Functional Characterization Of The 40 bp Internal Repeat Of Murine Gamma Herpesvirus-68

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JAI MA SATI

(Dedicated to my lovingly Family)

Declaration

I declare that this is my first dissertation work to pursue the PhD degree.

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Statement

I have written this thesis independently, without the help of others. The content of this thesis is based on experiments I performed by myself.

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List of Abbreviations

Abs	Antibodies
AIDS	Acquired immune deficiency syndrome
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
BL	Burkitt's lymphoma
bp	Base pairs
CTL	Cytotoxic T lymphocytes
dNTP	3' deoxyribonucleoside-5'-triphosphate
DIG	Digoxygenin
DMSO	Dimethyl sulphoxide
DMEM	Dulbecco's minimum essential medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EBER	Epstein-Barr virus encoded RNA
EBNA	Epstein-Barr Virus nuclear antigen
EBV	Epstein-Barr Virus
E. coli	Escherichia coli
EDTA	Ethylene diamine tetra-acetic acid
FACS	Fluorescence assisted cell sorting
FCS	Foetal calf serum
FITC	Fluorescein-isothiocyanat
GC	Germinal centre
GFP	Green fluorescence protein
HCMV	Human Cytomegalovirus
HD	Hodgkin's disease
HHV-4	Human herpesvirus 4
HHV-8	Human herpesvirus-8
HIV	Human immunodeficiency virus
HVS	Herpes virus saimiri
IFN	Interferon
IFN	Interferon
IL	Interleukin
IL	Interleukin
i.n.	intranasal
Kan	Kanamycin
kbp	Kilobase pair (s)
KSHV	Kaposi's sarcoma-associated herpesvirus
KS	Kaposi's sarcoma
LB	Luria-Bertani
LCL	Lymphoblastoid cell line
LMP	Latent membrane protein
LPD	Lymphoproliferative disease
MCD	Multicentric Castleman's Disease
MHC	Major histocompatibility complex
MHV-68	Murine gamma herpesvirus-68
MLN	Mediastinal lymph node
MOI	Multiplicity of infection

ml	Milliliter
µl	Microliter
NK cells	Natural killer cells
NPC	Nasopharyngeal carcinoma
oriLyt	Origin of lytic replication
oriP	Origin of plasmid replication
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEL	Primary effusion lymphoma
PFU	Plaque forming units
PMA	Phorbol myristate acetate
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Room temperature
SDS	Sodiumdodecylsulphate
SSC	Sodium chloride-sodium citrate buffer
TAE	Tris-acetate-EDTA buffer
TR	Terminal repeats
Tris	Tris-hydroxymethyl-aminomethan
vol.	Volume
v/v	Percentage volume to volume
w/v	Percentage weight to volume

1.0. Introduction

Gamma herpesviruses are lymphotropic viruses which establish lifelong infections in their hosts and are associated with cellular transformation and the development of malignancies, particularly in immunosuppressed individuals such as AIDS patients (Rickinson and Kieff 2001; Kieff and Rickinson 2001; Chang *et al.* 1994; Moore and Chang 1995). Until recently, mechanistic studies of the role of immunity in gamma herpesvirus infection have been hampered by the species specificity of human herpesviruses, Epstein-Barr virus (EBV) and human herpesvirus-8 (HHV-8) and the difficulties and expense inherent in analyzing pathogenesis, tumor induction, and mechanisms of immunity in primate models. Infection of laboratory mice with murine gamma herpesvirus-68 (MHV-68) provides a small animal model for addressing fundamental issues in gamma herpesvirus pathogenesis and immunity (Nash *et al.* 2001; Simas and Efstathiou 1998; Stevenson 2004; Stevenson *et al.* 2002 b; Speck and Virgin 1999; Virgin *et al.* 1997; Virgin and Speck 1999). MHV-68 was originally isolated from the bank vole (*Clethrionomys glareolus*) in Slovakia and is a natural pathogen for mice (Blaskovic *et al.* 1980). The overall similarities of MHV-68 to other gamma herpesviruses in terms of genomic organization, pathological symptoms and the establishment of both lytic and latent phase of infection make it a valuable model.

1.1. Herpesviruses

The family of herpesviruses is classified on the basis of the architecture of the virion. A typical herpesvirion consists of a core containing a linear double stranded DNA, an icosadeltahedral capsid approximately 100 to 110 nm in diameter containing 162 capsomeres with a hole running down the axis, an amorphous-appearing tegument which surrounds the capsid, and an envelope containing viral glycoprotein spikes on the surface (as reviewed by Roizman and Pellet 2001). All herpesviruses examined to date are able to remain latent in their natural hosts. In cells harboring latent virus, viral genomes persist as closed circular molecules, and only a small subset of viral genes is expressed. Latent genomes retain the capacity to replicate and cause disease upon reactivation. On the basis of their biological properties and DNA sequences, the members of the *Herpesviridae* family are divided into 3 subfamilies:

Alphaherpesvirinae, *Betaherpesvirinae* and *Gammaherpesvirinae* (Roizman and Pellet 2001).

1.1.1. The *Alphaherpesvirinae* subfamily

Alphaherpesvirinae have a broad host range and a relatively short reproductive cycle. They rapidly spread in culture and efficiently destroy infected cells. They have the capacity to establish latent infections primarily but not exclusively in sensory ganglia (as reviewed by Roizman and Pellet 2001). This subfamily contains the genera *Simplexvirus* (HSV-1, HSV-2), *Varicellovirus* (VZV), *Marek's disease like virus* and *Infectious laryngotracheitis-like virus*.

1.1.2. The *Betaherpesvirinae* subfamily

The members of the *Betaherpesvirinae* subfamily are characterized by a restricted host range and a long reproductive cycle. Infected cells frequently become enlarged (cytomegalia) and carrier cultures are readily established. Latent viruses can be found in secretory glands, lymphoreticular cells, kidneys, and some other tissues (as reviewed by Roizman and Pellet 2001). The members of this subfamily are *Human Cytomegalovirus* (HCMV), *Muromegalovirus* (murine cytomegalovirus), and *Roseolovirus* (HHV-7).

1.1.3. The *Gammaherpesvirinae* subfamily

The experimental host range of the members of the subfamily *Gammaherpesvirinae* is limited to the family or order to which the natural host belongs (as reviewed by Roizman and Pellet 2001). Most of these viruses are characterized by the establishment of lifelong infection in their host, association with cellular transformation and the development of malignancies (Simas and Efstathiou 1998). *In vitro*, all members replicate in lymphoblastoid cells, some also cause lytic infections in some types of epitheloid and fibroblastic cells. Viruses in this group are usually specific for either T or

B lymphocytes (as reviewed by Roizman and Pellet 2001). Members of the *Gammaherpesvirinae* subfamily are divided into two groups, gamma-1 herpes viruses, which are B-lymphotropic viruses, e.g., Epstein-Barr virus and genetically related viruses of old world primates, and the gamma-2 herpes viruses (rhadinoviruses), e.g., herpes virus saimiri (HVS) of new world monkeys, HHV-8 and MHV-68 (Simas and Efstathiou 1998). The sub-grouping of these viruses is based on the genomic organization and the gene content of representative sequenced members of gamma herpesviruses.

1.2. Human herpesvirus-8 (HHV-8)

Kaposi's Sarcoma-Associated Herpesvirus (KSHV) or HHV-8, which was discovered in 1994 (Chang *et al.* 1994), belongs to the subfamily of gamma-2 herpesviruses. HHV-8's full length genome is 165-170 kbp (as reviewed by Moore and Chang 2001) and contains a 140-145 kbp single continuous region flanked by repeats of 803 bp in length (Lagunoff and Ganem 1997). HHV-8 codes for several potential oncogenic proteins (Neipel and Fleckenstein 1999); however, the pattern of HHV-8 gene expression in *de novo* infection and during initial lymphomagenesis is not known.

