaloviruses as well as develop anti-viral therapies that can be extended to the treatment and prevention of HCMV infections. To this end, we have characterized a panel of monoclonal and polyclonal antibodies specific for a number of viral gene products (Loh *et al*., 1988; Loh and Qualtiere, 1988; Loh, 1989; Loh, 1991; Loh *et al*., 1991; Ph.D. thesis: Q. Wu, 1998; Scalzo *et al*., 2004). Using these reagents, we have identified MCMV gB, gH, and gp24 (gpM73.5) as targets for neutralizing monoclonal antibodies and potential candidates for vaccine development. In addition, both cDNA and genomic DNA libraries have been constructed and used successfully in the isolation and characterization of MCMV genes (Loh *et al*., 1994; Scalzo et al., 2004).

More recently, we have constructed recombinant MCMV by conventional *in vivo* complementation methods. In particular, we have constructed and characterized *M73.5* null and glycosylation mutants to study the role of this glycoprotein in the virus life cycle (manuscript in preparation). Furthermore, we have constructed a chimeric MCMV by replacing the MCMV *M44* (DNA polymerase processivity factor) gene with its HCMV homolog (*UL44*) in our initial attempt to replace MCMV replication genes with their HCMV homologs and facilitate the *in vivo* testing of anti-viral drugs targeting the HCMV DNA polymerase complex. We also anticipate that the production of similar chimeric MCMVs may play an important role in elucidating the mechanism of CMV host specificity. As a result, we began to explore and develop methodologies that would simplify and facilitate rapid and efficient construction of recombinant MCMV.

During the last 5 years, we have modified an existing method of constructing recombinant MCMV using BAC vectors. This strategy makes use of a recombinant MCMV where the unique *Swa*I site has been destroyed, and specific mutations can be introduced into any part of the MCMV genome in a 2-step process. The details of this procedure will be described in greater detail in the Results section. We have now successfully constructed *M73.5* mutants and *M44/UL44* chimeric MCMV with this approach. Preliminary results demonstrated that these recombinant MCMVs behaved similarly to those constructed by more "conventional" *in vivo* methods. However, the effect of the *Swa*I mutation introduced into the

“backbone” of these recombinant murine cytomegaloviruses has not yet been tested. In this thesis, I intend to provide preliminary evidence to demonstrate that the biological properties of the virus are not affected by the *Swa*I mutation.

2.0 RATIONALE AND OBJECTIVES

Our laboratory has used MCMV as a model for HCMV to explore the molecular mechanisms underlying the host specificity of cytomegaloviruses as well as develop anti-viral therapy/vaccines by studying the roles played by viral genes involved in these processes. Thus the ability to manipulate the MCMV genome and introduce specific mutations into viral genes efficiently became an important part of our research program. Recently, we have utilized a modified MCMV (Smith strain) genome in which the unique *Swa*I site has been destroyed as the "backbone" for specific mutagenesis of the viral genome. Subsequently we have successfully constructed several recombinant MCMV mutants and demonstrated the potential usefulness of this methodology.

In this thesis, I investigated whether the *Swa*I mutation introduced into the MCMV genome affects the growth properties of MCMV in tissue culture. In addition, I demonstrated that the *Swa*I mutation can be reversed, and the revertant virus has growth properties similar to the standard Smith strain of MCMV.

3.0 MATERIALS AND METHODS

3.1 Cell lines

3.1.1 Balb/3T3 clone A31 (ATCC CCL 163)

The Balb/3T3 cell line was originally derived from mouse embryo cultures of inbred Balb/c mice and is very similar to mouse 3T3 fibroblasts (Aaronson and Todaro, 1968). Since Balb/c mice were used in most laboratories as an animal model for studying permissive MCMV replication *in vivo*, these cells were used for experiments on the growth properties of recombinant and wild-type MCMV.

3.1.2 3T3-L1 (ATCC CL-173)

The 3T3-L1 cell line is a cloned subline of the mouse fibroblast line 3T3 that accumulates lipids (Green and Kehinde, 1974). These cells were used for plaque assays and the isolation of viral DNA from infected cells (sections 3.1.5 and 3.7) as well as large scale propagation of MCMV stocks.

3.1.3 COS-1 (ATCC CRL 1650)

COS-1 is a simian cell line that was developed through the transformation of CV-1 cells with a defective mutant of SV40 (Gluzman, 1981). These cells were used for the analysis of the growth of recombinant and wild-type MCMV in semi-permissive cells.

3.1.4 Cell culture and passage

Cell lines were maintained in Dulbecco's Minimum Essentials Media (DMEM, Invitrogen Canada, Inc) containing 10% Fetal Calf Serum (FCS, Invitrogen Canada, Inc) in a humidified NAPCO Incubator (Precision Scientific, Inc., Chicago, IL) at 37°C with 5% CO_2. Once cells were confluent, the old media were aspirated. Then cells were rinsed with GKNP balanced salt solution (0.8% (w/v) NaCl, 0.04% (w/v) KCl, 0.006% (w/v) $Na_2HPO_4 \cdot H_2O$, 0.006% (w/v) KH_2PO_4, 0.1% (w/v) glucose, 0.002% (w/v) phenol red, pH 7.0) and treated with trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA; 0.025% trypsin and 0.27 mM EDTA in GKNP). After cells were trypsinized, they were resuspended in fresh DMEM and generally split at a dilution of 1:10 at 3 day intervals.

3.2 Viruses

3.2.1 Virus strains

The Smith strain of MCMV (ATCC VR-194) was obtained from the American Type Culture Collection, Rockville, MD and used as a standard for comparison with recombinant virus in all experiments. The MCMV-EGFP virus was rescued after transfection of pMCMV_EGFP BAC DNA (obtained from Dr. Jay Nelson of Oregon Health Sciences University) into Balb/3T3 cells. The recombinant viruses MCMV-ETwt and MCMV-ETSwa$^-$ were recovered after transfection of pMCMV_ETwt and pMCMV_ETSwa$^-$* DNA into Balb/3T3 cells. Schematics for these recombinant viruses are shown in Figure 3.1.

3.2.2 Plaque assays

When 3T3-L1 cells in 35 mm tissue culture dishes were 75-80% confluent, they were infected in duplicate with at least two sets of 10 fold dilutions of each virus sample. After absorption for 1-2 hours at 37°C, the media were removed, and cells were overlaid with 2 mL of DMEM containing 0.5% (w/v) agarose, 2.5% (v/v) FCS, and 40 μg/mL gentamycin. The media were allowed to solidify and the plates incubated in a CO_2 incubator at 37°C. Plaques were generally counted between 5 to 7 days post-infection.

3.2.3 Analysis of viral growth properties

For the analysis of viral growth properties, Balb/3T3 or COS-1 cells in 35 mm tissue culture dishes were infected with wild-type or recombinant MCMV at an appropriate MOI when cells were about 60% confluent. The virus was allowed to absorb for 1 hour at 37°C before cells were washed four times with DMEM to remove unbound virus. Cells were subsequently incubated in DMEM containing 2.5% FCS at 37°C. At each time point, 1 mL of media was removed for plaque assays and cells were supplemented with 1 mL fresh media.

3.2.4 Virus production and purification

To produce high titer stocks of MCMV for infection experiments, 10 x 150 mm plates of 3T3-L1 cells were infected with recombinant or wild-type MCMV at low multiplicities of infection. Infection was allowed to proceed until virtually 100% of the cells exhibited cytopathic effects. Supernatants containing MCMV were collected and centrifuged at 4000 x g for 20 minutes to remove cellular debris. Then virus was pelleted at 15000 x g for 3 hours, resuspended in 5 mL of DMEM supplemented with 5% FCS, and stored in 1 mL aliquots at

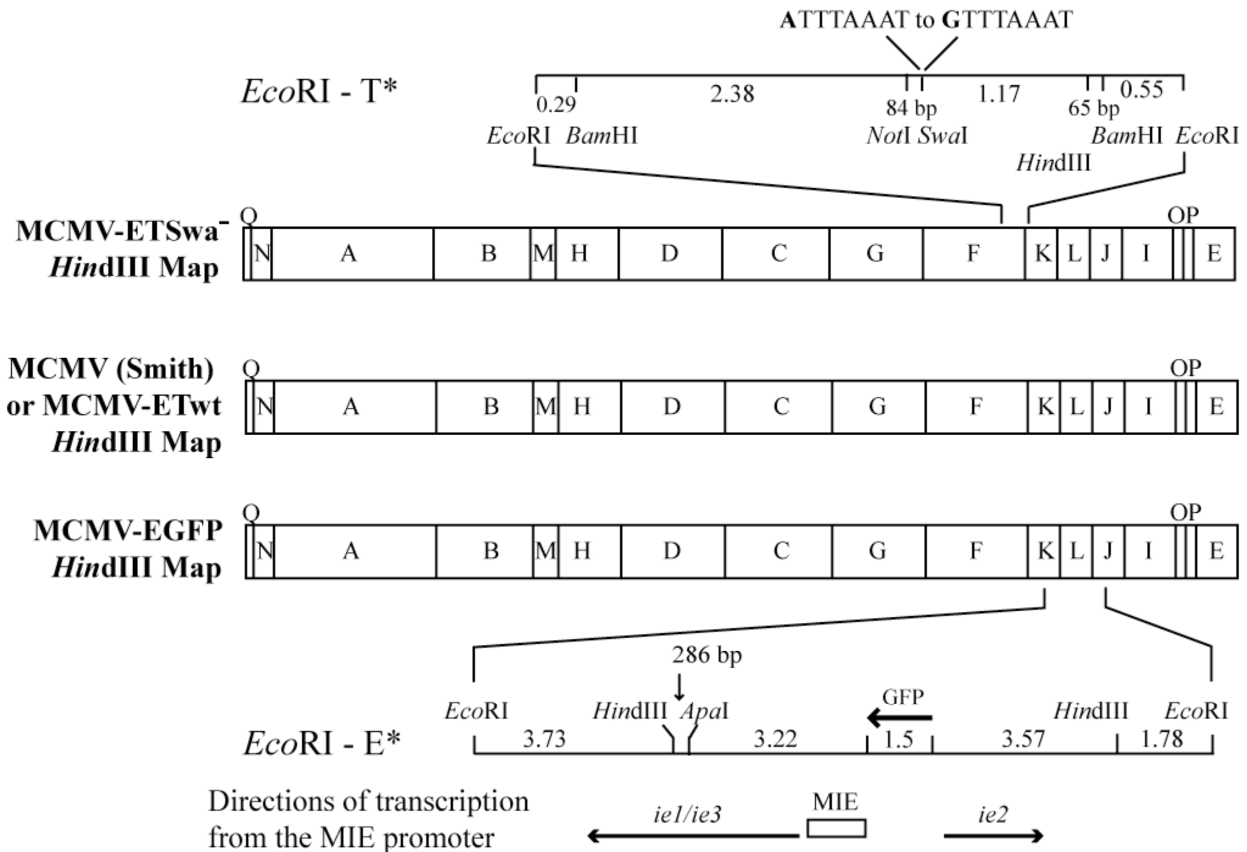

Figure 3.1 Schematics of recombinant MCMVs

The *Hin*dIII restriction maps of the recombinant MCMVs relevant to this thesis are shown. Expanded views of the modified *Eco*RI-T and *Eco*RI-E fragments in MCMV-ETSwa$^-$ and MCMV-EGFP are shown above or below the respective viral genomes. These fragments are labeled with an asterisk (*) to distinguish them from wild-type fragments. The numbers between restriction sites represent the sizes of the subfragments in kilobase pairs unless indicated otherwise. In particular, the A to G mutation introduced into the unique *Swa*I site in the *Eco*RI-T fragment of MCMV-ETSwa$^-$ is shown in bold at the top of the panel. The GFP (green fluorescent protein) cassette inserted into the modified *Eco*RI-E fragment of MCMV-EGFP contains an EF-1 promoter, the coding sequence of GFP, and a SV40 polyadenylation site. At the bottom of the panel, location of the major immediate-early promoter (MIE) is shown along with the bidirectional transcription that resulted in the spliced immediate-early transcripts *ie1/ie3* and *ie2*. Thus insertion of the GFP cassette disrupts *ie2* transcription in MCMV-EGFP.

-20°C. Finally, titers of the virus stocks were determined by plaque assays as described in section 3.2.2.

3.3 Bacteria

3.3.1 Bacterial strains and culture

Plasmids and Bacterial Artificial Chromosomes (BACs) were maintained in *Escherichia coli* strains DH5α [*supE*44 *lac*Ul69(80 *lac*ZM15)*hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi-1 relA*1] and DH10B [F$^-$ *endA*1 *recA*1 *galE*15 *galK*16 *nupG rpsL ΔlacX*74 *ϕ*80*lacZΔM*15 *araD*139 *Δ(ara, leu*)7697*mcrA Δ(mrr-hsdRMS-mcrBC)*λ^-], respectively. Both bacterial strains were obtained from Invitrogen (formerly Bethesda Research Laboratories, Maryland). Homologous recombination between BAC and complementing DNA was carried out in *E. coli* strain DY380 (obtained from Dr. Don Coen of Harvard Medical School) whose genotype is DH10B [*λcl857* (cro-bioA) <>tet]. The recombination genes *exo*, *bet*, and *gam* in DY380 are temperature-sensitive and can be activated at 42°C.

E. coli strains DH5α and DH10B were grown in Luria Broth (L-Broth) at 37°C with vigorous shaking at 200 rotations per minute in an environmental shaker (New Brunswick Scientific Co., Inc., Edison, NJ). *E. coli* strain DY380 was grown at 30°C in L-Broth containing tetracycline (12.5 µg/mL). When *E. coli* strain DH5α harbored plasmids containing the ampicillin resistance gene, L-Broth was supplemented with ampicillin (75 µg/mL). Similarly, when *E. coli* strain DH10B harbored a BAC containing a chloramphenicol resistance gene, L-Broth was supplemented with chloramphenicol (17 µg/mL).

3.3.2 Preparation of competent cells for electroporation

For routine preparation of competent bacteria, 500 mL of L-broth was inoculated with 1/100 volume for an overnight culture of bacteria and incubated at 37˚C with vigorous shaking until an OD_{600} of 0.7 was reached. The bacteria were then chilled on ice for 30 minutes and centrifuged at 4000 x g for 15 minutes. The bacteria pellet was resuspended in 500 mL of cold distilled water and pelleted again. The washing procedure was repeated 3 times by resuspending the bacteria in successively smaller volumes of water. The final pellet was resuspended in 2 to 3 mL of cold 10% glycerol and stored at -70˚C in 100 µL aliquots.

For homologous recombination in DY380, an overnight culture of the bacteria was diluted 1:50 into fresh L-Broth media and the fresh culture was allowed to grow at 30°C until an OD_{600} of 0.5-0.6 was reached. For each recombination reaction, 10 mL of culture was heat shocked at 42°C for exactly 15 minutes in a 50-mL Erlenmeyer flask at 200 rpm and quickly chilled in an ice water slurry for 10 minutes. The bacteria were pelleted by centrifugation at 5500 x g for 8 minutes at 4°C and washed 4 times with sterile, deionized water. Finally, competent bacteria were resuspended in 100 μL of deionized water and used for electroporation within an hour.

3.4 Cloning vectors and plasmids used in this study

The cloning vector pTZ18R was purchased from Invitrogen. All other plasmids used in this study were previously constructed in this laboratory and contain MCMV DNA fragments cloned into the pTZ18R vector (Table 3.1).

3.5 DNA purification and analysis

3.5.1 Small-scale purification of plasmid DNA by the "boiling" method

The procedure used was adapted from that described in Sambrook *et al.* (1989). Bacteria containing the plasmid of interest were grown in 3 mL of L-Broth supplemented with ampicillin (50 μg/ml) at 37°C for 12-16 hours until the stationary phase was reached. Then bacteria were pelleted by centrifugation at 15000 x g for 1 minute in a microfuge and resuspended in 350 μL of STET (0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 5% (v/v) Triton X-100, pH 8.0). Ten μL of lysozyme (20 mg/mL in STET) was added and the sample was boiled for 45 seconds. The lysed bacteria were then centrifuged at 15000 x g for 8 minutes at room temperature. Sixteen μL of 5 M NaCl and 420 μL of isopropanol were added to the supernatant. The solution was incubated for 5 minutes at room temperature before plasmid DNA was pelleted by centrifugation at 15000 x g for 15 minutes at room temperature. The DNA pellet was washed with 70% ethanol and resuspended in 50 μL of Tris-EDTA buffer (TE, 10 mM Tris-HCl and 0.1 mM EDTA, pH 8.0) containing Ribonuclease (RNAse, 50 μg/mL, Sigma Chemical Co., Missouri). Purified plasmid DNA was analyzed by restriction enzyme digestion and agarose gel electrophoresis as described in section 3.5.5.

Table 3.1- Plasmids Used in Recombinant MCMV BAC Construction

Plasmid	*Eco*RI fragment cloned	Description of the cloned *Eco*RI fragment
pTE66	*Eco*RI-T	Wild type sequence containing the unique *Swa*I site
pTE77	*Eco*RI-E	Wild type sequence
pTETSwa$^-$	*Eco*RI-T	*Swa*I site mutation and an unexpected T→ A mutation
pTETSwa$^-$*	*Eco*RI-T	*Swa*I site mutation
pTETzeo	*Eco*RI-T	Zeocin cassette replacing the *Not*I-*Bam*HI sequences
pTE-E/SwaZeo	*Eco*RI-E	Zeocin cassette replacing the *Apa*I-*Hpa*I sequences

Plasmids listed in this table contain wild-type or modified MCMV *Eco*RI-E or -T fragments cloned into the vector pTZ18R. Restriction maps for these two DNA fragments are shown in Figures 4.2, 4.4, and 4.6. Site-directed mutagenesis was used to destroy the unique *Swa*I site in the MCMV genome via an A to G mutation (ATTTAAAT to GTTTAAAT), and an unexpected T to A mutation (AATAAA to AAAAAA) was found near the *Swa*I site via nucleotide sequencing.

3.5.2 Large-scale purification of plasmid DNA

3.5.2.1 Preparation of plasmid DNA by the alkaline lysis method

This procedure was adapated from that described in Sambrook *et al.* (1989). Five hundred mL of L-Broth supplemented with the appropriate antibiotic was inoculated with 10 mL of an overnight culture of bacteria harboring the selected plasmid. The culture was allowed to grow at 37°C until the OD_{600} reached about 0.7, at which point 2.5 mL of chloramphenicol (34 mg/mL in ethanol) was added to prevent further bacterial growth, and the culture allowed to grow for an additional 12-16 hours. The bacterial culture was centrifuged at 2500 x g for 15 minutes and the pellet resuspended in 10 mL of cell suspension buffer (50 mM Tris-HCl and 10 mM EDTA, pH 8.0) containing lyzozyme (1 mg/mL). Ten mL of cell lysis solution (0.2 N NaOH, 1% SDS) was then added and the sample incubated at room temperature until lysis occurred, at which point 15 mL of neutralization buffer (3.1 M potassium acetate, pH 5.5) was added and the mixture allowed to incubate on ice for 10 minutes. The bacterial lysate was centrifuged for 20 minutes at 4100 x g and 4°C. Following removal of the precipitate, 0.7 x volume of isopropanol was added to the supernatant and the solution was centrifuged at 16000 x g for 25 minutes at room temperature. Finally, the DNA pellet was washed with 70% ethanol and resuspended in 5 mL TE, pH 8.0.

3.5.2.2 Purification of plasmid DNA by precipitation with polyethylene glycol

Plasmid DNA prepared by the alkaline lysis method was further purified by precipitation with polyethylene glycol (Sambrook *et al.,* 1989). Three mL of 5 M LiCl was added to the nucleic acid solution obtained in section 3.5.2.1 and centrifuged at 7800 x g for 10 minutes at 4°C. The DNA in the supernatant was recovered by the addition of an equal volume of isopropanol, incubation for 5 minutes at room temperature, and centrifugation at 7800 x g for 25 minutes at room temperature. The crude DNA pellet was washed with 70% ethanol and redissolved in 500 μL TE (pH 8.0) containing deoxyribonuclease-free pancreatic RNAase (20 μg/ml) and stored at room temperature for 30 minutes. Next, 500 μL of 1.6 M NaCl containing 13% (w/v) polyethylene glycol (PEG 8000) was added and the plasmid DNA precipitated by centrifugation at 12000 x g for 20 minutes at 4°C. The pelleted plasmid DNA was resuspended in 400 μL of TE (pH 8.0), extracted with phenol-chloroform and then chloroform, and precipitated for 10 minutes at room temperature by the addition of 100 μL of

10 M ammonium acetate and two volumes of 95% ethanol. The plasmid DNA was recovered by centrifugation at 12000 x g for 20 minutes at 4°C in a microfuge, washed with 70% ethanol, and dissolved in 500 µL of TE, pH 8.

3.5.3 Preparation of Bacterial Artificial Chromosome (BAC) DNA

A 10 mL culture of bacteria harboring a specific BAC was grown up overnight using appropriate temperatures and antibiotics. If the BAC was harbored in *E.coli* DY380, the culture was grown at 30°C in L-broth supplemented with tetracycline (12.5 µg/mL) and chloramphenicol (17 µg/mL). However, if the BAC was harbored in *E.coli* DH10B, the culture was grown at 37°C in L-broth supplemented with chloramphenicol. The procedure after this point was similar to that given in section 3.5.2.1 with the following modifications. First, the bacteria were initially pelleted at 2100 x g and resuspended in 2 mL of cell suspension buffer containing 100 µg/mL of RNAse. Two mL of cell lysis buffer was added, and the cleared lysate was centrifuged immediately following the addition of 2 mL of neutralization buffer. Secondly, after the BAC DNA was precipitated, it was resuspended in 50 µL TE, pH 8.0. Finally, all manipulations of BAC DNA were carried out carefully to avoid shearing of the large DNA molecules. Purified BAC DNA was analyzed by restriction enzyme digestion and agarose gel electrophoresis as described in section 3.5.5.

3.5.4 Determination of DNA concentration

The concentration of purified plasmid DNA was determined by measuring the OD_{260} and OD_{280} of an appropriately diluted DNA sample in a UV spectrometer. An absorbance of one at OD_{260} corresponds to approximately 50 µg/mL of double-stranded DNA. Thus, the plasmid DNA concentration was determined by the following formula:

$$\text{Concentration of DNA in } \mu g/mL = \text{dilution factor} \times 50\ \mu g/mL \times OD_{260}$$

If the ratio of the absorbance at 260 nm to that at 280 nm is greater than 1.8, the DNA sample is considered to be relatively free of contaminating proteins.

3.5.5 Restriction enzyme digestion and Agarose gel electrophoresis of purified DNA

Purified plasmid, BAC, or viral DNA was digested with restriction enzymes in appropriate buffers supplied by the manufacturers at 37°C for 2 hours. Following digestion, one-tenth volume of a 10X agarose gel-loading buffer (50% (v/v) glycerol, 100 mM EDTA,

and 1% (w/v) bromophenol blue) was added to DNA samples prior to separation via agarose gel electrophoresis. Typically, 0.7% or 1% agarose (Bio-Rad or Invitrogen) gels were cast in either a Horizontal Gel electrophoresis system model H6 (Bethesda Research Laboratories, Gaithersburg, MD) or an Easycast Horizontal System Model B1A (OWL, Portsmouth, NH) when plasmid DNA was analyzed. BAC or viral DNA samples were separated on 0.7% agarose gels on a BRL Horizon 11-14 gel system. Bacteriophage lambda DNA digested with *Eco*RI and *Hin*dlll was used as molecular weight standard markers. DNA fragments were separated at 70 volts in 1 X Tris-acetate-EDTA buffer (40 mM Tris-acetate, pH 8.0, and 1 mM EDTA, pH 8.3). Following electrophoresis, gels were stained in distilled deionized water containing ethidium bromide (0.1 μg/mL) until bands were readily visible on a Spectroline Model TC-302 ultraviolet transillumintaor (Spectronics Corporation, Westbury, NY). Images of agarose gel were recorded using the AlphaDigiDoc™ system from Alpha Innotech.

3.6 Construction of bacterial artificial chromosomes containing the MCMV genome by homologous recombination in DY380 cells

3.6.1 Purification of DNA fragments from agarose gels

Plasmid DNA was digested with suitable restriction enzymes. The desired DNA fragment was separated from the cloned vector by agarose gel electrophoresis and DNA was visualized as described in section 3.5.5. The DNA fragment was then excised from the agarose gel and purified using the GENECLEAN II Kit (BIO 101 Inc., La Jolla, CA) according to the manufacturer's instructions. Briefly, NaI (3 x w/v) was added the excised gel bands and the agarose was melted at 49°C for 5 minutes. Ten μL of GLASSMILK® was incubated with the DNA sample for 10 minutes at room temperature. The GLASSMILK®/DNA complexes were pelleted by brief centrifugation at 15000 x g at room temperature and washed with 1 mL of NEW WASH® Buffer 4 times. The pellet was allowed to dry for 5 minutes prior to resuspension in 5 to 10 μL of TE and elution at 49°C for 5 to 6 minutes. The purified DNA was recovered by centrifugation at 15000 x g for 20 seconds at room temperature to remove the precipitated GLASSMILK®. If it was necessary, the DNA was precipitated in 75% ethanol and resuspended in a volume suitable for a specific experiment.

3.6.2 Preparation of linear BAC DNA

Parental BAC DNA containing a zeocin cassette was purified as described in section 3.5.3 and digested with *Swa*I (New England Biolabs) for 2 hours at 25°C. Next, the restriction enzyme and residual protein contaminants were removed by phenol-chloroform extraction and the linearized DNA was precipitated in 0.2 M NaCl and 70% ethanol. Finally, the purified BAC DNA was resuspended in TE buffer containing the DNA fragment targeted for homologous recombination.

3.6.3 Transformation of DNA into competent cells and screening of BAC recombinants

Electroporation was performed using a Bio-Rad Gene pulser set at 25 μFD, 200 Ω, and 2.5 kilovolts according to the manufacturer's instructions. When we were replacing a zeocin cassette with a complementing DNA fragment, 1 μL of BAC DNA from section 3.6.2 was mixed with 40 μL of competent DY380 bacteria and added to a gene pulser cuvette. When we were inserting a zeocin cassette into the MCMV genome, 1 μL of a gel-purified DNA fragment containing the cassette was added to competent DY380 bacteria that harbored the MCMV BAC. Following successful electroporation, 0.5 mL of SOC media (2% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 0.05% (w/v) NaCl, and 20 mM glucose) was added to the transformed cells and the bacteria were allowed to recover at 30°C for 2 hours with gentle shaking. In cases where we were screening for zeocin sensitivity (loss of zeocin cassette), bacteria were spread on L-broth plates supplemented with chloramphenicol (17 μg/mL) and incubated at 30°C until colonies were clearly visible. These colonies were replica plated on L-broth plates supplemented with either chloramphenicol alone or chloramphenicol and zeocin and grown at 30°C. For homologous recombination of BAC DNA involving the insertion of a zeocin cassette into the MCMV genome, bacteria were plated on L-broth plates supplemented with chloramphenicol and zeocin (25 μg/mL). Subsequently, selected colonies were grown up in liquid culture containing appropriate antibiotics and BAC DNA was purified from these bacteria for analysis by restriction enzyme digestion and agarose gel electrophoresis as described in sections 3.5.3 and 3.5.5. Finally, bacteria harboring BAC DNA with the correct profiles were frozen at -70°C and BAC DNA from these colonies was transformed into competent DH10B bacteria for stable, long-term maintenance of BAC clones.

3.7 Rescue of recombinant virus in tissue culture

3.7.1 Purification of MCMV BAC DNA using the CONCERT high purity plasmid purification system

The Concert high purity plasmid purification system (Invitrogen) was used to purify MCMV BAC DNA to obtain DNA of sufficient purity for transfection. The initial steps were similar to those described in section 3.5.3. Bacteria harboring MCMV BACs were grown overnight in 10 mL of L-broth containing appropriate antibiotics. Bacteria were centrifuged at 9000 x g for 15 min and the pellet was resuspended in 2 mL of cell suspension buffer containing RNAse A (400 μg/mL). Resuspended cells were lysed for 5 minutes at room temperature by adding 2 mL of cell lysis solution. Two mL of neutralization buffer were added to the lysed bacteria and the mixture was centrifuged at 15000 x g for 10 minutes at room temperature. The supernatant was removed and added to a DNA-purification column that was previously equilibrated with equilibration buffer (600 mM NaCl, 100 mM sodium acetate, 0.5% (v/v) TritonX-100, pH 8.0). Subsequently, the column was washed with 2.5 mL of wash buffer (0.9 mM NaCl, 100 mM sodium acetate, pH 5.0) and BAC DNA was eluted from the column with 0.9 mL of elution buffer (1.25 mM NaCl, 100 mM Tris-HCl, pH 8.5) pre-warmed to 49˚C. Eluted DNA was precipitated by adding 0.7 x volumes of isopropanol and precipitated at 12000 x g for 30 minutes at 4°C. The DNA pellet was washed with 75% ethanol, centrifuged at 15000 x g at 4°C for 5 minutes, dried in air for 5 minutes, and dissolved in 25 μL of TE, pH 8.0.

3.7.2 Transfection of Balb/3T3 cells with MCMV BAC DNA

Balb/3T3 cells were transfected with purified BAC DNA using LipofectAMINE PLUS Reagent (Invitrogen) according to the manufacturer's instructions. Briefly, Balb/3T3 cells which were 70% confluent in 35 mm plates were washed and incubated in 0.8 mL of DMEM prior to transfection. Purified MCMV BAC DNA (section 3.7.1) was diluted into 100 μL of DMEM to maintain an appropriate concentration for transfection. Six μL of PLUS Reagent was added to the diluted DNA mixture and incubated at room temperature for 15 minutes. One hundred microliters of DMEM supplemented with 4 μL of LipofectAMINE reagent was added to the DNA/PLUS Reagent mixture and incubated at room temperature for a further 15 minutes. The DNA/PLUS reagent/LipofectAMINE complexes were then added to the cells prior to a 3-hour incubation at 37°C. Finally, 1 mL of DMEM supplemented with 10% FCS

was added to each plate and cells were allowed to recover overnight. Media were changed the next day and then once every 3 days.

Transfected cells were monitored everyday for signs of viral infection. Once foci of infection were present (usually 1-2 weeks post-transfection), media containing released virus particles were collected. Virus contained in the supernatant was subjected to 3 rounds of plaque-purification and at least two independently isolated virus clones from each transfection were kept for further experimentation. High titer virus stocks were produced for experimentation as described in section 3.2.4.

3.7.3 Isolation of viral DNA from infected cells

3T3-L1 cells in 60 mm plates were infected with recombinant MCMV at a MOI of one. Once most of the cells were in the late phases of infection, media were removed, and cells were rinsed 2-3 times with phosphate-buffered saline (PBS, 0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.02% (w/v) KH_2PO_4, 0.22% (w/v) $Na_2HPO_4 \cdot 7H_2O$, pH 7.4). Cells were then lysed in 1 mL of buffer A (0.6% (w/v) SDS, 10 mM EDTA, pH 7.5) supplemented with 0.66 mL of 5 M NaCl and the plate was incubated at 4°C overnight. Next, cellular DNA and proteins were precipitated by centrifugation at 15000 x g for 30 minutes at 4°C and the supernatant was extracted with phenol-chloroform and then chloroform. Once extraction was complete, 2.5 x volumes of 95% ethanol was added and viral DNA was pelleted by centrifugation at 15000 x g for 20 minutes at 4°C. The DNA pellet was washed with 70% ethanol and dissolved in 100 μL of TE, pH 8.0. Purified viral DNA was analyzed by restriction enzyme digestion and agarose gel electrophoresis.

3.8 Analysis of MCMV proteins

3.8.1 Antibodies

All monoclonal antibodies were generated in our laboratory previously. Monoclonal antibody 9D3.22A is specific for *M112-113 (E1)* gene products with molecular weights of 33 kDa, 36 kDa, 38 kDa, and 87 kDa (Loh *et al.,* 1991). Monoclonal antibody 3B9.22A recognizes the early-late protein pp50 (50 kDa) encoded by *M44* (Loh *et al.*, 1991). Monoclonal antibody 2E8.12A is specific for the envelope glycoprotein gB (Loh *et al.,* 1988). It recognizes an epitope present on the 128 kDa gB precursor, the 150 kDa uncleaved form of gB, and the 100 kDa subunit of the gB glycoprotein.

3.8.2 Preparation of whole cell extracts

Cells grown in a 35 mm plate were infected at a MOI of 5 with MCMV (Smith strain) or recombinant MCMV when they were 60% confluent. At each time point, cells were washed 3 times with cold PBS and solubilized in 0.35 mL of radioimmunoprecipitation assay buffer (RIPA, 50 mM Tris-HCl, 150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) deoxycholate, and 0.1% (w/v) SDS, pH 8.0) for 1 hour on ice. Soluble proteins were collected following centrifugation at 12000 x g for 15 minutes at 4°C and analyzed by western blot as described in section 3.8.4.

3.8.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). Protein samples solubilized in SDS-PAGE sample buffer (50 mM Tris-HCl, 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue, pH 6.8) were heated at 100°C for 5 minutes and analyzed on either 7.5% or 9% polyacrylamide gels in a mini-PROTEIN II electrophoresis cell (Bio-Rad). Pre-stained molecular weight standards (Bio-Rad) were used to determine the sizes of protein bands. Gels were run at 100 volts in Tris-glycine electrophoresis buffer (10% (v/v) 10x Tris-glycine buffer, 0.1% (w/v) SDS, pH 8.3) until the dye front migrated to within 1 cm of the bottom edge of the gel.