HHV-8 is associated with Kaposi's Sarcoma (KS), Multicentric Castleman's Disease (MCD) and Primary Effusion Lymphoma (PEL). KS is a tumor of endothelial cell origin, whereas PEL is a B-cell lymphoma, and MCD is B-cell lymphoproliferative disorder. These tumors are most frequently found in immunosuppressed patients (as reviewed by Moore and Chang 2001; Dourmishev *et al.* 2003). Compared to most of the herpesviruses, HHV-8 is not ubiquitous, and only small minorities of people in most developed countries are HHV-8 positive (as reviewed by Moore and Change 2001). The primary reservoir for persistent asymptomatic HHV-8 infection appears to be $CD19^+$ B cells (Ambroziak *et al.* 1995). However, the natural infection of endothelial cells, $CD68^+$ monocyte-macrophage cells, prostate epithelia, and dorsal root sensory ganglion cells has been reported (as reviewed by Moore and Chang 2001). HHV-8 establishes latency in B cells, endothelial cells, monocytes/macrophages, and dendritic cells (as reviewed by Moore and Chang 2001).

Within KS tumors virtually all spindle cells have detectable viral DNA and protein, but most of the cells are latently infected and do not generate lytic virus (Boshoff *et al.*

1995; Dupin *et al.* 1999; Orenstein *et al.* 1997). However, HHV-8 can be reactivated *in vitro* from PEL B-cell lymphomas and from peripheral blood cells of PEL patients by treating the latently infected cells with chemicals like phorbol myristate acetate (PMA) and butyrate (Wang *et al.* 1998; Yu *et al.* 1999). Although HHV-8 encodes a large number of transforming genes, HHV-8-associated malignancies are often seen in the context of immune suppression. This suggests that the normal host immune surveillance mechanisms in healthy individuals are generally able to keep HHV-8 in check.

1.3. Epstein–Barr virus

EBV or human herpesvirus 4 (HHV-4) was discovered in 1965 (Epstein *et al.* 1965) and belongs to the genus *Lymphocryptovirus* within the subfamily of gamma herpesviruses. More than 90% of the world's population is latently infected with EBV.

The EBV genome is composed of a linear, double-stranded DNA of 184 kbp and approximately 60% (G+C) content (as reviewed by Kieff and Rickinson 2001). The *in vitro* host range for efficient EBV infection is restricted to primary human B lymphocytes. However, B lymphocytes derived from peripheral blood, tonsils, or fetal cord blood, T cells, natural killer cells, squamous epithelial cells, glandular epithelium, smooth muscle cells and follicular epithelial cells can be infected although with a lower efficiency compared to primary B cells (as reviewed by Kieff and Rickinson 2001; Volpi 2004). Upon EBV infection, primary B cells become activated and undergo morphological changes. These morphological changes are also associated with the entry of cells into the cell cycle, continuous proliferation and often establishment of latently infected lymphoblastoid cell lines (LCLs) (as reviewed by Bornkamm and Hammerschmidt 2001). EBV-immortalized cells or LCLs express a limited number of genes, which include six nuclear antigens EBNA-1, 2, 3A, 3B, 3C and EBNA-LP, two latent membrane proteins 1 and 2, and two small nuclear non-polyadenylated RNAs (as reviewed by Bornkamm and Hammerschmidt 2001; Kieff and Rickinson 2001).

EBV is primarily transmitted by saliva. Primary infection of EBV is marked by active viral replication in the epithelial cells of the oropharynx and subsequent infection of recirculating B lymphocytes (as reviewed by Rickinson and Kieff 2001). Most of the time, primary infections in children are asymptomatic. However, infection during adolescence or young adulthood leads to infectious mononucleosis (IM) in 35-50% of

cases (Evans *et al.* 1968; Henke *et al.* 1973; as reviewed by Rickinson and Kieff 2001). The symptoms of IM are fever, sore throat, swollen lymph nodes, splenomegaly and malaise (Bailey 1994).

EBV is associated with human tumours like Burkitt's lymphoma, nasopharyngeal carcinoma, lymphoproliferative disease in transplant patients and X-linked lymphoproliferative disorder. EBV also has been linked to other neoplasias including Hodgkin's disease, peripheral T cell tumours and gastric cancer (as reviewed by Damania 2004; Kuppers 2003; Rickinson and Kieff 2001; Young and Rickinson 2004; Volpi 2004).

Considerable progress has been made in understanding EBV infection and the immune responses against it. However, the role of viral genes and the immune response to the pathogenesis of EBV infection is limited, due to the species specificity of the virus and the lack of a proper experimental model. Although transgenic mice models expressing genes of EBV (Baichwal and Sugden 1988; Caldwell *et al.* 1998; Fu *et al.* 2004; Kulwichit *et al.* 1998; Wang *et al.* 1985), and NOD/SCID mice engrafted with human $CD34^+$ cells which are reconstituted mainly with human B lymphocytes (Islas-Ohlmayer *et al.* 2004) have been used to study EBV infection and pathogenesis, these models do not reflect a natural infection, pathogenesis and immunity in the natural host. Therefore, a small animal model that reproduces the biology of EBV infection would be beneficial for hypothesis testing, in particular for the analysis of individual viral gene functions *in vivo* (Rickinson and Kieff 2001).

1.4. Murine gamma herpesvirus-68 (MHV-68)

MHV-68 was originally isolated from the bank vole (*Clethrionomys glareolus*) in Slovakia (Blaskovic *et al.* 1980) during a study on the ecology of arboviruses. However, Blasdell *et al.* found that wood mice (*Apodemus sylvaticus*) are the natural host for MHV-68 and not the two species of voles (*Clethrionomys glareolus* and *Microtus agrestis*) (Blasdell *et al.* 2003). Other related murine gamma herpesviruses are MHV-60 and MHV-72 which were isolated from bank voles, and MHV-76 and MHV-78 which were isolated from wood mice. MHV-68 shares similar serological and biological properties with other virus isolates recovered from wood mice (Apodemus *flavicollis*) in Northern Europe. Intranasal infection of mice with MHV-68 leads to an acute,

productive infection in alveolar epithelial and mononuclear cells in the lung and to a persistent infection in B-lymphocytes, macrophages, dendritic cells and epithelial cells of the lung. The key features of the MHV-68 that are shared by other gamma herpesviruses and by which it can be defined as a model for gamma herpesvirus pathogenesis and immunity are:

1.4.1. Features of the MHV-68 genome

The MHV-68 genome consists of a unique DNA sequence, approximately 118 kbp in length, with a G+C content of 46%. It is flanked by multiple copies of terminal repeats of 1.23 kbp in length, having a G+C content of 77.6% (Efstathiou *et al.*1990 a & b). The G+C contents of the unique region as well as of the terminal repeats of MHV-68 are comparable with that of HHV-8 (Fig. 1.1).

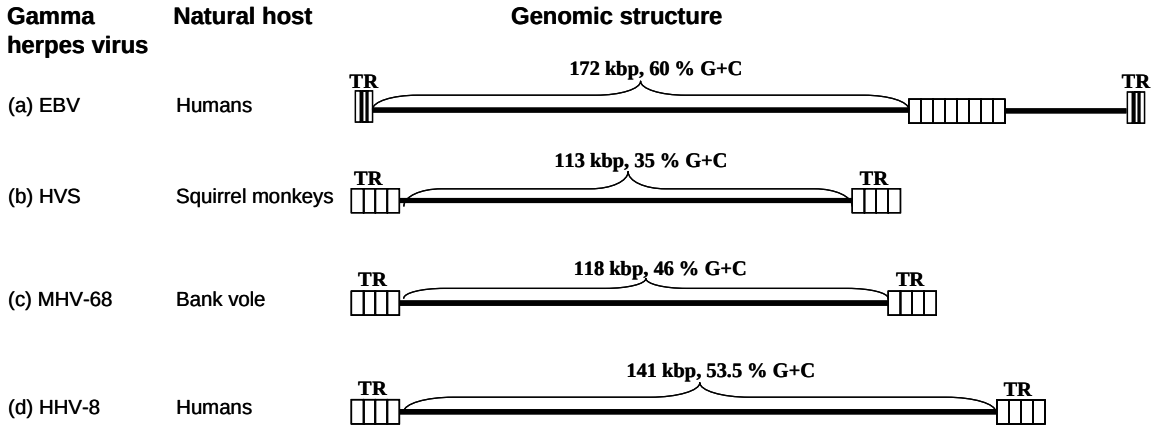


Fig. 1.1. The genomic structure of representative members of the gamma herpesvirus family: (a) Epstein-Barr virus (EBV); (b) herpesvirus saimiri (HVS); (c) murine gamma herpesvirus-68 (MHV-68); (d) human herpesvirus-8 (HHV-8). Characteristically, the gamma herpesviruses consist of a central unique segment of low G+C DNA (L-DNA) ranging from 110-175 kbp, flanked by repetitive high G+C DNA (H-DNA).

(modified from Simas and Efstathiou 1998)

The MHV-68 genome has been sequenced by two laboratories (Nash *et al.* 2001; Virgin *et al.* 1997). Virgin *et al.* estimated that MHV-68 codes for at least 80 ORFs, out of which 63 ORFs are collinear and homologous to other gamma herpesviruses. MHV-68

shares blocks of conserved genes with other gamma herpesviruses and these blocks of conserved genes are interspersed by open reading frames (ORFs) that are in general unique to each individual family member (Fig. 1.2). These genes are thought to be responsible for modulating the immune response, cell transformation and latency (Simas and Efstathiou 1998). It appears that each virus has developed unique mechanisms to evade the immune response and establish latency and malignancies (Nash *et al*. 2001; Simas and Efstathiou; Speck and Virgin 1999; Virgin *et al.* 1997; Virgin and Speck 1999).

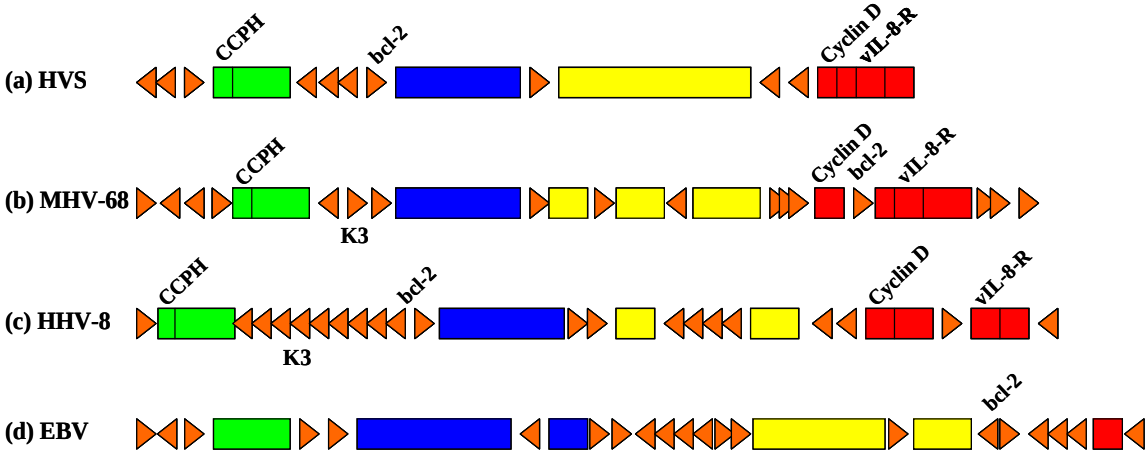


Fig. 1.2. A comparison of the genetic organization among the fully sequenced gamma herpesviruses. The genomes of (a) herpes virus saimiri (HVS), (b) murine gamma herpesvirus-68 (MHV-68), (c) human herpesvirus-8 (HHV-8), and (d) Epstein-Barr virus (EBV) are shown. The genome of EBV has been inverted relative to its conventional orientation. The conserved gene blocks I (green), II (blue), III (yellow) and IV (red) are shown within these boxes and virus specific genes are shown in triangles. Abbreviations: bcl-2, B cell lymphoma gene 2; CCPH, complement control protein homologue; Cyclin D, Cyclin D homologue; vIL-8-R, viral IL-8 receptor.

(modified from Simas and Efstathiou 1998)

During the lytic phase of herpesvirus infection, DNA replication proteins assemble at an origin of lytic replication (*ori* Lyt) and initiate the replication process, resulting in "head-to-tail" concatemers (as reviewed by Kieff and Rickinson 2001; Mocarski and Courcelle 2001; Roizman and Knipe 2001). The *ori* Lyt regions of rhesus macaque rhadinovirus, EBV, bovine herpesvirus-4 and HHV-8 have been identified (AuCoin *et al.* 2002; Hammerschmidt and Sugden 1988; Pari *et al.* 2001; Zimmermann *et al.* 2001). All these herpesviruses have two copies of *ori* Lyt. In case of MHV-68, one copy of *ori* Lyt has been identified at the right end of the genome (Deng *et al.* 2004), and a second

copy has been proposed to be situated at the left end of the genome (Coleman *et al.* 2003). Both *ori* Lyt regions have A/T-rich and G/C-rich regions characteristic of herpesvirus lytic origins of replication. Also, the function of *ori* Lyt has been confirmed for the equivalent regions of HHV-8 and rhesus monkey rhadinovirus (RRV) (AuCoin *et al.* 2002; Pari *et al.* 2001).

The MHV-68 genome, like other gamma-2 herpesviruses, has a marked suppression of CpG dinucleotides. This suppression of CpG dinucleotides might be due to the mutagenic effects of genomic methylation and viral DNA maintenance in dividing cell populations (Honess *et al.* 1989; Simas and Efstathiou 1998). All known gamma herpesviruses encode small non-coding RNAs. MHV-68 is no exception and encodes eight unique tRNA like sequences. These tRNA like sequences are expressed in both lytic and latent infection (Bowden *et al.* 1997; Virgin *et al.* 1997). The function of these uncharged viral tRNAs is unknown. However, the viral tRNA transcript expression can be detected up to 70 days post infection in the germinal centers of latently infected spleens of mice and therefore represents a marker for latent infection in the same way as the EBV encoded RNAs (EBERs) and HVS U-RNAs.

1.4.2. Infection of cells *in vitro*

In contrast to the two human gamma herpesviruses HHV-8 and EBV, *in vitro* MHV-68 infects and replicates in a broad range of cell types from both humans and mice, including fibroblasts, epithelial cell lines and primary macrophages (Blaskovic *et al*. 1980; Svobodova *et al.* 1982). MHV-68 infects primary B cells efficiently, leading to the formation of clumps of enlarged, activated cells within three to four days (Dutia *et al.* 1999 b; Stevenson and Doherty 1999). However, these cells do not become transformed and usually die within two weeks.