3.8.4 Western blotting analysis

Following SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Bio-Rad, Canada) electrophoretically in transfer buffer (10% (v/v) 10x Tris-glycine buffer, 20% (v/v) methanol, 70% (v/v) ddH_2O) at 100 volts for two hours as described by Towbin *et al.* (1979). The membrane was blocked overnight in Tris-buffered saline (TBS, 50 mM Tris base and 150 mM NaCl, pH 7.4) containing 1% Bovine Serum Albumin (blocking buffer). Next, the nitrocellulose membrane was incubated for three hours with an appropriate dilution of a specific monoclonal antibody in blocking buffer. Following three washes in TBS containing 0.05% (v/v) Tween 20 (TBST), the membrane was incubated for one hour with alkaline phosphatase conjugated goat anti-mouse antibody (BioRad) at a 1:3000 dilution. Subsequently, the membrane was washed 4 times in TBST and once with TBS before 10 mL of alkaline phosphatase buffer (0.1 M Tris-HCl, 0.1 M NaCl, and 50 mM $MgCl_2$, pH 9.5)

containing 44 μL of p-nitro blue tetrazolium chloride (NBT) and 33 μL of 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt (BCIP) were added to allow the visualization of protein bands reactive with the monoclonal antibody. The reaction was stopped by washing the membrane in ddH_2O.

4.0 RESULTS

4.1 Development of a modified protocol to produce recombinant virus

4.1.1 Rationale and strategy for recombinant MCMV construction using a modified MCMV genome

Our laboratory has made use of the fact that bacteria can be transformed more efficiently by circular rather than linear plasmids to develop a modified method for BAC mutagenesis and recombinant virus production. The protocol depends on the introduction of unique restriction sites into the viral genome so that the BAC containing the entire MCMV genome can be linearized at the region targeted for mutagenesis. Subsequent homologous recombination with a viral DNA fragment containing the desired mutation allows re-circularization of the BAC and efficient transformation of the host bacteria. Because of the size of the MCMV genome, we were unable to find a restriction site that is absent from pMCMV-EGFP, the BAC containing the entire viral genome (originally obtained from Dr. Jay Nelson, Oregon Health Sciences University). However, a unique *Swa*I site is present in a region of the MCMV genome (*Eco*RI-T fragment) that does not appear to contain predicted open reading frames of significant sizes (<300 bp). Therefore, the unique *Swa*I site was destroyed by site-directed mutagenesis (<u>A</u>TTTAAAT to <u>G</u>TTTAAAT, Figure 3.1) and the resulting BAC (pMCMV-ETSwa$^-$) was used as the "backbone" for the construction of recombinant viruses. Although the *Swa*I site is within a 63 bp open reading frame located between *m119* and *m119.1* in the MCMV genome (Rawlinson *et al.,* 1996), the mutation introduced did not alter the amino acid sequence of the short ORF. Nevertheless, the effect of the *Swa*I mutation on MCMV growth properties needs to be tested experimentally to ensure that any as yet uncharacterized control sequence, intron, or exon is not affected. It should be noted that the *Swa*I site can easily be restored using the same mutagenesis procedure mentioned above after all the desired mutations have been introduced into the genome.

As shown in Figure 4.1, our modified BAC construction protocol began with the homologous recombination between a MCMV BAC and a zeocin (zeo) cassette flanked by two *Swa*I sites and homologous viral sequences. This resulted in the insertion of the zeocin

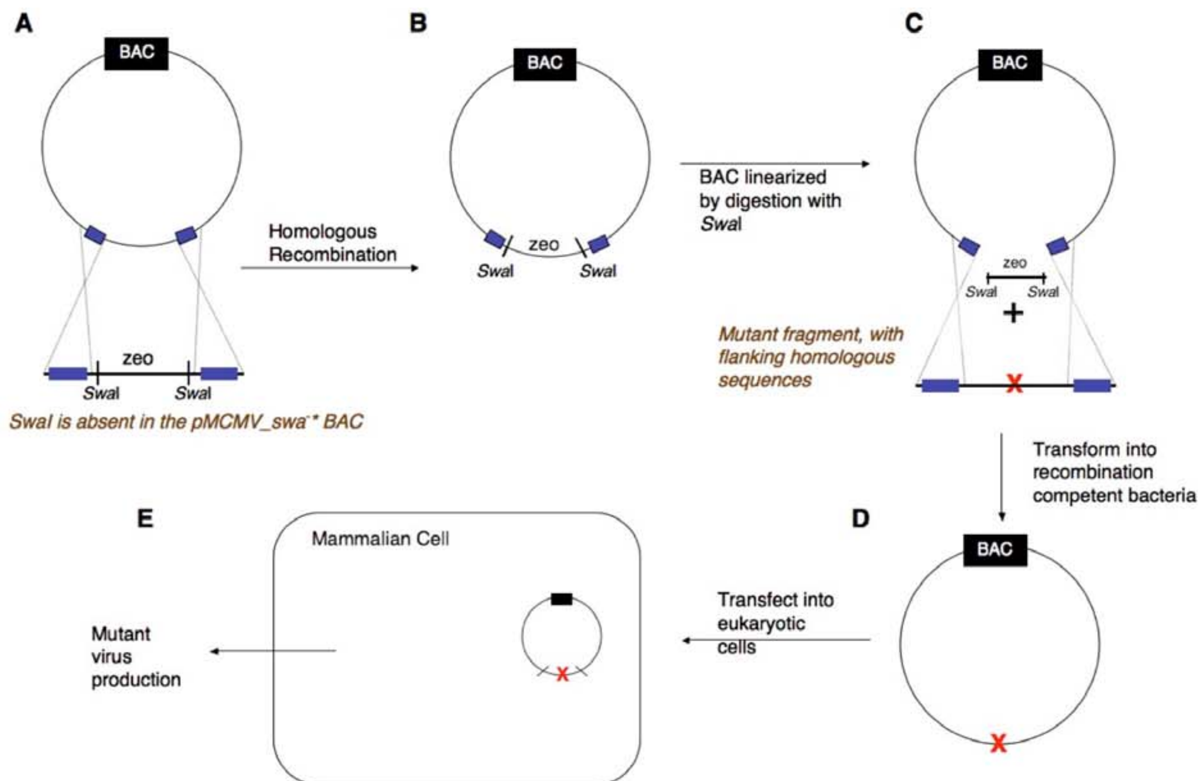

Figure 4.1: System for genetic engineering of large viral genomes using homologous recombination and linearized DNA (modified from Figure 2 of Bryan Weber's NSERC USRA poster, 2004)**.**

The steps involved in the mutagenesis procedure are summarized below:

A. Homologous recombination between MCMV BAC and a zeocin (zeo) cassette flanked by two *Swa*I sites and homologous viral sequences (shaded boxes). **B**. MCMV BAC containing zeocin cassette flanked by two *Swa*I sites. **C.** Homologous recombination between linearized BAC DNA and DNA fragment containing the desired mutation X.
D. Re-circularization of mutated MCMV BAC after homologous recombination.
E. Transfection of mutated MCMV BAC into eukaryotic cells and production of recombinant virus.

cassette into a region of the MCMV genome (lacking *Swa*I site) that we wished to manipulate. Correct recombinants were isolated as zeocin-resistant clones and analyzed by restriction endonuclease digestion. Then the MCMV BAC was linearized by digestion with *Swa*I, resulting in the excision of the zeocin cassette. The linearized BAC and a viral DNA fragment containing the desired mutation were transformed into recombination-competent bacteria where homologous recombination could take place. The loss of zeocin resistance was selected for by replica plating and potentially correct recombinants were screened by restriction endonuclease analysis. Then DNA from BAC clones with the expected restriction profiles were transfected into mammalian cells and recombinant virus were recovered, assuming that the mutation did not affect the viability of the virus.

The original pMCMV_EGFP BAC from Dr. Jay Nelson contained a GFP cassette (coding sequence of the green fluorescence protein) inserted within the MIE region of the genome (Figures 3.1 and 4.2). This insertion resulted in the disruption of murine *ie*2 gene expression. Although *mie*2 is dispensable for MCMV replication, MCMV-EGFP appeared to have a reduced capacity to replicate in COS-1 cells (unpublished results in our laboratory), suggesting that *mie2* may play a role in host specificity. Therefore, we made an initial attempt to test this hypothesis by replacing the GFP cassette with wild-type *Eco*RI-E sequences through homologous recombination using our modified BAC construction method.

To determine whether the biological properties of MCMV are affected by the destruction of the *Swa*I site or the presence of the GFP cassette, the growth properties of MCMV_ETSwa$^-$ or MCMV-EGFP need to be compared to the Smith strain of MCMV and MCMV-ETwt where the *Swa*I site has been restored and the GFP cassette replaced with wild-type sequences (Figure 3.1). For example, if recombinant MCMV containing the *Swa*I mutation were to behave the same way as their parental counterparts, it would suggest that additional steps to restore the *Swa*I site might not be necessary.

4.1.2 Construction of recombinant viruses

The list of BACs relevant to this thesis is shown in Figure 4.3 and Table 4.1. Prior to my involvement in this project, pMCMV_EGFP BAC has been modified by the insertion of a zeocin cassette into the *Eco*RI-T region, creating pMCMV_EGFP_ETzeo. The zeocin cassette was then replaced with a modified *Eco*RI-T region containing a *Swa*I$^-$ mutation (Figure 4.2), resulting in pMCMV_EGFP_ETSwa$^-$. Part of the MIE region of the *Eco*RI-E fragment of

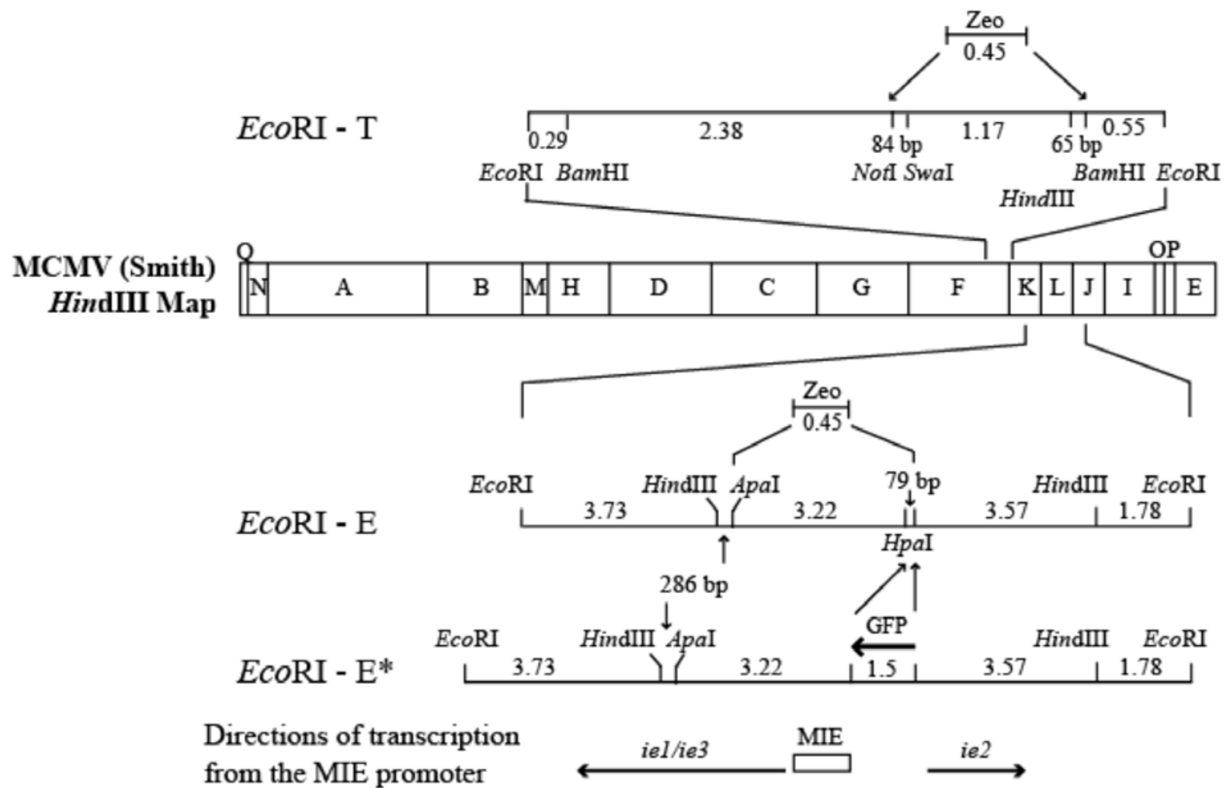

Figure 4.2: Locations of the *Eco*RI-T and *Eco*RI-E fragments on the MCMV genome and features relevant to this report.

The *Hin*dIII restriction map of the MCMV genome is shown in the middle with expanded views of the *Eco*RI-T and *Eco*RI-E fragments directly above or below it. The numbers along the line represent DNA fragment sizes in kilobases unless indicated otherwise. The sites of insertion of the zeocin (zeo) cassette (flanked by *Swa*I sites) into the *Eco*RI-T and *Eco*RI-E fragments are indicated. Furthermore, in pMCMV_EGFP, the wild type *Eco*RI-E fragment was replaced by the *Eco*RI-E* fragment (shown below the restriction map for the *Eco*RI-E fragment) where the 79 bp sequence between the 2 *Hpa*I sites was replaced by a GFP cassette which contained the EF-1 promoter, the coding sequence for GFP, and a SV40 polyadenylation site. At the bottom of the panel, the location of the major immediate-early promoter (MIE) is shown along with the bidirectional transcription that resulted in the spliced immediate-early transcripts *ie*1/*ie*3 and *ie*2.

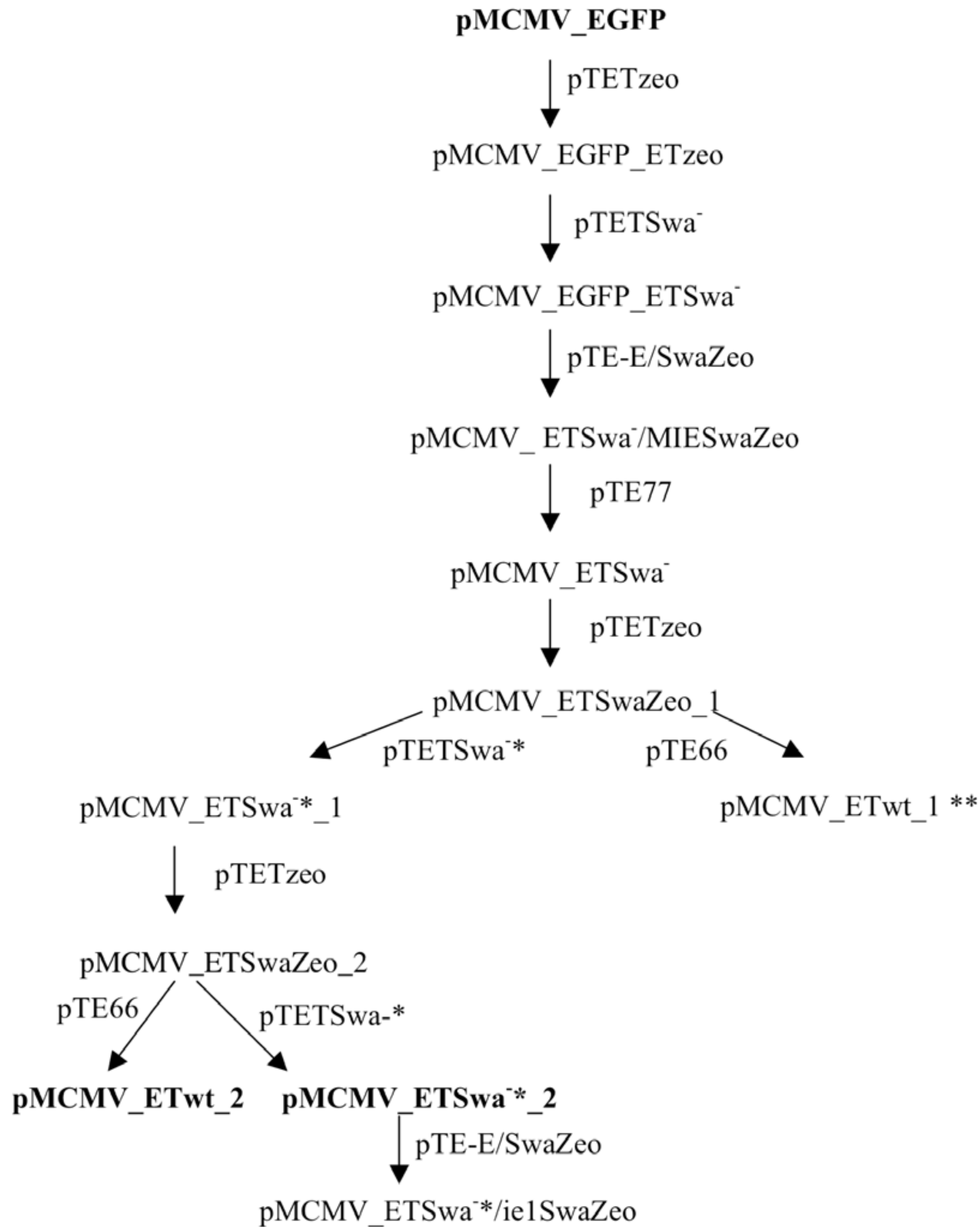

Figure 4.3: Flowchart illustrating the Construction of Recombinant MCMV BACs. Descriptions of the plasmids and BACs included in this flowchart are shown in Table 3.1 and Table 4.1, respectively. Starting with the parental BAC pMCMV_EGFP at the top of the chart, the plasmid whose viral DNA insert was used for the homologous recombination reaction in the DY380 strain of *E. coli* to produce the next BAC is shown next to the arrow. The three MCMV BACs whose properties were characterized in this thesis are shown in **bold**. Virus derived from pMCMV_ETwt_1 (marked with **) was not further characterized for reasons discussed in the text.