1.4.3. *In vivo* infection

The natural route of infection with MHV-68 is not known. Other gamma herpesviruses are transmitted through the respiratory tract, therefore it is likely that this is also true for MHV-68 (Simas and Efstathiou 1998). Intranasal infection (i.n.) of MHV-68 in 4-6

week old mice leads to productive infection in the lungs (Cardin *et al.* 1996; Sunil-Chandra *et al.* 1992 a) followed by virus dissemination and establishment of persistent, life-long latent infection in B lymphocytes (Fig. 1.3). Alternative routes of infection, including intravenous, intraperitoneal (i.p.), and gastric instillation have also been used experimentally (Sunil-Chandra 1992 a; Weck *et al.* 1996; Peacock and Bost 2000). *In vivo*, MHV-68 infects a broad range of cells of different tissue types including epithelial cells from the oral and nasal cavities, lung and gastrointestinal tracts, B cells, macrophages, dendritic cells in the spleen and liver cells (Flano *et al.* 2000; Stewart *et al.* 1998; Sunil-Chandra *et al.* 1992 b; Weck *et al.* 1999).

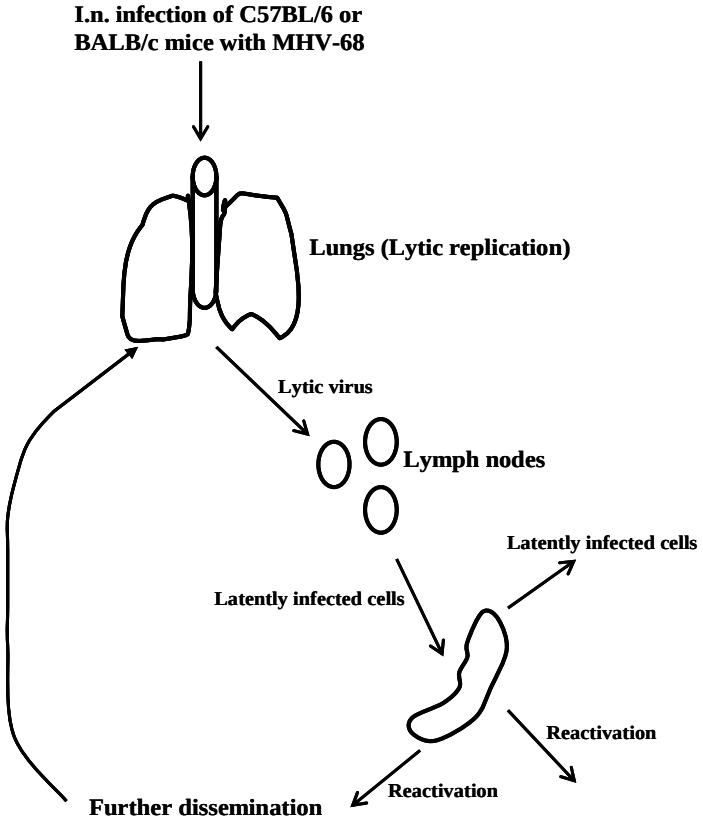


Fig. 1.3. *In vivo* replication. After i.n. infection, MHV-68 replicates lytically in the respiratory tract. This is followed by seeding to lymphoid tissue, where there is amplification of latent virus and further dissemination, probably via latently infected memory B cells. Latent virus persists in the lung, in lymphoid tissue and also in other sites.

(modified from Stevenson *et al.* 2002 b)

1.4.3.1. Lytic Replication

Introducing virus intranasally into 5-6 weeks old inbred mice results in an acute, productive infection of alveolar epithelial cells, with some mononuclear cells in the lung also positive for late virus gene expression (Sunil-Chandra, *et al.* 1992 a). The virus titer in the lungs reaches the peak around day 6 post infection and within two weeks post infection acute infection gets resolved from the lungs of mice (Fig. 1.4). Acute infection in the lung is resolved by $CD8^+$ T cells with or without the help of $CD4^+$ T cells, which is followed by a life-long latent infection in lymphoid tissue (Cardin *et al.* 1996; Ehtisham *et al.* 1993). Infectious virus can also be consistently detected during acute infection in the adrenal glands and heart tissues, indicating that MHV-68 has a hematogenous spread (Sunil-Chandra *et al.* 1992 a; Cardin *et al.* 1996). Infection of the lung is accompanied by bronchiolitis (interstitial pneumonia) (Sunil-Chandra *et al.* 1992 a; Cardin *et al.* 1996).

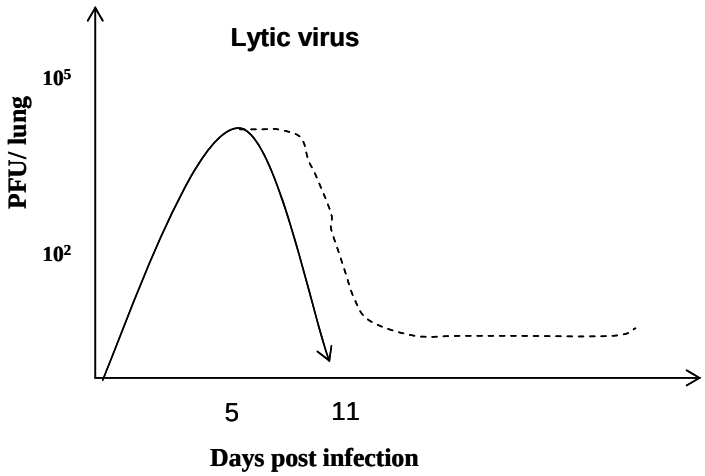


Fig.1.4. Lytic replication of MHV-68 in the lungs of mice. Following i.n. infection, the virus replication in the lungs of immunocompetent mice (solid line) and immunocompromised mice e.g. $CD4^+$ T cell-deficient mice ($I\text{-}A^{b-/-}$) mice (dashed line) is shown.

(modified from Stevenson *et al.* 2002 b)

1.4.3.2. Latency

Like all herpesviruses, MHV-68 establishes latency in its host. Latency is usually assessed by an "infectious center assay" or the "*ex vivo* reactivation assay", which measures the ability of virally-infected cells to reactivate *in vitro* and form plaques on a permissive monolayer (Sunil-Chandra *et al.*1999 a & b; Cardin *et al.* 1996; Weck *et al.* 1996). The establishment and the maintenance of latency are independent of infectious dose or route of infection (Tibbetts *et al.* 2003 a). Latency is established by virus traveling from the lung to the local lymph node [the mediastinal lymph node (MLN)], where dendritic cells, macrophages and B cells are infected. Dendritic cells are believed to be responsible for the initial transport of virus to the draining lymph node, where they seed B cells and establish latent infection (Nash *et al.* 2001). Upon the infection in the MLN, B cells undergo a rapid expansion accompanied by an increase in the number of latently infected B cells. B cells from the MLN then traffic to the spleen and other lymphoid organs and establishment of life-long latency takes place (Nash *et al.* 2001).

Like in the EBV infection, B lymphocytes are the major reservoir harboring latent MHV-68 (Weck *et al*. 1996; Sunil-Chandra *et al.* 1992 b), although macrophages (Flano *et al.* 2000; Weck *et al.* 1999 b), splenic dendritic cells (Flano *et al.* 2000) and lung epithelial cells (Stewart *et al.* 1998) also have been shown to harbor latent infection. Memory B cells are the major reservoir for long term latency of MHV-68 (Flano *et al.* 2002; Willer and Speck 2003) as well as of EBV (Babcock *et al.* 1997; Miyashita *et al.* 1997).

The establishment of latency in lymphoid tissue is characterized by a transient splenomegaly and lymph node enlargement that peaks 2-3 weeks post-infection (Fig. 1.5). The transient splenomegaly phase resembles the infectious mononucleosis syndrome seen after EBV infection in adolescence. The hallmarks of this syndrome include splenomegaly, polyclonal B cell activation and associated autoantibody production, and a $CD8^+$ T cell lymphocytosis in peripheral blood (Tripp *et al.* 1997; Blackman *et al.* 2000; Doherty *et al.* 1997; Sangster *et al.* 2000). Splenomegaly is due to increased numbers of cycling $CD4^+$ T cells, $CD8^+$ T cells and B cells in the spleen. Lymphocytosis in the peripheral blood is characterized by increased numbers of activated $CD8^+$ T cells. The activated T cells in the peripheral blood are predominately $CD8^+$ and express an $CD62L^{lo-}$, $CD44^{hi-}$ activated phenotype with as many as 75% of the $CD8^+$ T cells expressing $V\beta4^+$TCR (Ehtisham *et al.* 1993; Sunil-Chandra *et al.* 1992

a & b; Tripp *et al.* 1997). There are however, key differences in the MHV-68 and EBV-induced $CD8^+$T cell responses. Whereas the expanded $CD8^+$T cells associated with EBV-induced mononucleosis are largely the outgrowth of T cells responding to lytic viral epitopes elicited during the acute phase of the response, the $CD8^+$T cell lymphocytosis associated with MHV-68-induced infectious mononucleosis is dominated by an oligoclonal population of T cells expressing $V\beta4^+$T cell receptors that are not reactive to acute viral epitopes (as reviewed by Flano *et al.* 2002).

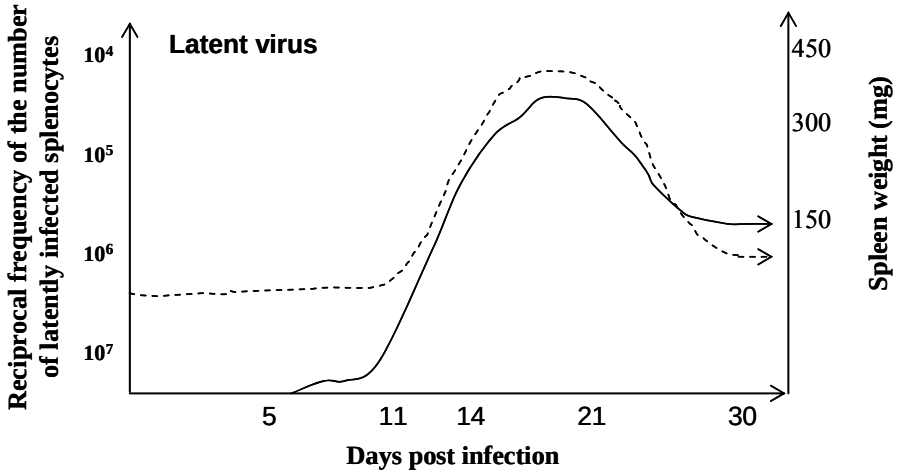


Fig. 1.5. Establishment of latency in the spleen of mice infected with MHV-68. Following i.n. infection, MHV-68 establishes latency in the spleen which is characterized by transient splenomegaly (dashed line) and amplification of latency, i.e., increase of the number of latently infected cells in the spleen (solid line).

(modified from Stevenson *et al.* 2002 b)

Also, during the transient splenomegaly phase, the amplification of latency takes place, i.e., the number of latently infected cells increases from 1 per 10^7 to 1 per 10^4 spleen cells (Fig. 1.5). Following the amplification of latency, the number of latently infected splenocytes decreases to 1 per 10^6 spleen cells by the third to fourth week post infection followed by the splenomegaly then decreasing back to a basal level (Sunil-Chandra *et al.* 1992 a; Usherwood *et al.* 1996 a). The rise and fall of the number of latently infected cells can be explained by the observation that, during the splenomegaly, many latently infected cells can be detected within germinal centers (GCs). As the B-cell response matures, GCs regress and there is a constant decline in the level of latency. Multiple immune mechanisms including $CD8^+$ T cells, $CD4^+$ T cells and antibodies contribute to the control of latency and prevent recrudescence of lytic virus (Christensen *et al.* 1999; Stevenson *et al.* 1999 b; Stewart *et al.* 1998).

The latent MHV-68 genome can be reactivated (i.e., emerge from latency) and enter the lytic phase of infection, but little is known about how latent reservoirs are maintained or which signals trigger reactivation from latency. Analogous to EBV and HHV-8, reactivation of MHV-68 *ex vivo* can be stimulated by the treatment of latently infected splenocytes with either anti-IgG-IgM and anti-CD40 or LPS (Moser *et al.* 2005).

1.4.4. Pathogenesis

Infection of adult mice with MHV-68 can lead to severe exudative pneumonia, vasculitis of the great arteries, infectious mononucleosis like syndrome and splenic fibrosis (Ehtisham *et al.* 1993; Sunil-Chandra *et al.* 1992 a; Usherwood *et al.* 1996 a; Weck *et al.* 1996). Chronic infection of mice with MHV-68 can lead to inflammation of large arteries, splenic fibrosis and fatal encephalitis in neonatal mice (Blaskovic *et al.* 1980; Rajcani *et al.* 1985; 1987).

It has been shown that gamma herpesviruses are associated with lymphoproliferative diseases of B and/or T cells. Additionally, EBV is associated with epithelial cancers, the most prominent is nasopharyngeal carcinoma (NPC), and HHV-8 is linked to vascular endotheliosarcomas such as Kaposi's sarcoma. A striking property that is shared by many members of the gamma herpesvirus family, including EBV, HHV-8 and HVS is their ability to induce neoplasias in the natural or experimental hosts (Table. 1).

It has been shown that MHV-68 infection can also lead to the development of tumors (Sunil-Chandra *et al.* 1994). However, these studies have not been reproduced by other groups. Balb/c mice infected with MHV-68 for period of nine months or longer have been shown to develop lymphomas and approximately 10% of infected mice developed tumors in both lymphoid and non-lymphoid tissues (lung, liver, kidney and heart). 50% of the lymphoid tumors were classified as high-grade lymphomas (Sunil-Chandra *et al.* 1994). In another study, five cell lines were derived from persistently infected mice exhibiting lymphoproliferative disease (LPD); however, only one such line carried the virus genome (Usherwood *et al.* 1996 b). Transgenic expression of MHV-68 ORF 72 (cyclin D homologue) in mice can also induce the development of high grade lymphoblastic lymphomas (van Dyk *et al.* 1999).

Table. 1. Malignancies associated with gamma herpesvirus infection.

Primate gamma herpesviruses linked to neoplastic disease			
Gamma-herpesvirus	**Natural Host**	**Associated malignancies**	**References**
Epstein-Barr virus	Human	Burkitt's lymphoma	Facer and Playfair (1989)
		Hodgkin's lymphoma	Weiss *et al.* (1989)
		Post-transplant lymphoma	Gratama *et al.* (1988)
		X-linked lymphoproliferative syndrome	Purtilo (1985)
		T-cell lymphomas	Ott *et al.* (1992)
		Nasopharyngeal carcinoma	Wolf *et al.* (1973)
		Gastric carcinoma	Imai *et al.* (1994)
Kaposi's sarcoma-associated herpesvirus	Human	Kaposi's sarcoma	Beral *et al.* 1990
		Primary effusion lymphomas	Cesarman *et al.* 1995; Nador *et al.* 1996
		Multicentric castleman's disease	Parravicini *et al.* 1997
Herpesvirus saimiri	Squirrel monkey	T-cell lymphomas in non-natural host	Daniel *et al.* 1974; Melendez *et al.* 1969

1.4.5. Immune response to MHV-68 infection

The immune system plays a very important role during gamma herpesvirus infection which is highlighted by the fact that most people worldwide are EBV carriers and normally remain asymptomatic unless they become immunocompromised, which exposes them to an increased risk of developing EBV-induced lymphoproliferative diseases and lymphomas (as reviewed by Penn 1986). In the case of MHV-68, both innate and adaptive immune responses are involved in recovery after infection as described below.