Table 4.1 Descriptions of MCMV BACs

MCMV BAC	*Eco*RI fragments modified	Description of the modifications
pMCMV_EGFP	*Eco*RI-E	GFP cassette inserted between the 2 *Hpa*I sites
pMCMV_EGFP_ETzeo	*Eco*RI-E *Eco*RI -T	GFP cassette inserted between the 2 *Hpa*I sites in the *Eco*RI-E fragment Zeocin cassette replacing the *Not*I-*Bam*HI fragment of the *Eco*RI-T fragment
pMCMV_EGFP_ETSwa$^-$	*Eco*RI-E *Eco*RI -T	GFP cassette inserted between the 2 *Hpa*I sites in the *Eco*RI-E fragment *Swa*I mutation and an unexpected T→ A mutation in the *Eco*RI-T fragment
pMCMV_ETSwa$^-$/MIESwaZeo	*Eco*RI-E *Eco*RI -T	Zeocin cassette replacing the *Apa*I-*Hpa*I fragment in the *Eco*RI-E fragment *Swa*I mutation and an unexpected T→ A mutation in the *Eco*RI-T fragment
pMCMV_ETSwa$^-$	*Eco*RI-T	*Swa*I mutation and an unexpected T→ A mutation in the *Eco*RI-T fragment
pMCMV_ETSwaZeo	*Eco*RI-T	Zeocin cassette replacing the *Not*I-*Bam*HI fragment
pMCMV_ETSwa$^-$*	*Eco*RI-T	*Swa*I mutation
pMCMV_ETwt	*Eco*RI-T	Wild type sequences
pMCMV_ETSwa$^-$*/ie1SwaZeo	*Eco*RI-E *Eco*RI-T	Zeocin cassette replacing the *Apa*I-*Hpa*I fragment in the *Eco*RI-E fragment *Swa*I mutation in the *Eco*RI-T fragment

This table lists the names of the MCMV BACs relevant to this study, the *Eco*RI fragment that was modified in a particular BAC, and a brief description of the specific modifications. Restriction maps of the *Eco*RI-E and -T fragments are shown in Figures 4.2, 4.4, and 4.6. Site-directed mutagenesis was used to destroy the unique *Swa*I site (ATTTAAAT to GTTTAAAT), whereas the unexpected T to A mutation occurred in a nearby sequence (AATAAA to AAAAAA).

pMCMV_EGFP_ETSwa$^-$ was then replaced with a zeocin cassette to yield pMCMV_ETSwa$^-$ /MIESwaZeo.

4.1.2.1 Construction of pMCMV_ETSwa$^-$

The purpose of inserting a zeocin cassette into the MIE region was to replace the modified *Eco*RI-E, which contained a GFP cassette, with wild-type sequences. This was accomplished by homologous recombination between pMCMV_ETSwa$^-$/MIESwaZeo, which was linearized by digestion with *Swa*I, and the *Eco*RI-E fragment excised from pTE77 according to protocols described in section 3.6. The GFP cassette was inserted within the MIE region of the MCMV *Eco*RI-E fragment, causing a 1.5 kb insertion in this fragment as well as the *Hin*dIII-L fragment which is part of this region (Figure 4.2 and Figure 4.4 panel B). Replacement of the zeocin cassette with the wild-type *Eco*RI-T fragment, and thus removal of the GFP cassette, should result in a decrease in the size of the *Eco*RI-E fragment from 14.1 kb to 12.7 kb, and a decrease in the size of the *Hin*dIII-L fragment from 8.56 kb to 7.16 kb (Figure 4.4, panels A and B). Following selection, replica plating and restriction analysis, two zeocin-sensitive colonies (Table 4.2) yielded the expected restriction profile for pMCMV_ETSwa$^-$ (Figure 4.5). Differences in the *Eco*RI restriction profiles were not readily apparent on the gel (Figure 4.5, Lanes 4-6) due to the reduced resolution of fragment sizes at that range of molecular weights. However, more obvious differences were demonstrated by digestion with *Hin*dIII, where the expected changes in the restriction profiles were observed (Figure 4.5, Lanes 1-3).

4.1.2.2 Construction of pMCMV_ETSwaZeo_1

Once pMCMV_ETSwa$^-$ was constructed, nucleotide sequencing revealed an unexpected AATAAA to AAAAAA mutation in the modified *Eco*RI-T fragment that might affect properties of the virus by altering a putative polyadenylation signal. Therefore, we decided to remove this point mutation. The first step involved the replacement of the 1.32 kb *Not*I-*Bam*HI fragment (Figures 4.2 and 4.6) in the *Eco*RI-T region of the genome with a zeocin cassette. Homologous recombination between the *Eco*RI-T fragment from pTETzeo (Table 3.1) and pMCMV_ETSwa$^-$ resulted in the BAC pMCMV_ETSwaZeo_1. This construct should contain a modified 3.67 kb *Eco*RI-T fragment that replaced the 4.54 kb *Eco*RI-T fragment from pMCMV_EGFP_ETSwa$^-$ (Figure 4.2 and Figure 4.6, panels B and C). Insertion of

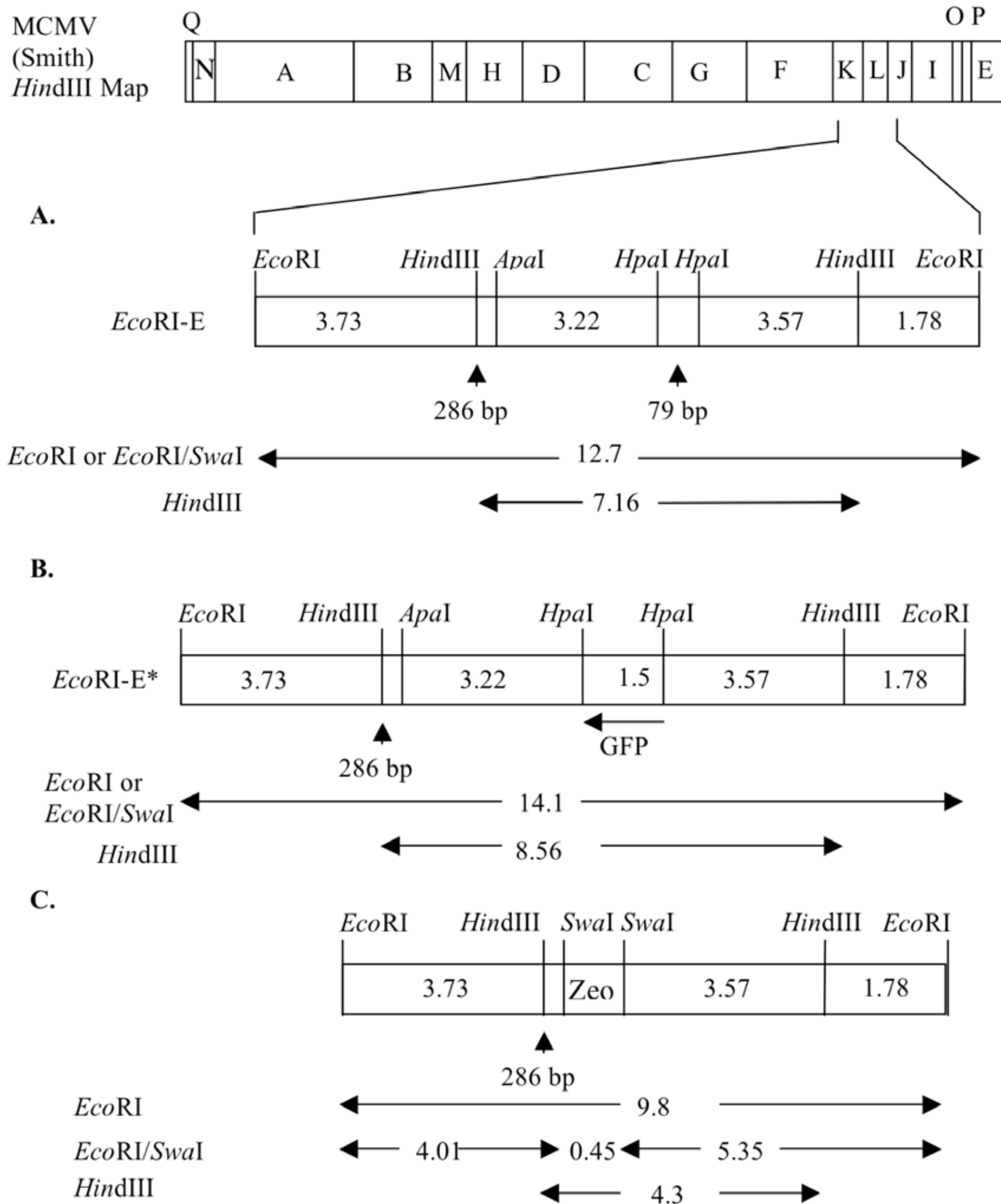

Figure 4.4: Restriction maps of the *Eco*RI-E region of the MCMV genome

The *Hin*dIII map of the MCMV genome is shown on the first line.

A: Wild-type *Eco*RI-E region; **B:** *Eco*RI-E* region containing GFP; **C:** *Eco*RI-E region containing a zeocin cassette in place of the *Apa*I-*Hpa*I region. The restriction sites relevant to this report are shown, with sizes of DNA fragments indicated in kilobase pairs (kbp) or base pairs. The GFP cassette contains an EF-1 promoter, the coding sequence for GFP, and a SV40 polyadenylation site. Numbers beneath each map indicate the sizes of DNA fragments in kbp obtained after digestion with *Eco*RI, *Eco*RI/*Swa*I or *Hin*dIII.

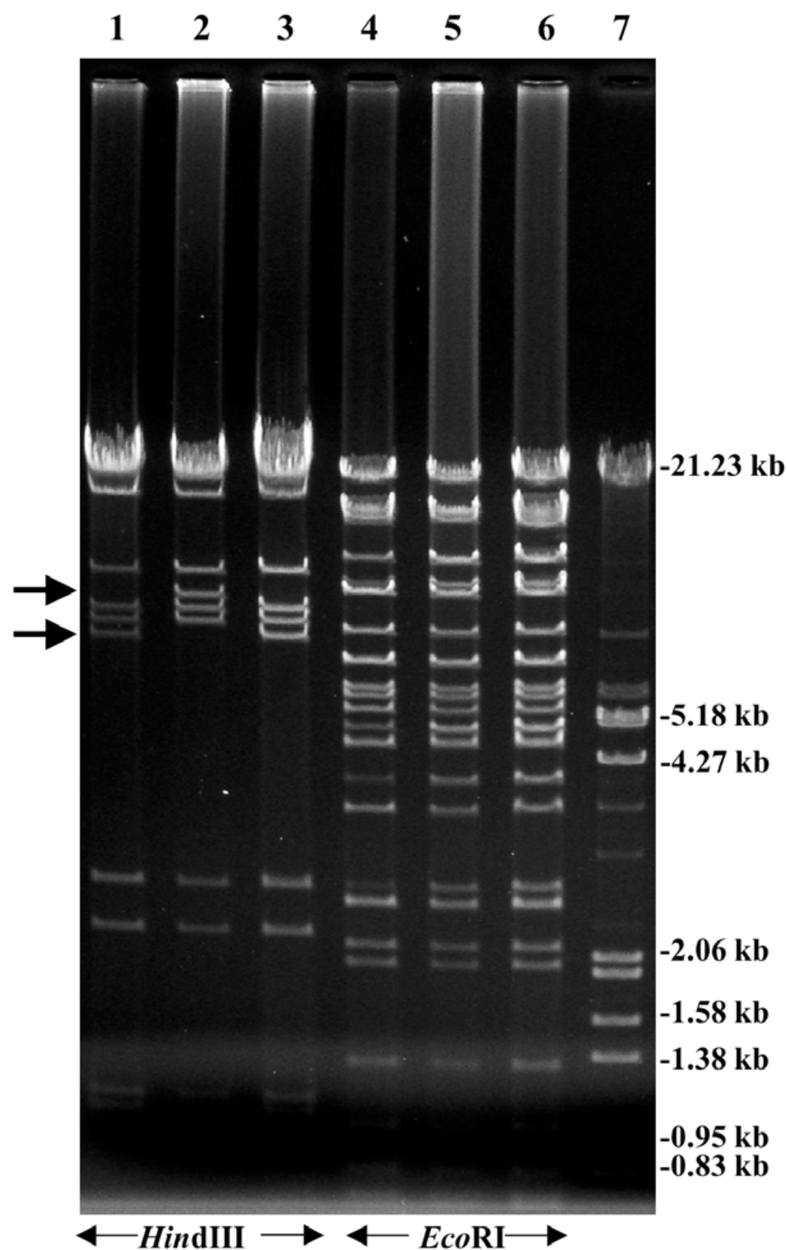

Figure 4.5: Restriction Analysis of pMCMV_ETSwa$^-$

DNA from pMCMV_ETSwa$^-$ clone 45 (lanes 3 and 6), clone 63 (lanes 1 and 4) and pMCMV_EGFP_ETSwa$^-$ (lanes 2 and 5) were digested with *Eco*RI or *Hin*dIII as indicated, run on a 0.7% agarose gel, and visualized by staining with ethidium bromide. Arrows on the left denote the positions of the 8.56 kb and 7.16 kb DNA bands. Numbers on the right represent the sizes of λ DNA fragments after digestion with *Eco*RI and *Hin*dIII (lane 7).

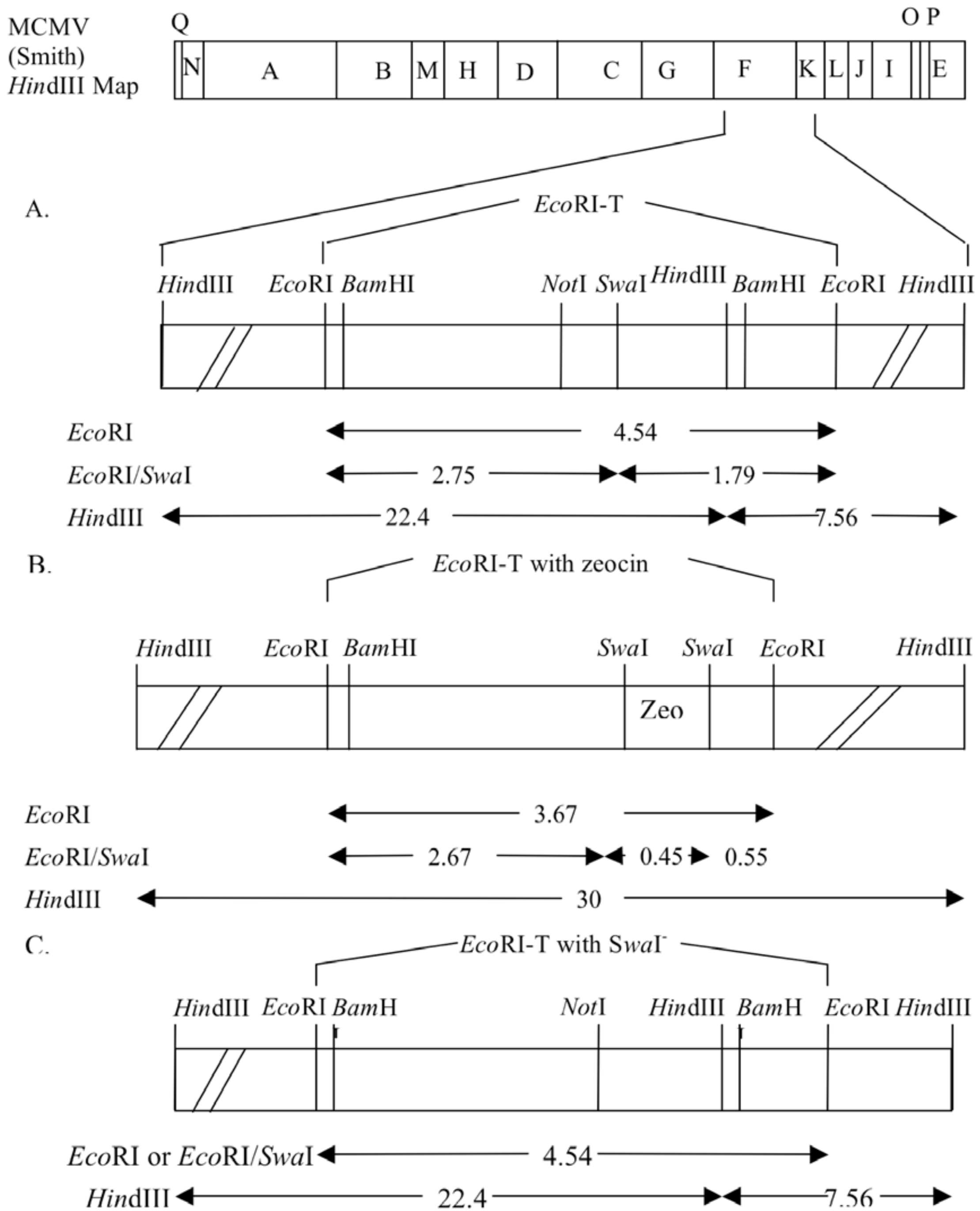

Figure 4.6: Diagrams of the *Hin*dIII-F and -K regions of the MCMV genome
The *Hin*dIII map for MCMV is shown on the first line.
A: Wild-type *Eco*RI-T region; **B:** *Eco*RI-T region containing a zeocin cassette in place of the *Not*I-*Bam*HI region; **C:** *Eco*RI-T region containing the *Swa*I$^-$ mutation. The restriction sites relevant to this report are shown, with DNA fragment sizes indicated in kbp. Numbers beneath the maps indicate the sizes of DNA fragments in kbp obtained after digestion with *Eco*RI, *Eco*RI/*Swa*I, or *Hin*dIII as indicated. The "double diagonal" lines represent a break in the map.

the zeocin cassette also resulted in the fusion of the 22.4 kb *Hin*dIII-F and 7.56 kb *Hin*dIII-K fragments, creating a 30.0 kb fragment via the removal of the *Hin*dIII site between these fragments (Figure 4.2, and Figure 4.6, panel B). All 4 zeocin resistant clones tested showed the expected restriction profile for pMCMV_ETSwaZeo_1 (Figure 4.7, and Table 4.2). After digestion with *Eco*RI, the 4.54 kb *Eco*RI-T fragment was lost (reduced intensity of the 4.54 kb DNA band in Figure 4.7, lanes 5 and 7, compared to the 4.54 kb band representing 2 co-migrating fragments in lane 6) and a 3.67 kb fragment appeared (Figure 4.7, lanes 5 and 7). In addition, the 7.56 kb *Hin*dIII-K fragment was replaced with the expected 30.0 kb fragment (cannot be resolved adequately from other high molecular weight DNA fragments of similar sizes on a 0.7 % gel) after digestion of pMCMV_ETSwaZeo_1 DNA with *Hin*dIII (arrow in Figure 4.7, lanes 1 and 3; Figure 4.6, panels B and C).

4.1.2.3 Construction of pMCMV_ETSwa$^-$*_1

Once we had successfully inserted the zeocin cassette into the *Eco*RI-T region of the MCMV genome, we wanted to replace the zeocin cassette with the modified 1.32 kb *Not*I-*Bam*H1 sequence containing the correct *Swa*I mutation. The BAC pMCMV_ETSwaZeo_1 was digested with *Swa*I to remove the zeocin cassette and co-transformed with the modified *Eco*RI-T fragment from pTETSwa$^-$* (Table 3.1) into the DY380 strain of *E. coli* to yield pMCMV_ETSwa$^-$*_1. The loss of the zeocin cassette and the incorporation of the *Swa*I mutation should result in a 4.54 kb *Eco*RI-T region and the restoration of the *Hin*dIII-F/K site (Figure 4.6, panel C). Following selection, replica plating, and restriction analysis, two zeocin sensitive clones had the expected restriction profile for pMCMV_ETSwa$^-$*_1 (Table 4.2). After digestion with *Eco*RI/*Swa*I, the 2.67 kb band was lost, and a 4.54 kb fragment appeared, resulting in a more intense DNA band at that position (arrows in Figure 4.8, lanes 1 and 2; Figure 4.6, panels B and C). The 0.45 kb and 0.55 kb bands were probably too faint to be seen at the bottom of the gel. In addition, digestion of pMCMV_ETSwa$^-$*_1 with *Hin*dIII resulted in the appearance of a 7.56 kb fragment not present during digestion of pMCMV_ETSwaZeo_1 (arrows in Figure 4.8, lanes 6 and 7).

4.1.2.4 Reconstruction of pMCMV_ETSwaZeo (pMCMV_ETSwaZeo_2)

Although we had previously constructed the two BACs pMCMV_ETwt_1 and pMCMV_ETSwa$^-$*_1, initial isolations of pMCMV_ETwt_1 from pMCMV_ETSwaZeo_1

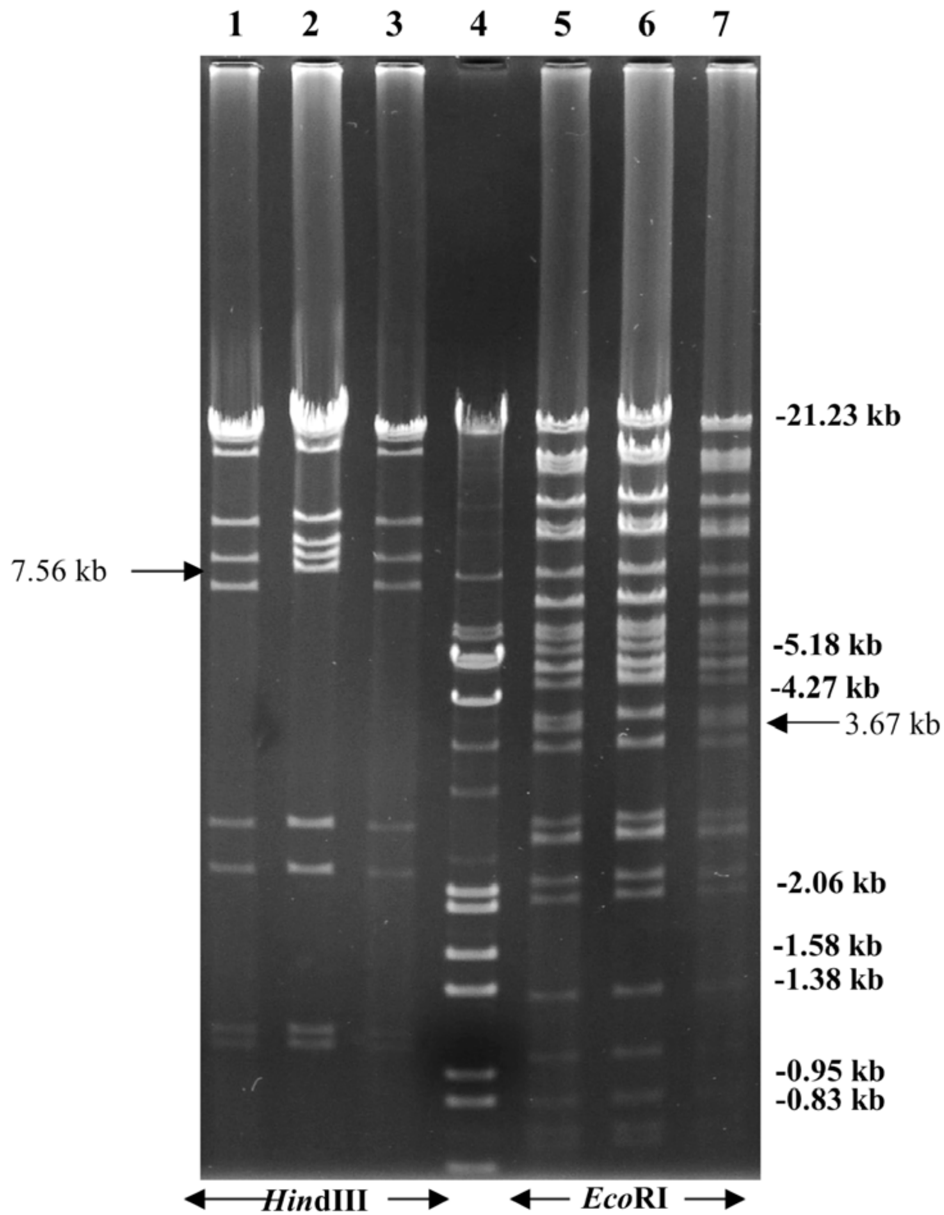

Figure 4.7: Restriction analysis of pMCMV_ETSwaZeo_1

DNA from pMCMV_ETSwaZeo_1, clone 1.4 (lanes 1 and 5), clone 1.1 (lanes 3 and 7) and pMCMV_EGFP_ETSwa$^-$ clone 39 (lanes 2 and 6) were digested with *Eco*RI or *Hin*dIII as indicated, run on a 0.7% agarose gel, and visualized by staining with ethidium bromide. Numbers on the right represent the sizes of λ DNA fragments after digestion with *Eco*RI and *Hin*dIII (lane 4).

Table 4.2 Analysis of MCMV BAC clones

MCMV BAC	Total number of zeocin sensitive clones	Number of clones tested	Number of clones with correct profiles	% of tested clones with correct profiles
pMCMV_ETSwaZeo_1	ND	4	4	100%
pMCMV_ETSwa^{-}	66	15 (23%)	4	27%
pMCMV_ETwt_1	32	4 (13%)	1	25%
pMCMV_ETSwa^{-}*_1	73	23 (32%)	2	8.7%
pMCMV_ETSwaZeo_2	ND	4	4	100%
pMCMV_ETwt_2	75	17 (23%)	5	29%
pMCMV_ETSwa^{-}*_2	145	8 (5.5%)	2	25%
pMCMV_ETSwa^{-}*/ie1SwaZeo	ND	4	4	100%

This table provides a summary of the data from my analysis of MCMV BAC clones relevant to this thesis. Briefly, zeocin sensitive or resistant MCMV BAC clones were isolated by replica plating and BAC DNA from these clones were tested for the expected profile by restriction analysis. When a zeocin cassette was inserted into a BAC (pMCMV_ETSwaZeo_1, pMCMV_ETSwaZeo_2, and pMCMV_ETSwa^{-}*/ie1SwaZeo), the total number of zeocin resistant clones were not determined (ND) since these numbers were typically quite large, and every clone tested was shown to possess the correct restriction profile. In addition, only a percentage of the zeocin sensitive clones were analyzed (shown inside brackets in column 3).

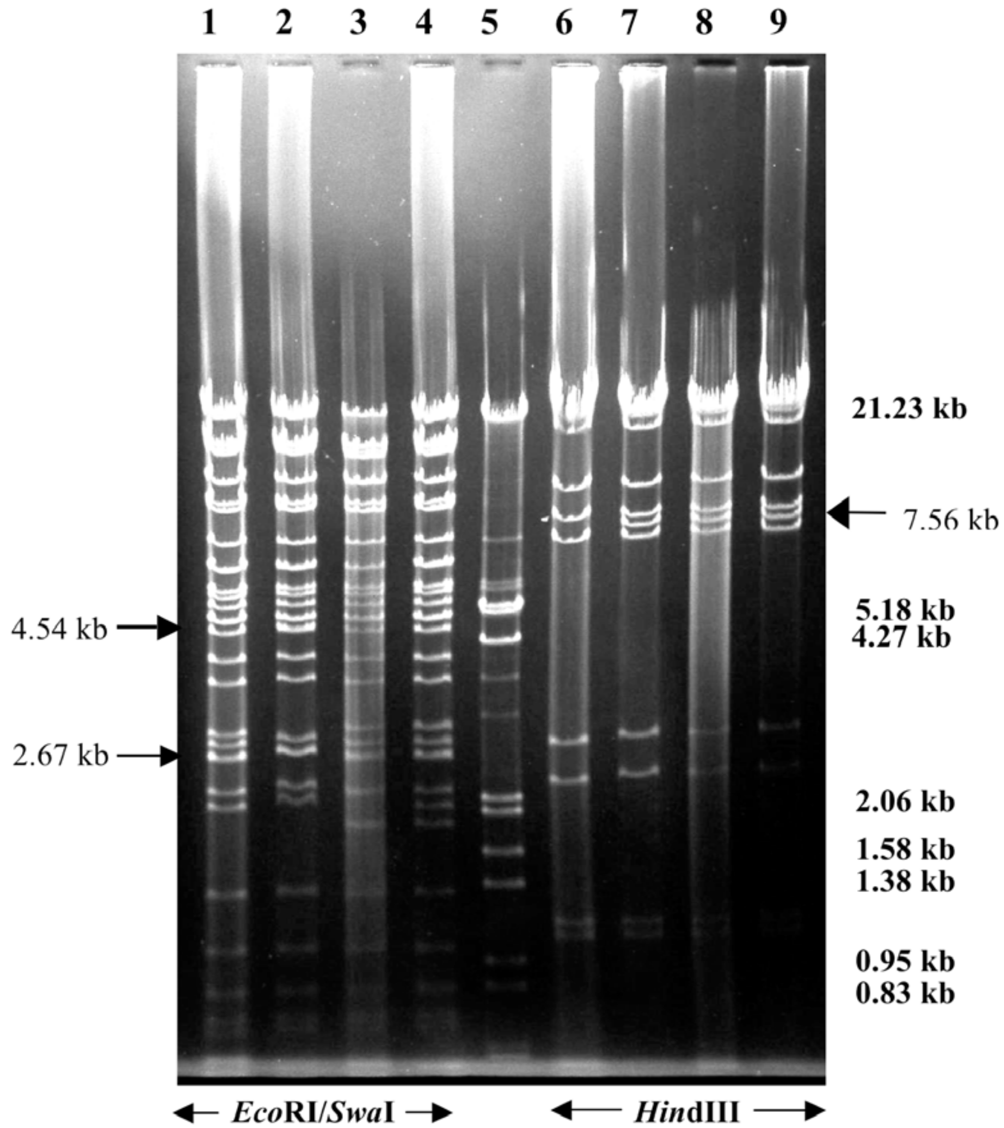

Figure 4.8: Restriction Analysis of pMCMV_ETSwa^{-}*_1

DNA from pMCMV_ETSwaZeo_1 clone 1.4 (lanes 1 and 6), pMCMV_ETSwa^{-}*_1 clone 1.16 (lanes 2 and 7), pMCMV_ETwt clone 1.27 (lanes 4 and 9) and viral DNA extracted from MCMV (Smith strain) virions (lanes 3 and 8) were digested with *Eco*RI and *Swa*I or *Hin*dIII as indicated, run on a 0.7% agarose gel, and visualized by staining with ethidium bromide. Arrows indicate the positions of DNA bands discussed in the text. Numbers on the right represent the sizes of λ DNA fragments after digestion with *Eco*RI and *Hin*dIII (lane 5).

(marked with ** in Figure 4.3) were very inefficient and inevitably resulted in the production of at most one correct clone per experiment. This was a concern as multiple clones with the correct profiles were generally obtained for all other recombinants constructed in this laboratory (Table 4.2 and unpublished results). Moreover, our observation that growth properties of recombinant MCMVs derived from these pMCMV_ETwt clones were not comparable to wild-type MCMV (data not shown) led us to believe that these BACs might contain genomic alterations that were not readily detectable through restriction analysis. Multiple attempts to construct correct pMCMV_ETwt clones from pMCMV_ETSwaZeo_1 clone 1.4 yielded similarly disappointing results, suggesting that perhaps the mutation resided in this particular construct. However, virus rescued from pMCMV_ETSwa$^-$*_1 clone 1.16 appeared to replicate normally in tissue culture. Therefore, we decided to use this BAC as a starting point to reconstruct pMCMV_ETwt (pMCMV_ETwt_2 in Figure 4.3).

First, the 4.54 kb *Eco*RI-T fragment in pMCMV_ETSwa$^-$*_1 clone 1.16 BAC was replaced with the 3.67 kb modified *Eco*RI-T fragment in the pTETzeo (Table 3.1; Figure 4.6, panels B and C). All four zeocin resistant clones tested had the expected profile for pMCMV_ETSwaZeo (Table 4.2). These BACs were designated pMCMV_ETSwaZeo_2. As shown in Figure 4.9, after digestion of pMCMV_ETSwaZeo_2 with *Eco*RI, one 4.54 kb fragment was lost (reduced intensity of the 4.54 kb band in lane 5 compared to the 4.54 kb band representing 2 co-migrating fragments in lane 4) and a new 3.67 kb fragment appeared (lane 5). Digestion with *Eco*RI and *Swa*I resulted in three bands from the *Eco*RI-T region of pMCMV_ETSwaZeo_2 (2.67 kb, as well as 0.45 kb and 0.55 kb bands which have probably run off the gel) in contrast to the 4.54 kb band representing the *Eco*RI-T region of pMCMV_ETswa$^-$*_1 clone 1.16 (Figure 4.9, lanes 1 and 2; Figure 4.6, panels B and C).