1.4.5.1. Role of B cells as host cells and in the immune response to MHV-68 infection

B cells play important roles in the MHV-68 infection, for example as a site of latency, spread of virus in the host, regulation of latency and control of chronic infection. MHV-68 readily establishes latency in B cells, memory B cells and in germinal centers (Ehtisham *et al.* 1993; Flano *et al.* 2002; Bowden *et al.* 1997; Simas *et al.* 1999). The inefficient latent infection in the spleen after intranasal infection (Usherwood *et al.* 1996 c; Weck *et al.* 1999 a; Willer and Speck 2003) and the inefficient acute infection in the spleen after intraperitoneal infection (Weck *et al.* 1996) in B cell deficient mice point to a role of B cells in the spread of virus through the host.

As described above, B cells are host cells for MHV-68. In addition they play an important role in controlling the acute infection. B cell deficient mice have a continuous productive infection in the lung and die after prolonged infection with MHV-68 (Stewart *et al.* 1998; Weck *et al.* 1999 a).

Induction of neutralizing antibodies can mediate an attenuated latent infection (Stewart *et al.* 1999). A direct role for protective antibodies (Abs) was demonstrated by the ability of passively transferred immune serum to prevent viral recrudescence in $CD28^{-/-}$ mice after T cell depletion (Kim *et al.* 2002). Finally, using reactivation deficient MHV-68 for the vaccination, McClellan *et al.* have shown that an intact humoral response, even in the absence of $CD4^+$ or $CD8^+$ T cells, can provide protection against the establishment of latent infection in mice (McClellan *et al.* 2004). However, antibodies are apparently not essential for the resolution of acute infection (Cadrin *et al.* 1996; Usherwood *et al.* 1996 c; Ethisham *et al.* 1993).

1.4.5.2. Role of $CD4^+$ T cells in the immune response to MHV-68 infection

$CD4^+$ T cells are required for B cell proliferation, maturation, activation and antibody response (Stevenson and Doherty 1999; Sangster *et al.* 2000). The induction of splenomegaly after MHV-68 infection is driven by $CD4^+$ T cells (Cardin *et al.* 1996; Usherwood *et al.* 1996 a). $CD4^+$ T cells can resolve the acute infection, prevent lethal infection and inhibit the establishment of latency of MHV-68 in combination with $CD8^+$ T cells (Ehtisham *et al.* 1993) or in the absence of $CD8^+$ T cells (Christensen *et al.* 1999;

McClellan *et al.* 2004; Stevenson *et al.* 1999 b). It has been found that in MHC class II knockout mice, there was little to no effect on clearance of acute infection of the lung and spleen. However, these mice were prone to recurrent virus infection in the lungs that is largely unresolved and progresses towards a chronic lung disorder (Cardin *et al.* 1996). In agreement with EBV (Nikiforow *et al.* 2003), $CD4^+$ T cells play an important role to contain the persistent MHV-68 infection (Tripp *et al.* 1997), probably by IFN-γ production (Christensen *et al.* 1999; Weck *et al.* 1997). Also, the ineffectiveness of antibodies and cytotoxic lymphocytes (CTLs) against lytic cycle proteins indicates the importance of $CD4^+$ T cells in containing the persistent MHV-68 infection (Belz *et al.* 2000; Liu *et al.* 1999; Stewart *et al.* 1999; Usherwood *et al.* 2001).

1.4.5.3. Role of $CD8^+$ T cells in the immune response to MHV-68 infection

Similar to other gamma herpesviruses, $CD8^+$ T cytotoxic lymphocytes play a major role in controlling MHV-68 acute infection. However, $CD8^+$ T cells are not required for survival following acute infection depending on the mouse strain and the conditions of infection (Stevenson *et al.* 1999 b). Depletion of $CD8^+$ T cells prior to infection results in a severe infection in the lung and dissemination of virus via the blood to infect spleen, liver and adrenal glands (Ehtisham *et al.* 1993). In BALB/c mice, MHV-68 infection after $CD8^+$ T cell depletion can be lethal (Ehtisham *et al.* 1993; Stevenson *et al.* 1999 b). In C57BL/6 mice depleted of $CD8^+$ T cells, acute infection in the lungs is still cleared, although the transient splenomegaly period is extended (Weck *et al.* 1996).

One striking aspect of the response of $CD8^+$ T cells to MHV-68 infection is the dramatic expansion of $V\beta4^+$ $CD8^+$ T cells (Tripp *et al.* 1997); however, the mechanism underlying this selective $V\beta4^+$ T cells expansion and the physiologic importance of this phenomenon is currently undefined. It has been suggested that $V\beta4^+$ $CD8^+$ T cells can acquire effector function, including cytotoxicity and the capacity to secrete IFN-γ, although they have an atypical activation profile compared with well-characterized $CD8^+$ T cells specific for conventional viral epitopes (Flano *et al.* 2004).

1.4.5.4. Role of cytokines and chemokines in the immune response to MHV-68 infection

MHV-68 infection increases the expression of several chemokines and cytokines, which could potentially play important roles in controlling lytic and latent infection. MHV-68 infection leads to the production of IL2, IL-6, IL-10 and IFN-γ (Sarawar *et al.* 1996). It has been shown that type I interferons (IFN α/β) play a key role in controlling acute viral replication and dissemination prior to the development of adaptive immunity (Dutia *et al.* 1997). Lack of the IFN-α/β receptor results in 100% mortality in mice within four days of MHV-68 infection (Weck *et al.* 1997). IFN-γ seems to be nonessential for clearance of an acute infection and control of latent virus following intranasal infection (Dutia *et al.* 1997; Sarawar *et al.* 1997); however, $CD4^+$ T cell immunity is mediated by IFN-γ (Christensen *et al.* 1999). IL-6 is not essential for the development of an effective immune response to MHV-68 and does not influence viral replication and latency (Sarawar *et al.* 1998), whereas IL-10 has a dichotomous role, both in protection and pathogenesis (Peacock and Bost 2001). It has been demonstrated that the MHV-68 infection induces the expression of IL-12 in macrophages and dendritic cells and this cytokine plays an important role in the protective host response and in virus-induced splenomegaly (Elsawa and Bost 2004).

It has been shown that the infection of mice with MHV-68 lead to an increase in the expression of RANTES, eotaxin, MIP-1α, MIP-1β, IP-10, KC, and MCP-1 by day 7 post infection (Sarawar *et al.* 2002; Weinberg *et al.* 2002). It was proposed that chemokines play an important role in lymphocyte trafficking to the lung during the MHV-68 infection (Sarawar *et al.* 2002).

1.4.6. Immune evasion by MHV-68

MHV-68 infection leads to a massive proliferation of $CD8^+$ T cells, which clear the lytic replication in the lungs with or without the help of $CD4^+$ T cells. However, the T cell response is ineffective in preventing the massive proliferation of latently infected B cells in germinal centers, indicating the existence of immune evasion by MHV-68. This amplification and lymphoproliferation is accompanied by antigen-nonspecific B cell activation which is important for host colonization (Stevenson 2002 a & b; 2004).

Gamma herpesviruses encode several proteins (HSV, ICP47; HCMV, pp65, US6, US11, US2, US3; EBV, EBNA-1; HHV-8, K3, K5; MHV-68, MK3, M3) that play a role in immune evasion (as reviewed by Alcami and Koszinowski 2000; Hegde *et al.* 2003; McFadden *et al.* 1996; Ploegh 1998; Yewdell and Hill 2002). So far two immune evasion genes (M3 and MK3) have been identified in MHV-68.