4.1.2.5 Reconstruction of pMCMV_ETwt (pMCMV_ETwt_2)

The pMCMV_ETSwaZeo_2 clone 2.4 BAC was used to reconstruct pMCMV_ETwt, a BAC that should contain "wild-type" MCMV sequences identical to the Smith strain of MCMV. To accomplish this, pMCMV_ETSwaZeo_2 was digested with *Swa*I and co-transformed into the DY380 strain of *E. coli* with the *Eco*RI-T fragment from pTE66 (Table 3.1) under conditions that promoted homologous recombination. Following replica plating and restriction analysis, five of the zeocin-sensitive colonies (Table 4.2) had the expected restriction profile for pMCMV_ETwt_2 (Figures 4.10 and 4.11). As expected (Figure 4.6,

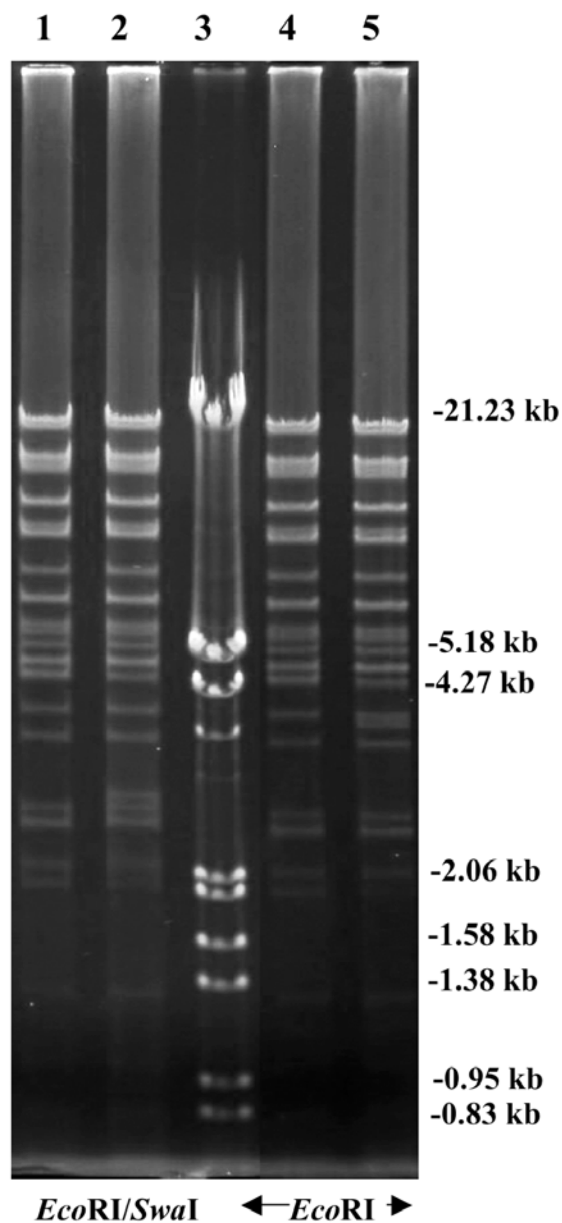

Figure 4.9: Restriction analysis of pMCMV_ETSwaZeo_2

DNA from pMCMV_ETSwaZeo_2, clone 2.1 (lanes 2 and 5), and pMCMV_ETSwa^{-}* clone 1.16 (lanes 1 and 4) were digested with *Eco*RI/*Swa*I or *Eco*RI as indicated at the bottom of the gel, run on a 0.7% agarose gel, and visualized by staining with ethidium bromide. Numbers on the right represent the sizes of λ DNA fragments after digestion with *Eco*RI and *Hin*dIII (lane 3).

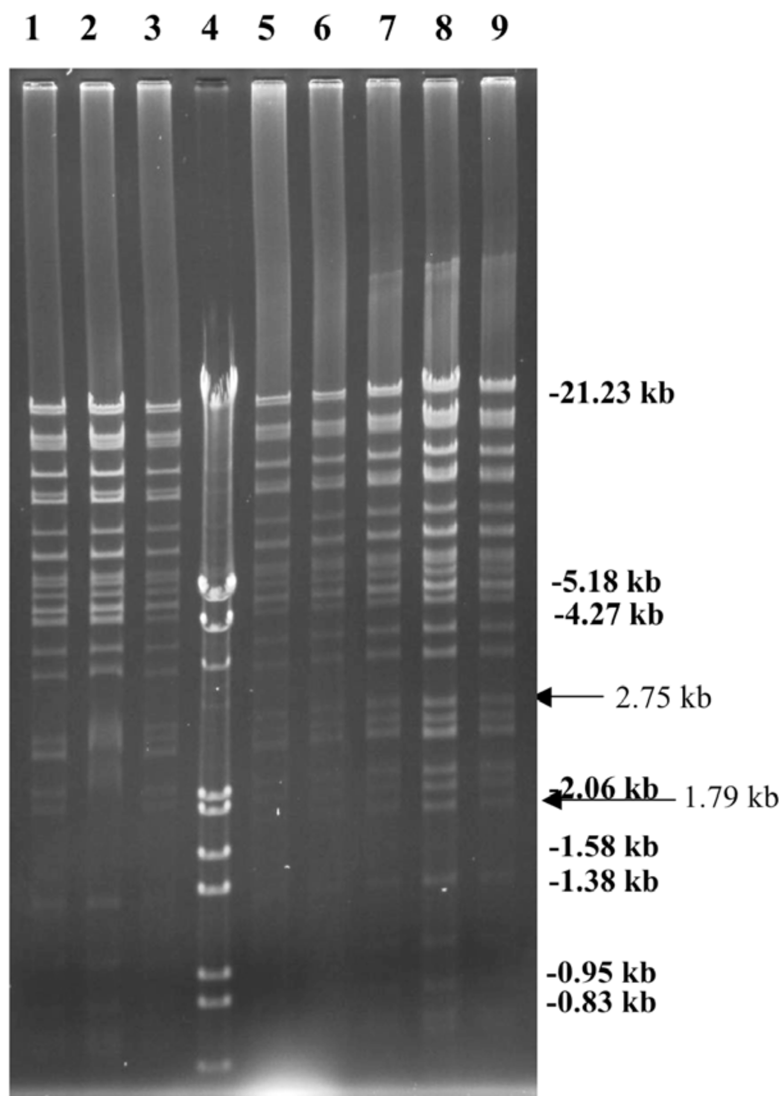

Figure 4.10: Restriction analysis of pMCMV_ETwt and pMCMV_ETSwa⁻*

DNA from pMCMV_ETSwa⁻*_2 clone 2.25 (lane 2) and clone 2.58 (lane 1), pMCMV_ETSwaZeo_2 (lane 3), and pMCMV_ETwt_2 clone 2.63 (lane 9), clone 2.71 (lane 8), clone 2.73 (lane 7), clone 2.107 (lane 6) and clone 2.119 (lane 5) were digested with *Eco*RI and *Swa*I, run on a 0.7% agarose gel, and visualized by staining with ethidium bromide. Numbers on the right represent the sizes of λ DNA fragments after digestion with *Eco*RI and *Hin*dIII (lane 4).

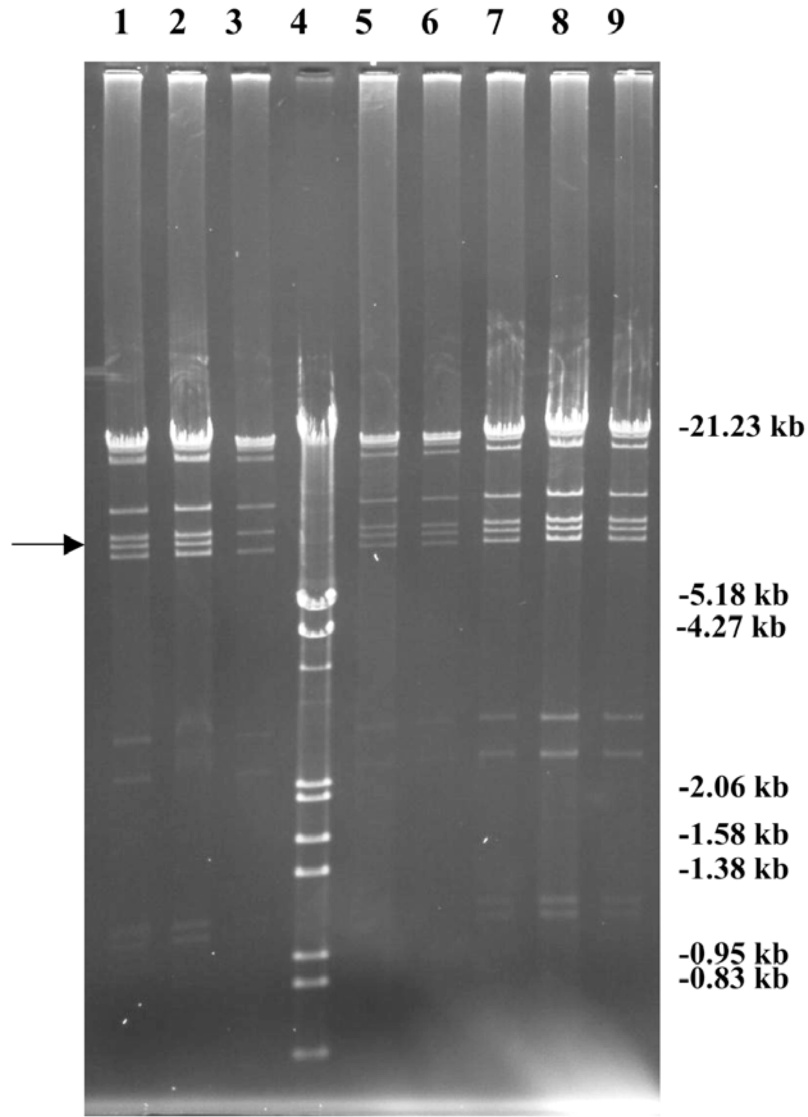

Figure 4.11: Restriction analysis of pMCMV_ETwt and pMCMV_ETSwa$^-$* with *Hin*dIII

DNA from pMCMV_ETSwa$^-$*_2 clone 2.25 (lane 2) and clone 2.58 (lane 1), pMCMV_ETSwaZeo_2 (lane 3), and pMCMV_ETwt_2 clone 2.63 (lane 9), clone 2.71 (lane 8), clone 2.73 (lane 7), clone 2.107 (lane 6) and clone 2.119 (lane 5) were digested with *Hin*dIII, run on a 0.7% agarose gel, and visualized by staining with ethidium bromide. The arrow on the left points to the position of the 7.56 kb band discussed in the text. Numbers on the right represent the sizes of λ DNA fragments after digestion with *Eco*RI and *Hin*dIII (lane 4).

panels A and B), digestion of pMCMV_ETwt_2 with *Eco*RI and *Swa*I resulted in the appearance of 2.75 kb and 1.79 kb bands (Figure 4.10, lanes 5-9) that were not present in pMCMV_ETSwaZeo_2 (Figure 4.10, lane 3). Moreover, the *Hin*dIII-F/K site should be restored in pMCMV_ETwt_2 (Figure 4.6, panels A and B). Thus digestion of pMCMV_ETwt_2 with *Hin*dIII resulted in the appearance of a 7.56 kb band (arrow in Figure 4.11, lanes 5-9) that was absent in pMCMV_ETSwaZeo_2 (Figure 4.11, lane 3). This was a vast improvement over previous attempts at constructing pMCMV_ETwt where only one recombinant had the expected restriction profile.

4.1.2.6 Reconstruction of pMCMV_ETSwa^{-}* (pMCMV_ETSwa^{-}*_2)

The BAC pMCMV_ETSwaZeo_2 was used to construct pMCMV_ETSwa^{-}*_2 so that it shared the same genetic background as pMCMV_ETwt_2 other than the *Swa*I mutation. The pMCMV_ETSwaZeo_2 BAC (clone 2.4) was digested with *Swa*I to remove the zeocin cassette and co-transformed with the modified *Eco*RI-T fragment from pTETSwa^{-}* (Table 3.1) into the DY380 strain of *E. coli*. Following selection, replica plating, and restriction analysis, two zeocin sensitive clones (Table 4.2) had the expected *Eco*RI/*Swa*I and *Hin*dIII restriction profiles for pMCMV_ETSwa^{-}*_2 (Figure 4.6, panels B and C). The loss of the zeocin cassette and the presence of the *Swa*I mutation should result in the appearance of a 4.54 kb fragment and the disappearance of the 2.67 kb fragment from this region following digestion of pMCMV_ ETSwa^{-}*_2 with *Eco*RI and *Swa*I (Figure 4.10, lanes 1 and 2). In addition, restoration of the *Hin*dIII-F/K site in pMCMV_ETSwa^{-}*_2 should result in the appearance of a 7.56 kb *Hin*dIII-K fragment (arrow in Figure 4.11, lanes 1 and 2). This BAC will be used as the "backbone" for the construction of MCMV mutants in the future using the protocol described in this thesis.

4.1.2.7 Construction of pMCMV_ETSwa^{-}*/ie1SwaZeo

As a first step towards constructing MCMV *mie1* mutants that could be useful for studying CMV host specificity, we replaced the 3.3 kb *Hpa*I-*Apa*I segment of the wild-type *Eco*RI-E fragment in pMCMV_ETSwa^{-}*_2 with the zeocin cassette from pTE-E/SwaZeo to create pMCMV_ETSwa^{-}*/ie1SwaZeo. The changes made to the *Eco*RI-E fragment could be seen in Figure 4.4 (panels A and C). Experimentally, this was accomplished via homologous recombination between the modified *Eco*RI-E fragment from pTE-E/SwaZeo and

pMCMV_ETSwa$^-$*_2 in DY380 cells as described in section 3.6. All four zeocin-resistant clones selected for BAC DNA purification and analysis showed the expected restriction profile for pMCMV_ETSwa$^-$*/ie1SwaZeo. As shown in Figure 4.4 (panels A and C), digestion of pMCMV_ETSwa$^-$*/ie1SwaZeo with *Eco*RI/*Swa*I resulted in the appearance of bands of 5.35 kb, 4.01 kb and 0.45 kb, and the disappearance of a 12.7 kb band (Figure 4.12, lanes 1-5). On the other hand, digestion of pMCMV_ETSwa$^-$*/ie1SwaZeo with *Hin*dIII resulted in the disappearance of the 7.16 kb *Hin*dIII-L fragment, and the appearance of a 4.3 kb band (Figure 4.12, lanes 7-11) when the 3.3 kb *Hpa*I-*Apa*I fragment was replaced with the 0.45 kb zeocin cassette (Figure 4.4, panels A and C). In the future, the pMCMV_ETSwa$^-$*/ie1SwaZeo BAC would be used for the construction of specific MCMV *mie*1 mutants by replacing the zeocin cassette with viral DNA fragments containing defined mutations or alterations of the MCMV *mie*1 gene via the homologous recombination methodology described in this thesis.

4.2 Characterization of Recombinant Viruses

Recombinant MCMVs were recovered from Balb/3T3 fibroblasts transfected with MCMV BACs as described in section 3.7, and recombinant viruses that are relevant to the studies described in this thesis are listed in Table 4.3.

4.2.1 Analysis of recombinant Viral DNA restriction patterns

Although the restriction profiles for pMCMV_EGFP, pMCMV_ETwt_2 and pMCMV_ETSwa$^-$*_2 were correct (section 4.1.2), we needed to ascertain that viral DNA purified from MCMV-EGFP, MCMV-ETwt, and MCMV-ETSwa$^-$ showed the expected restriction profiles. To this end, permissive Balb/3T3 fibroblasts were infected with these recombinant viruses as well as the Smith strain of MCMV, and viral DNA was purified from infected cells during the late phase of the replication cycle. The DNA was analyzed by digestion with either *Eco*RI/*Swa*I or *Hin*dIII and the results are shown in Figure 4.13. As predicted in Figure 4.6 (panels A and C), the major observable difference between MCMV-ETSwa$^-$ and the other 3 viruses lied in the *Eco*RI-T fragment. The MCMV (Smith), MCMV-EGFP, and MCMV-ETwt viruses should show very similar restriction profiles, with the *Eco*RI-T fragment being cleaved into 2.75 kb and 1.79 kb fragments during digestion with *Eco*RI/*Swa*I, whereas in MCMV-ETSwa$^-$, with the destruction of the *Swa*I site, the intact 4.54 kb *Eco*RI-T fragment would be seen. Although the insertion of the GFP cassette in

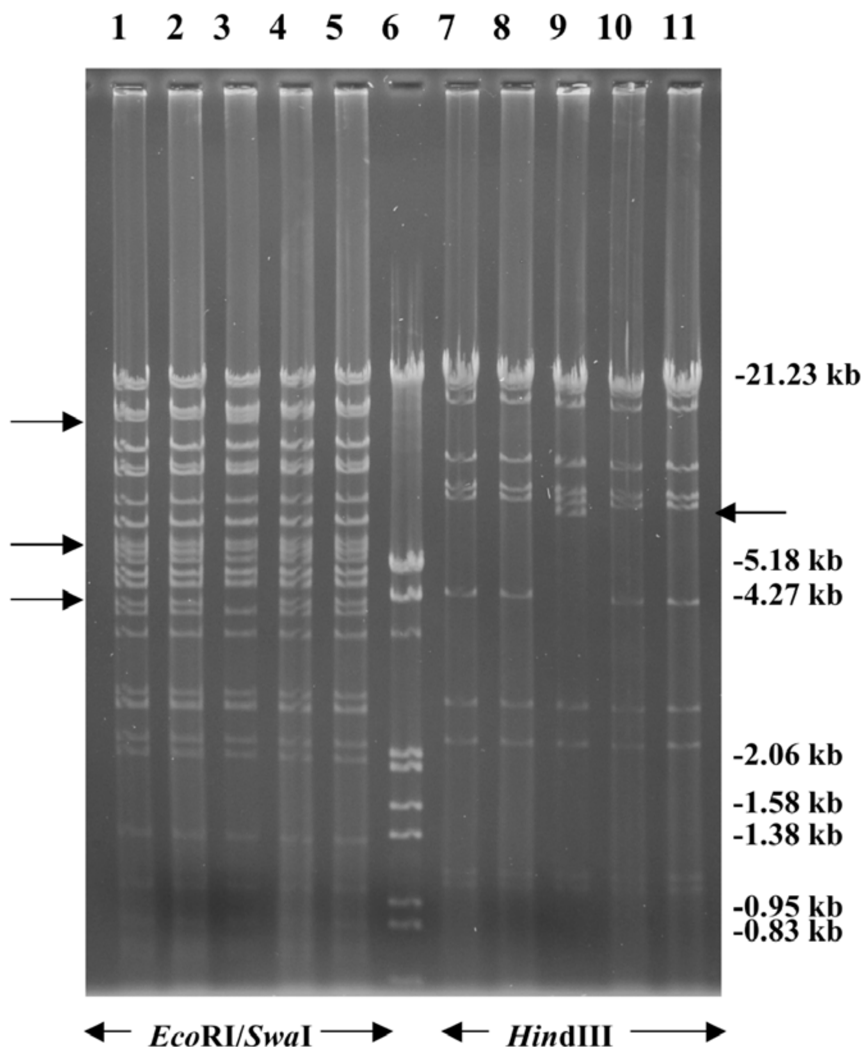

Figure 4.12: Restriction analysis of pMCMV_ETSwa⁻*/ie1SwaZeo

DNA from pMCMV_ETSwa⁻*/ie1SwaZeo clone 1 (lanes 1 and 7), clone 2 (lanes 2 and 8), clone 3 (lanes 4 and 10), clone 4 (lanes 5 and lane 11) and pMCMV_ETSwa⁻*_2 (lanes 3 and 9) were digested with *Eco*RI/*Swa*I or *Hin*dIII as indicated, run on a 0.7% agarose gel, and visualized by staining with ethidium bromide. Arrows on the left point to the locations of the 12.7 kb, 5.35 kb, and 4.01 kb fragments. The arrow on the right points to the location of the 7.16 kb fragment. Numbers on the right represent the sizes of λ DNA fragments after digestion with *Eco*RI and *Hin*dIII (lane 6).

Table 4.3 Production of recombinant MCMV stocks

Virus stock	MCMV BAC DNA
MCMV_ETSwa^{-}	pMCMV_ETSwa^{-}*_2
MCMV_ETwt	pMCMV_ETwt_2
MCMV_EGFP	pMCMV_EGFP

Each of the recombinant MCMVs listed on the left hand column was recovered from Balb/3T3 fibroblasts transfected with DNA purified from the MCMV BAC listed in the column immediately to its right using protocols described in section 3.7.

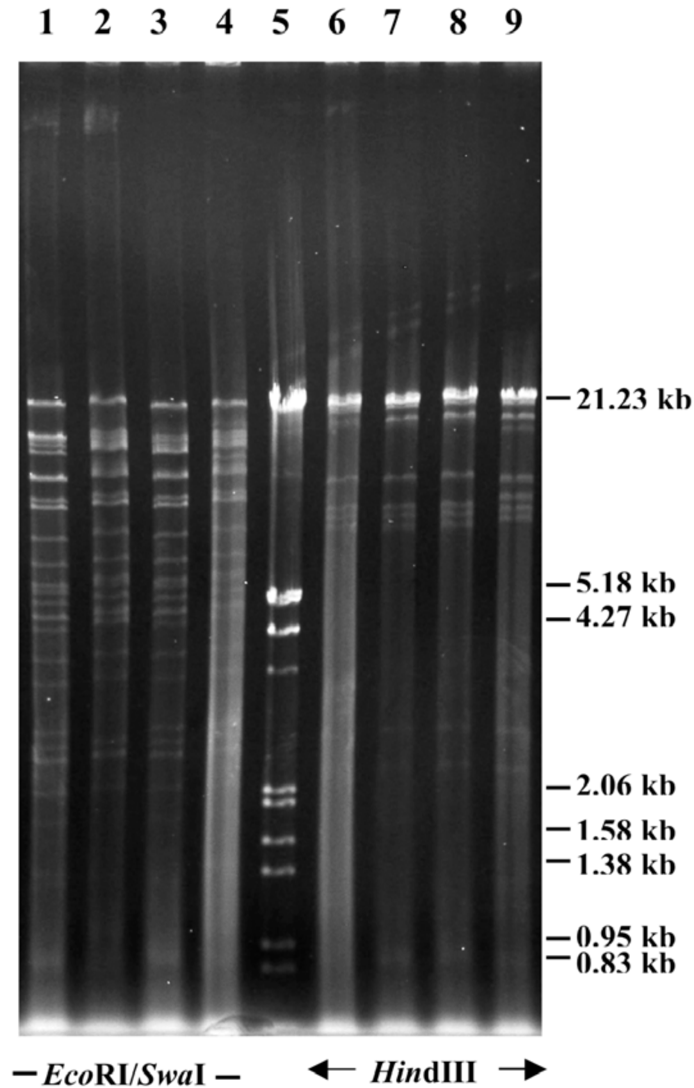

Figure 4.13: Restriction analysis of viral DNA isolated from MCMV-infected Balb/3T3 cells

Balb/3T3 cells were infected with the wild-type (Smith strain) or recombinant MCMV at a MOI of 1 when cells were about 70% confluent. When all cells showed cytopathic effects, viral DNA was isolated from cells infected with MCMV-EGFP (lanes 1 and 9), MCMV-ETSwa$^-$ (lanes 2 and 8), MCMV-ETwt (lanes 3 and 7) and the Smith strain of MCMV (lanes 4 and 6), and digested with *Eco*RI/*Swa*I or *Hin*dIII as indicated, run on a 0.7% agarose gel, and visualized by staining with ethidium bromide. Numbers on the right represent the sizes of λ DNA fragments after digestion with *Eco*RI and *Hin*dIII (lane 5).

MCMV-EGFP produced a slightly larger *Eco*RI-E fragment (14.1 kb versus 12.7 kb), the difference would not be obvious on a 0.7% agarose gel. This was exactly what was observed in Figure 4.13 (lanes 1-4). It was noted that the DNA fragments from MCMV (Smith strain) were not particularly well resolved even though they were all present (lane 4). In contrast, MCMV-EGFP was most easily distinguishable from the other 3 viruses by its *Hin*dIII restriction profile (predicted in Figure 4.4, panels A and B). As shown in Figure 4.13 (panels 6-9), the modified *Hin*dIII-L fragment of MCMV-EGFP, with the insertion of the GFP cassette, was 8.56 kb, whereas the unaltered fragments from the other 3 viruses were 7.16 kb. Therefore it is likely that no major genome rearrangements have occurred in the recombinant viruses rescued from cells transfected with the 3 selected MCMV BACs based on the restriction analyses performed here.

4.2.2 Expression of representative MCMV genes

I then proceeded to explore whether there were differences exhibited in the progress of these recombinant viruses through their life cycles. The time course and level of expression of viral proteins belonging to different kinetic classes during MCMV infection was monitored and no significant differences between MCMV (Smith strain), MCMV-EGFP, and MCMV-ETSwa$^-$ (Figure 4.14) were found. For example, the M112-113 proteins were already highly expressed at 7 hours post-infection (panel A) and persisted throughout the infection. The major gene products with sizes of 36 kDa and 38 kDa were readily detectable in infected cells, whereas bands representing the 33 kDa and 87 kDa proteins were faint, but still visible. Meanwhile, ppM44, a delayed early protein was barely detectable at 12 hours post-infection, but expressed at high levels only after DNA replication had commenced (panel B). Finally, the 128 kDa gB precursor, the most abundant form of the glycoprotein in infected cells, was faintly detectable during the late phase of infection (panel C). Thus, the level of accumulation of viral proteins in infected cells did not differ significantly between the different MCMV recombinants, indicating that neither the *Swa*I mutation nor the presence of the GFP insertion affected the expression of viral proteins belonging to different kinetic classes. In addition, the coding sequences for the viral proteins monitored in this experiment are located in different parts of the viral genome. Therefore, my results provided additional support for the absence of aberrant genome rearrangements during the construction of MCMV mutants.

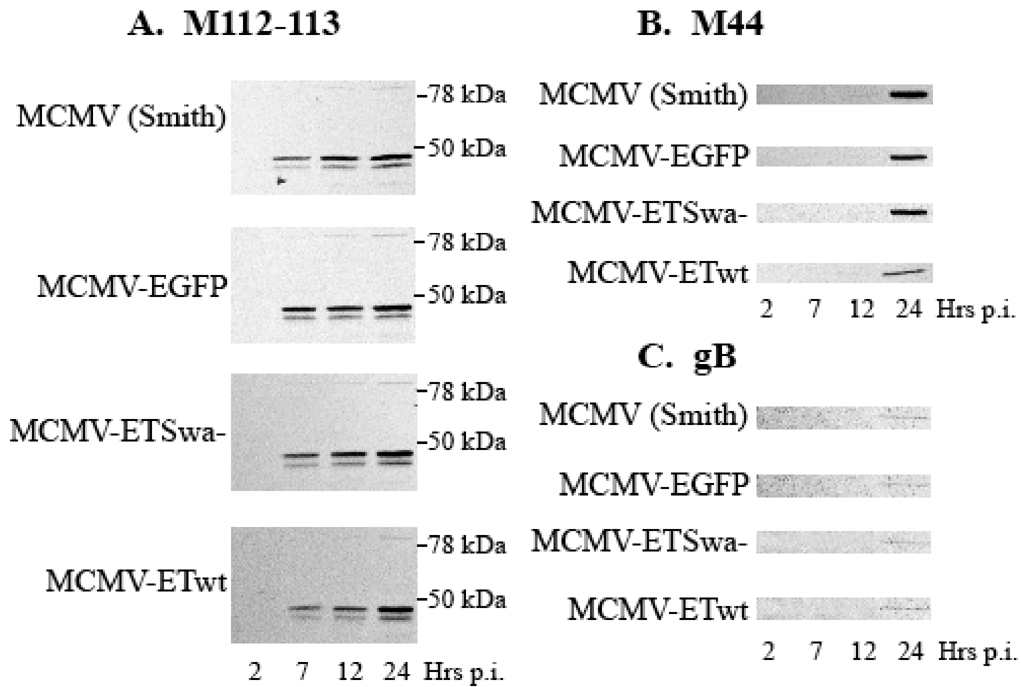

Figure 4.14: Expression of viral proteins during infection by recombinant murine cytomegaloviruses

Balb/3T3 fibroblasts infected by recombinant MCMV (MOI = 5) were harvested at 2, 7, 12, and 24 hours post-infection (Hrs p.i.) and processed for Western blotting. The viral gene products M112-113 (A), M44 (B), and gB (C) were detected by their reactivity with MAbs 9D3.22A, 3B9.22A, and 2E8.21A+8G5.22A, respectively. The numbers to the right of panel A represent the sizes of pre-stained molecular weight markers in kiloDaltons. The recombinant MCMVs used were listed to the left of each panel. The different panels representing the Western blot analyses were prepared in Adobe Photoshop CS3.

4.2.3 Comparison of recombinant viral growth in different cell lines

One of the ways I compared the recombinant viruses MCMV-ETwt, MCMV-ETSwa$^-$ and MCMV-EGFP was to analyze their growth properties by monitoring virus production in both permissive (Balb/3T3) and semi-permissive (COS-1) cells via plaque assays. These viruses were selected for analysis based on the rationale presented in section 4.1.1. In the permissive Balb/3T3 cell line, recombinant virus production was analyzed at both high and low MOI. During high MOI infections, the intention was to infect all the cells in a culture simultaneously. This ensured that the virus replication cycle was synchronized and data for a one-step growth curve could be analyzed. In contrast, during low MOI infection, only a small fraction of the total cell population was initially infected, and thus the ability of the virus to spread from cell to cell during infection could be monitored. In this instance, infected Balb/3T3 cells were monitored until most of the cells were destroyed by the virus spread over the course of infection. Finally, because growth of MCMV is abortive in COS-1 cells at low MOI, viral growth was only examined through high MOI infections.

The overall trend of viral growth in Balb/3T3 cells at low MOI demonstrated that MCMV-EGFP, MCMV-ETwt and MCMV-ETSwa$^-$ all behaved very similarly to MCMV (Smith), the virus which served as the wild-type control (Figure 4.15). All four viruses showed similar growth kinetics with peak titers at 5 days post-infection of between $1.27 \times 10^7 \pm 1.41 \times 10^5$ (MCMV-EGFP) and $1.68 \times 10^7 \pm 9.90 \times 10^5$ (MCMV-ETwt) PFU/10^6 cells. After this point, there was a slow drop in virus titers to between $2.16 \times 10^6 \pm 8.72 \times 10^4$ (MCMV-ETwt) and $4.05 \times 10^6 \pm 7.78 \times 10^5$ (MCMV-EGFP) PFU/10^6 cells.

Comparison of viral growth at high MOI in Balb/3T3 cells also demonstrated that the four viruses had very similar growth kinetics (Figure 4.16). However, the virus titers varied to a greater extent than that observed during low MOI infection of Balb/3T3 cells. However, in plaque assays, a difference in virus titers of 2-3 fold is not very significant and is more likely to be due to normal experimental variations rather than real differences between the recombinant viruses themselves unless these differences can be demonstrated consistently over multiple experiments. Virus production peaked around 3 days post-infection for all 4 viruses. Overall, it would appear that during both high and low MOI infection in permissive cells, MCMV-ETwt and MCMV-ETSwa$^-$ both behaved very similarly to MCMV (Smith), indicating that the *Swa*I$^-$ mutation did not appear to have a significant effect on virus growth. Thus, the

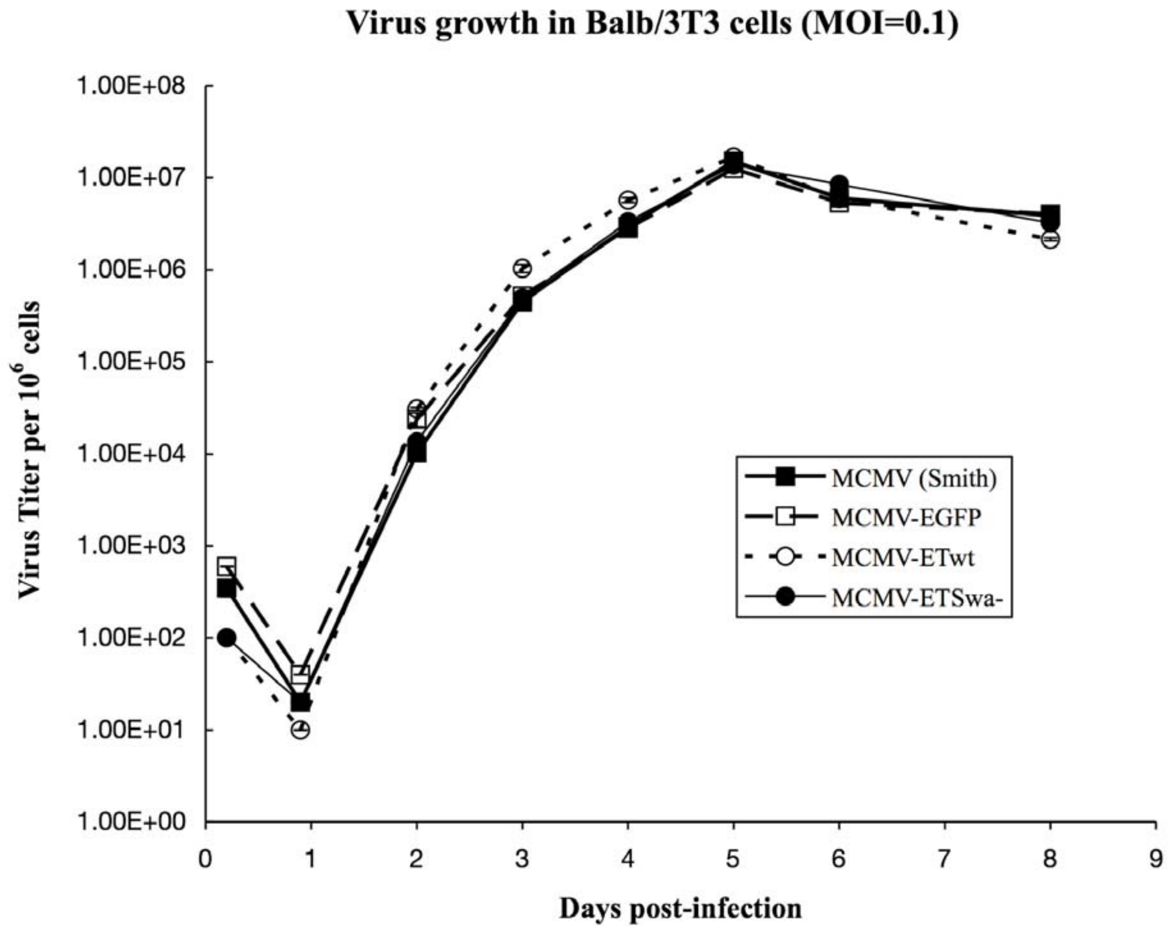

Figure 4.15: Replication of recombinant MCMVs in Balb/3T3 cells at low MOI

Balb/3T3 cells were infected with either the Smith strain of MCMV, MCMV-EGFP, MCMV-ETwt, or MCMV-ETSwa$^-$ at a MOI of 0.1. Virus released into the media at appropriate times was quantified by plaque assays. Each data point was calculated as the mean of 2 to 4 determinations, with error bars representing the standard deviations. Some of the standard deviations are so small that the error bars are hidden beneath the symbols representing the data points. The legend for the recombinant viruses used is shown in the graph.