1.4.6.1. Immune evasion mediated by the M3 protein of MHV-68

Herpesviruses encode a variety of proteins with the potential to disrupt chemokine signaling, and hence immune organization (Alcami and Koszinowski 2000; Lalani *et al.* 2000). The MHV-68 M3 gene encodes a secreted 44-kD broad spectrum chemokine-binding protein that neutralizes cellular responses to a broad spectrum of chemokines *in vitro* (Parry *et al.* 2000; van Berkel *et al.* 2002). The M3 protein has no sequence similarity to known chemokine receptors. It binds CC, CXC, C, and CX_3C chemokines, but does not bind human B-cell specific nor mouse neutrophil-specific CXC chemokines (van Berkel *et al.* 2002). *In vivo*, disruption of M3 has little effect on lytic replication or the initial spread of virus to lymphoid tissues. However, there is no amplification of latently infected B cells in the spleen that normally drives MHV-68-induced infectious mononucleosis and a marked reduction in latent virus load is observed. This defect in the amplification of latency was largely reversible by $CD8^+$ T cell depletion (Bridgeman *et al.* 2001).

1.4.6.2. Immune evasion mediated by the MK3 protein of MHV-68

A major arm of host immunity to viral infection are cytotoxic T lymphocytes (CTL), which recognize viral antigens presented as peptides bound to MHC class I molecules on the surface of infected cells. Therefore, evasion of CTL recognition is thought to play a key role in the establishment of systemic viral infection. Many viruses including the herpesviruses have evolved complex strategies for such evasion, usually centering on down-regulation of cell surface MHC class I display (Boname *et al.* 2004 a; Coscoy *et al.* 2001; Coscoy and Ganem 2000; Hewitt *et al.* 2002; Ishido *et al.* 2000; Lorenzo *et al.*

2002; Stevenson *et al.* 1999a; 2002 a). Both MHV-68 and HHV-8 encode membrane bound Ring finger proteins that act as E3 ubiquitin ligases for MHC class I called MK3, K3 and K5. The HHV-8 proteins K3 and K5 act late in the secretory pathway and direct MHC class I to lysosomes (Coscoy and Ganem 2000; Hewitt *et al.* 2002; Ishido *et al.* 2000; Lorenzo *et al.* 2002). The MHV-68 MK3 protein remains in the endoplasmic reticulum (ER) and ubiquitinates MHC class I heavy chains that associate with the transporter associated with antigen processing (TAP), leading to proteasome-dependent degradation (Lybarger *et al.* 2003). The association of MK3 with the peptide-loading complex also allows it to degrade TAP, thereby broadening its inhibitory repertoire to include peptide loading of non-classical MHC class I glycoproteins with short or absent cytoplasmic tails that are not accessible to ubiquitination (Boname *et al.* 2004 a). The TAP degradation by the MK3 protein also provides a remarkable resistance to MHC class I up-regulation by IFN-γ (Boname *et al.* 2004 a). *In vivo*, the infection of mice with mutant virus for MK3 protein has little effect on lytic replication in the lungs. However, the disruption of the MK3 protein leads to enhanced antiviral CTL responses, impaired expansion of latently infected cells in lymphoid germinal centers and an attenuation of the MHV-68-induced infectious mononucleosis. This defect in the amplification of latency was largely reversible by $CD8^+$ T cell depletion as now the MHC class I dependent defense has disappeared (Stevenson *et al.* 2002 a).

Both the MK3 and M3 genes are transcribed during acute lytic infection in the lung as well as during establishment of latency in lymphoid tissue. The tissue damage caused by productive MHV-68 replication is presumably a potent inflammatory stimulus and therefore, the immune activation overcomes the immune evasion mediated by MK3 or M3, an effect that is well documented with other herpesviruses (Hengel *et al.* 1999; Posavad *et al.* 1998). Latent virus, unlike lytic replication, probably causes minimal tissue disruption, and in this setting, evasion seems to be dominant. Also, during the viral reactivation, when tissue damage will be minimal, the MK3 expression may contribute to immune evasion (Stevenson *et al.* 2002 b).

1.4.7. Exploring vaccination strategies against gamma herpesviruses using the MHV-68 system

Due to the species specificity of the human gamma herpesviruses, strategies for vaccinating against human gamma herpesviruses have been difficult to study. Therefore, several groups have used MHV-68 infection of mice as an important model in defining novel vaccination strategies against human and animal gamma herpesviruses (Belz *et al.* 2000; Liu *et al.* 1999; Stevenson *et al.* 1999; Stewart *et al.* 1999; Usherwood *et al.* 2001). Several vaccination studies have demonstrated effective reduction in both acute infection and early forms of latency. Vaccination against the lytic cycle protein peptide epitopes $ORF6_{487-495}/D^b$ and $ORF61_{425-531}/K^b$ and latency-associated antigen M2 induces strong $CD8^+$ T-cell responses and significantly reduces acute infection and early latency (Belz *et al.* 2000; Liu *et al.* 1999; Stewart *et al.* 1999). Vaccination against the cell membrane and virus particle antigen gp150 induces neutralizing antibody and reduces acute infection and early stages of latency (Liu *et al.* 1999; Stewart *et al.* 1999). Despite the effectiveness of these strategies against acute infection and/or early latency, none was effective against MHV-68 latency beyond 20 days of infection.

In contrast to MHV-68 proteins or peptide vaccines, latency or reactivation-deficient MHV-68 viruses serve as better vaccines, and it has been shown that they largely prevent subsequent wild type latency establishment (Boname *et al.* 2004 b; Fowler and Efstathiou 2004; McClellan *et al.* 2004; Tibbetts *et al.* 2003 b). Using reactivation-deficient MHV-68 as vaccine, McClellan *et al.* have shown that $CD4^+$ T cells play a critical role in immune surveillance of gamma herpesvirus latency and can mediate vaccination against latency, even in the absence of an antibody response (McClellan *et al.* 2004). Therefore, successful vaccination strategies against gamma herpesviruses will depend upon defining the molecular mechanism that underlie vaccine-induced protection, in particular the potential of $CD4^+$ T cells to control *in vivo* MHV-68 latency (Stevenson 2004).

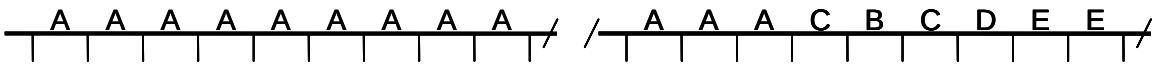
No sequence homologues to the EBV latency and transformation-associated proteins [EBV nuclear antigens (EBNAs), latent membrane protein 1 and 2 (LMP-1 and 2)] or the EBV-encoded transactivator, BZLF-1, are present in the genome of MHV-68 (Simas and Efstathiou 1998). Yet, the similarities of MHV-68 to other gamma herpesviruses in terms of its genomic organization, pathological symptoms and the establishment of both lytic and latent phase of infection, provide a valuable tool to study gamma herpesvirus

pathogenesis and immunity. The strength of this model lies in its capacity to reproduce key common features of gamma herpesvirus infection in a natural host, including primary lytic replication, latency establishment and maintenance, and viral reactivation. This, combined with the availability of genetically defined mice and the capacity to manipulate the MHV-68 genome, provides the basis for the comprehensive description of gamma herpesvirus biology.

1.5. Aim of the project

One of the characteristics of gamma herpesviruses is the presence of various kinds of internal repeats in their genome. The human herpesviruses, EBV and HHV-8, possess internal repeats of different numbers and size in their genome; however, it is not known if variations in the number of repeats play a role in the pathogenesis. *In vitro*, internal repeats (IR) of EBV (IR2) and IR 4 are part of an origin of lytic replication (*ori* Lyt) and in addition, these repeats contain enhancer-like sequences (Cox *et al.* 1990; Hammerschmidt and Sugden 1988). Also, some of the repeats of the HHV-8 and MHV-68 genome are part of an *ori* Lyt, and these repeat structures act as an auxiliary element to enhance replication efficiency (AuCoin *et al.* 2002; Deng *et al.* 2004). The internal repeats of the herpes simplex virus play an important role in pathogenesis. Loss of the internal repeat resulted in lower virulence, a decreased spread of virus from the cornea to sensory ganglia and a decreased establishment of reactivable latent virus in the trigeminal ganglion (Jenkins *et al.* 1996).