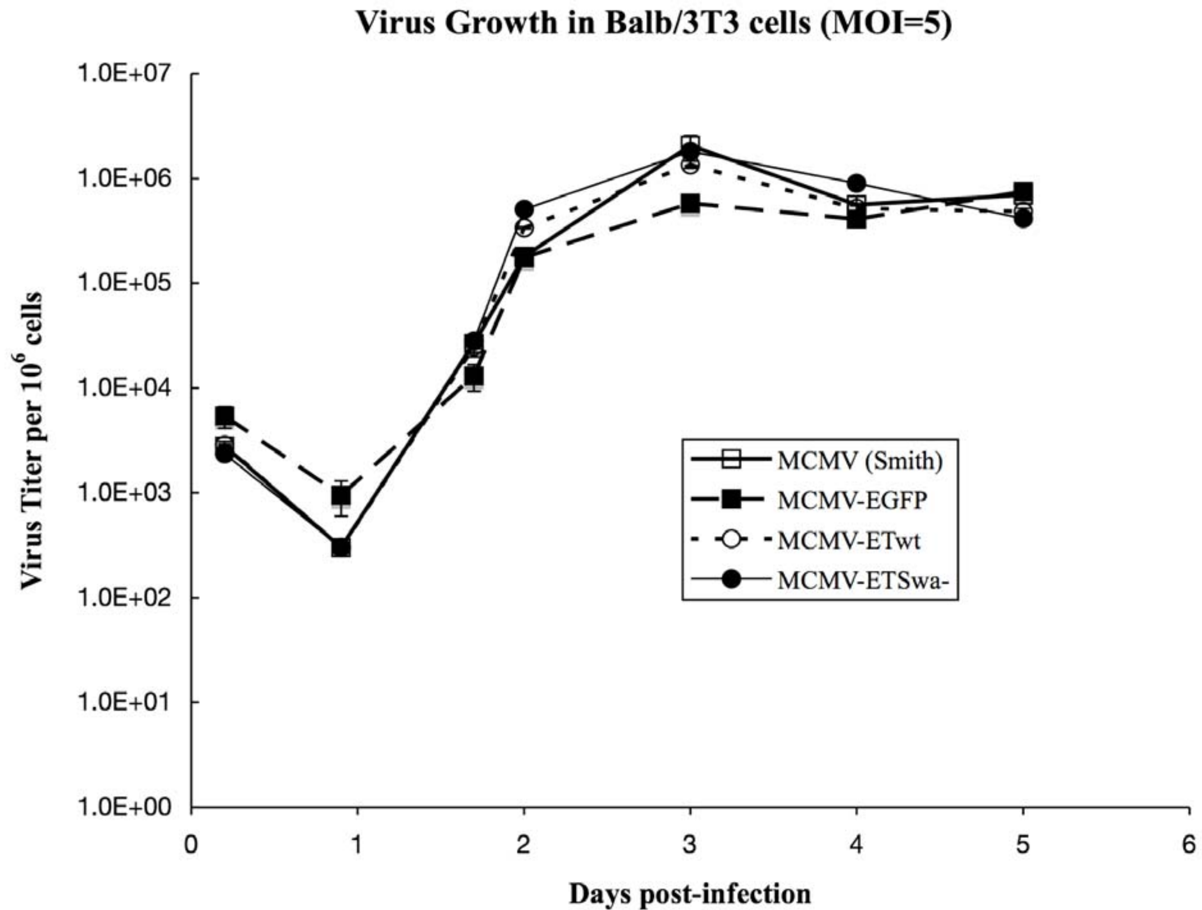

Figure 4.16: Replication of recombinant MCMVs in Balb/3T3 cells at high MOI

Balb/3T3 cells were infected with either the Smith strain of MCMV, MCMV-EGFP, MCMV-ETwt, or MCMV-ETSwa$^-$ at a MOI of 5. Virus released into the media at appropriate times was quantified by plaque assays. Each data point was calculated as the mean of 2 to 4 determinations, with error bars representing the standard deviations. Some of the standard deviations are so small that the error bars are hidden beneath the symbols representing the data points. The legend for the recombinant viruses used is shown in the graph.

phenotypes of both recombinant MCMVs resembled that of the Smith strain of MCMV. In addition, the growth of MCMV-EGFP was very similar to both MCMV-ETwt and MCMV (Smith) indicating that in permissive cells the insertion of the GFP cassette into *mie2* does not appear to have a significant affect on viral growth kinetics.

Virus growth at high MOI in COS-1 cells, a non-murine semi-permissive cell line, was also examined (Figure 4.17). Upon observation under the microscope, the number of cells showing cytopathic effects was significantly less than that observed in Balb/3T3 cells even when the same high MOI was used. All virus infections still followed the same pattern of the MCMV (Smith) strain, with an eclipse phase following the initial infection, virus titers that peaked at 3 days post-infection, and slowly declined throughout the infection after this point. Interestingly, the MCMV-EGFP virus had higher virus titers than the other 3 viruses, particularly between 4 and 5 days post-infection (Figure 4.17). This differed from observations made from similar experiments conducted previously in our laboratory. One plausible explanations for this observation is that the COS-1 cell line might have mutated during multiple cell passages. As a result, cells could become confluent much faster, at which point virus production would decrease rapidly. Therefore, in order to obtain results similar to those observed previously in this laboratory, a higher dosage of virus may be necessary, so that a larger percentage of COS-1 cells would exhibit cytopathic effects early in infection.

The results of COS-1 infection indicated that the growth properties of MCMV-ETwt were similar to that of MCMV (Smith), and thus the “wild-type” phenotype of the virus appeared to have been restored. MCMV-ETSwa$^-$ also behaved very similarly to MCMV (Smith), indicating that the *Swa*I$^-$ mutation did not appear to have an affect on the growth properties of MCMV in COS-1 cells.

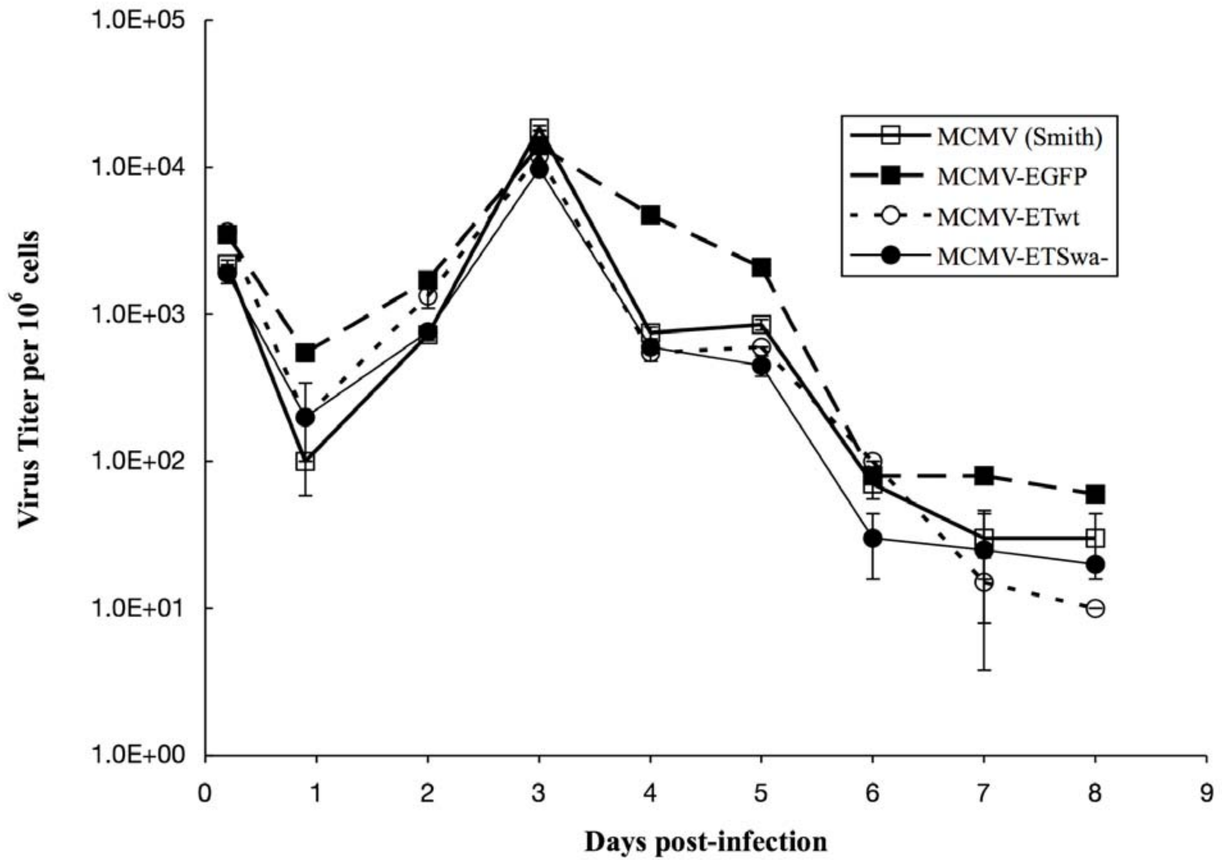

Figure 4.17: Replication of recombinant MCMVs in COS-1 cells at high MOI

COS-1 cells were infected with either the Smith strain of MCMV, MCMV-EGFP, MCMV-ETwt, or MCMV-ETSwa$^-$ at a MOI of 5. Virus released into the media at appropriate times was quantified by plaque assays. Each data point was calculated as the mean of 2 to 4 determinations, with error bars representing the standard deviations. Some of the standard deviations are so small that the error bars are hidden beneath the symbols representing the data points. The legend for the recombinant viruses used is shown in the graph.

5.0 DISCUSSION

5.1 Feasibility of using a modified viral genome for the construction of recombinant murine cytomegaloviruses

In this thesis, I have provided evidence that our method of constructing recombinant MCMV using a modified viral genome is viable and efficient. This method is reliant upon the replacement of a specific region on the genome with a zeocin cassette flanked by *Swa*I sites (Figure 4.1), necessitating the removal of the unique *Swa*I site from the MCMV (Smith strain) genome. To this end, I constructed pMCMV_ETSwa$^-$* which contained a single point mutation in the unique recognition sequence for *Swa*I. In addition, the GFP cassette in pMCMV_EGFP, which represented the start of our BAC construction lineage, was removed. Furthermore, I constructed pMCMV_ETwt where the mutated *Swa*I site in pMCMV_ETSwa$^-$* was restored. The successful construction of this BAC using the aforementioned methodology demonstrated that after successful introduction of specific mutations into the viral genome, I could revert the mutated *Swa*I site in the *Eco*RI-T fragment of the MCMV genome to wild-type sequences if the necessity arose (see sections 5.2 and 5.3).

An advantage of our method of constructing viral recombinants is that co-transformation of the linearized MCMV BAC genome with a mutated viral DNA fragment into a recombination competent *E. coli* strain such as DY380 results in efficient recovery of MCMV BACs incorporating the desired mutation via homologous recombination. This is due to the fact that bacterial transformation efficiency is much higher with circular rather than linear plasmids. Thus, upon homologous recombination, the re-circularized MCMV BACs containing the mutation will be recovered at higher frequencies than linearized BACs. Our experimental results support this prediction since the percentage of MCMV BAC clones that lost zeocin resistance, and thus presumably has incorporated the desired mutation, was fairly high (Table 4.2). The initial screening for loss of zeocin resistance ensured that the number of recombinant clones that needed to be tested was manageable (Table 4.2). Subsequently, correct MCMV recombinants were identified by restriction analysis of MCMV BACs selected

for zeocin-sensitivity (Figures 4.5, 4.7-4.12). This analysis also allowed me to ascertain that no major rearrangements of the MCMV genome had occurred after the introduction of the desired mutations or alterations. In general 25% or higher of zeocin sensitive BAC clones had the correct profile (Table 4.2), indicating that our method of selecting recombinant BACs was quite efficient.

Another potential advantage of our method of recombinant virus construction is that, unlike some of the approaches described in the introduction section, no foreign sequences are left at the site where the desired mutations are introduced. This may be important in studies on gene expression and DNA replication where sequences affecting promoters, enhancers, or origin of replication could be altered.

5.2 Effect of the *Swa*I mutation on the biological properties of MCMV

The biological properties of the recombinant MCMV-ETwt and MCMV-ETSwa$^-$ viruses and the standard Smith strain of MCMV were compared through analysis of viral DNA restriction patterns (Figure 4.13), protein expression (Figure 4.14), and viral growth curves in both permissive and semi-permissive cell lines (Figures 4.15-4.17). In so doing, I have provided preliminary evidence that the *Swa*I mutation did not appear to have an effect on selected biological properties of MCMV. Furthermore, the similarities between MCMV-ETwt and the Smith strain of MCMV suggested that our method of constructing recombinant MCMVs did not appear to result in unintentional alterations to the MCMV genome. However, since the experiments outlined in sections 4.2.1-4.2.3 have been carried out only once, they should be repeated to confirm the observations described in this thesis.

5.3 Future experiments to analyze the effect of the *Swa*I mutation on the biological properties of MCMV

Additional comparisons between MCMV-ETSwa$^-$, MCMV-ETwt and the Smith strain of MCMV during infection of Balb/3T3 cells can be made by carrying out indirect immunofluorescence assays on infected cells with monoclonal antibodies specific for MCMV proteins expressed at different stages of the virus life cycle. These assays will be used to analyze the expression of immediate-early, early and late MCMV genes, the intracellular locations of these gene products, and the development of replication compartments at different times post-infection. Results from these experiments may provide additional evidence to support my aforementioned conclusions.

One of the assumptions that I made during my studies on infectious virus production is that MCMV released into the media is representative of total virus production during the virus life cycle. However, it is possible that the *Swa*I mutation could affect the release of MCMV into the media. Thus, it may be prudent to monitor the amount of cell-associated virus during infection by recombinant MCMVs before reaching a definitive conclusion.

Although *in vitro* experiments described in this thesis have provided evidence to suggest that the *Swa*I mutation did not have an effect on selected biological properties of MCMV, further experimentation needs to be carried out to better evaluate the potential biological effects of this mutation *in vivo*. There are several examples of CMV genes that are conserved between different virus strains, but do not affect replication in permissive tissue culture cells. However, they are hypothesized to be required for successful *in vivo* infections. For example, in HCMV, both *US3* and *US11* genes play a role in immune evasion, a function not required *in vitro* (see section 1.2.4.2.2). Human CMV *US10* and MCMV *ie2*, whose potential roles in the viral life cycle have not yet been determined (Manning and Mocarski, 1988; Jones *et al.,* 1991; Greaves *et al.,* 1995), do not appear to be essential for infection of permissive cells *in vitro*. Although the point mutation introduced into the MCMV genome that destroyed the *Swa*I site did not interrupt any known genes, the possibility remains that it could disrupt an unknown control sequence, an intron, or an exon that is critical for successful *in vivo* infection.

The biological effects of the *Swa*I mutation on *in vivo* MCMV infection can be determined by infecting Balb/C mice with the recombinant viruses MCMV-ETSwa$^-$, MCMV-ETwt, and the Smith strain of MCMV. Virus infection can be monitored in the spleen, kidneys, lungs, and salivary glands by harvesting these organs and performing plaque assays as well as histochemical examinations of infected tissues. As our method of manipulating the viral genome did not appear to affect the biological properties of MCMV-ETwt *in vitro*, we expect that this virus would have a similar course of infection as the Smith strain of MCMV. If results from these experiments with mice infected with MCMV-ETSwa$^-$ and the Smith strain of MCMV differ significantly, the *Swa*I site will need to be restored following future manipulations of the MCMV genome. I have already demonstrated this that is possible through the construction of pMCMV_ETwt. The results of these *in vivo* studies will provide more conclusive evidence regarding the biological effects of the *Swa*I mutation.

5.4 Utility of the *Swa*I$^-$ MCMV backbone to future research in our laboratory

Once *in vivo* testing is completed to ensure that there are no obvious biological effects due to the *Swa*I mutation, the pMCMV_ETSwa$^-$* backbone will be very useful for the construction of recombinant MCMV. This will enable the construction of recombinant MCMV with specific alterations/mutations in any region of the viral genome. Alternatively, chimeric viruses where MCMV coding sequences are replaced by those from corresponding HCMV homologs can be constructed to investigate the mechanisms of host specificity or facilitate the testing of anti-HCMV therapies.

As a specific example of this, the pMCMV_ETSwa$^-$*/ie1SwaZeo BAC (section 4.1.2.7) can be used to construct a *mie1* null mutant containing a stop codon at the beginning of exon 4, which is unique to this gene. In addition, the *mie*1 exon 4 sequences could be replaced with the corresponding sequences from HCMV *ie1*. We shall leave the splicing signals intact so that the structures of immediate-early transcripts would not be altered. The resultant recombinant MCMVs could be used to investigate the role of *mie1* in host specificity by monitoring infection and gene expression in murine and human cells. The pMCMV_ETSwa$^-$*/ie1SwaZeo BAC could also be used to incorporate specific mutations into *mie*1 or HCMV *ie*1 sequences that are required for interactions with cellular proteins such as PML, DAXX, and HDAC, and facilitate the mapping of functional domains that may be involved in these interactions. Thus, their effects on cytomegalovirus host specificity can be evaluated.

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