MHV-68 also contains two internal repeats in its genome, i.e., the 40 bp repeat and 100 bp repeat. The presence of these internal repeats in the genome of MHV-68 provides an opportunity to address the role of these repeats in gamma herpesvirus pathogenesis. The 100 bp repeat is present at the right side of the genome (nucleotide positions 98981-101170) and consists of approximately 21 copies of 100 bp repeat units. The 100 bp repeat is a part of three overlapping ORFs: ORF M10a, ORF M10b and ORF M10c. In addition, the 100 bp repeat contributes to the lytic origin of replication at the right side of the MHV-68 genome (Deng *et al.* 2004). The 40 bp internal repeat of MHV-68 is present at the left side of the genome (nucleotide positions 26778-28191). It consists of approximately 36 copies of repeat units, some of which can vary in their sequence (Virgin *et al.* 1997) (Fig. 1.6).



Type A repeat – 40mer with *Bam HI* site

CCGACCTCCC CGGCCCCTC CCGAGCTGGG GCCCGGATCC

Type B repeat – 41mer with *Bam HI* site

CCCGACCTCC CGGCCCCTC CCGAGCTGGG GCCCGGATCC

Type C repeat – 41mer with *Bam HI* site

CCCGACCTTC CGGCCCCTC CCGAGCTGGG GCCCGGATCC

Type D repeat – 51mer with *Bam HI* site

CCCCGGTTCC CCCGACCTTC CGGCCCCTC CCGAGCTGGG GCCCGGATCC

Type E repeat – 41mer without *Bam HI* site

CCCGACCTCCC CGGCCCCTC CCGAGCTGGG GCCCGGTTCC

Fig. 1.6. Organization and sequence of the 40 bp internal repeat present in the MHV-68 genome. The 40 bp repeat is estimated to contain 27 copies of a type A repeat units, linked to several degenerated forms of the repeat located at the right-hand end (type B to E repeats), as illustrated. The sequence of each type of repeat is shown below the schematic diagram of the repeat structure. Residues which differ from the type A repeat are underlined, and the presence of *BamH* I sites is indicated by open rectangles.

(modified from Virgin *et al.* 1997)

Although the complete sequence of MHV-68 has been determined, the precise role of many viral genes or family of genes is not well known. One way to determine the function of a viral gene in the context of the whole viral genome is mutagenesis of the gene of interest. Recently, MHV-68 genomic DNA was cloned as a bacterial artificial chromosome (BAC) (Adler *et al.* 2000; 2001; 2003), which provides an efficient, fast and valuable tool for mutagenesis. However, during mutagenesis, many clones lose some of their 40 bp internal repeat units, due to recombination between direct repeats. These size variations due to alterations in the repeat structures also occur naturally in other gamma herpes viruses. Therefore, it is interesting to determine whether the variability of the MHV-68 internal repeat structures has any biological effect *in vivo*.

It has been suggested that MHV-68 can be used for the development of amplicon vectors (Deng *et al.* 2004). Since the 40 bp internal repeat and its neighbouring region in the MHV-68 genome might contain a lytic origin of replication (Coleman *et al.* 2004), it is critical to define and check the stability of putative origin of lytic replication sequences required for viral DNA replication and packaging. This will help to define the parameters for the development of MHV-68-based amplicon vectors.

The 40 bp internal repeat is also a part of the hypothetical ORF M6. Micro-array analysis (Ahn *et al.* 2002) and genome sequencing of MHV-68 (Virgin *et al.* 1997) predicted the existence of ORF M6, however, it was also proposed that it is unlikely to encode for any protein (Nash *et al.* 2001; Ebrahimi *et al.* 2003). Therefore, it is important to define the existence of ORF M6 protein expression.

We used infection of mice with murine gamma herpesvirus-68 (MHV-68) as a small animal model to study the biological significance of the 40 bp internal repeat and/or ORF M6 of MHV-68 both *in vitro* and *in vivo*. In order to achieve this goal, we carried out the following steps:

A) Construction of the 40 bp repeat and ORF M6 mutant viruses

6 independent virus mutants were constructed to determine the role of the 40 bp repeat and/or ORF M6 in the context of the whole MHV-68 genome.

B) *In vitro* characterization of the 40 bp repeat and ORF M6 mutants

The *in vitro* characterization of the 40 bp and ORF M6 mutants was carried out by analyzing their growth properties in cell culture.

C) *In vivo* characterization of the 40 bp repeat and ORF M6 mutants

Mice were infected with the mutant viruses and their phenotype was analyzed *in vivo* by determining different parameters including lytic virus titers in the lung, spleen weight and *ex vivo* reactivation.

2.0 Materials

2.1. Bacterial strains

E. coli DH10B	F-, *mcr*A, (*mrr-hsd*RMS-*mcr*BC), Φ80d*lac*ZΔM15, Δ*lac*X74,*deo*R, *rec*A1, *end*A1, *ara*D139, Δ(*ara*, *leu*)7697, *gal*U, *gal*K, λ-, *rps*L, *nup*G, used for propagation and recombination of MHV-68 BAC.
E. coli JC8679	*rec* BC, *sbc* A, used for ET cloning

2.2. Existing cell lines

293	Human embryonic kidney epithelial cell line (Graham *et al.* 1977)
Ag8	Mouse B cell line (ATCC No. CRL-1580)
BHK-21	Baby hamster kidney cell line (ATCC No. CCL-10)
NIH 3T3	Mouse fibroblast cell line (ATCC No. CRL-1658)
Ref/Cre	rat embryonal fibroblast cell line expressing Cre recombinase

2.3. Mice strains

C57BL/6 wild type	Charles River Laboratory, Sulzfeld, Germany
C57BL/6 wild type	Jackson Laboratory, Bar Harbor, ME, USA
C57BL/6 β2 micoglobulin-/-	Jackson Laboratory, Bar Harbor, ME, USA
C57BL/6 CD8-/-	Jackson Laboratory, Bar Harbor, ME, USA

2.4. Materials used for mice maintenance, anesthesia

Autoclaved chow

Ketamine/Xylazine

2.5. Materials for bacterial and eucaryotic cell culture

2.5.1. Microbial nutrients

Bacto-agar	Invitrogen, Germany
Bacto-tryptone	Invitrogen, Germany
Bacto-yeast extract	Invitrogen, Germany

2.5.2. Growth media and accessories for mammalian cell culture and transfection

DMEM High Glucose Medium	Invitrogen, Germany
Foetal calf serum	Invitrogen/Gibco, Germany
Glasgow`s MEM (GIBCO)	Biochrom AG, Germany
HEPES	Invitrogen, Germany
L-glutamine	Invitrogen, Germany
RPMI - 1640 medium	Invitrogen, Germany
Superfect transfection reagent	Qiagen, Germany

2.5.3. Antibiotics

Ampicillin	Sigma-Aldrich, USA
Chloramphenicol	Sigma-Aldrich, USA
Kanamycin	Sigma-Aldrich, USA
Penicillin/Streptomycin	Invitrogen, Germany

2.6. Plasmids

2.6.1. Existing plasmids used during this work

pST 76 K-SR	shuttle plasmid, used for mutagenesis of MHV-68 (Adler *et al.* 2003)
pUC19	a small, high copy number *E. coli* plasmid
MHV-68 BAC	MHV-68 genome, cloned as BAC (Adler *et al.* 2003)

2.6.2. Plasmids produced during the course of this work

pST 76 derived plasmids:

pST 76 K-SR M6	a pST 76 K-SR derivative plasmid containing a *Sma I* fragment of MHV-68 (nucleotide position 24918-30834)
pST 76K-SR M6 Stop	a pST 76K-SR derived plasmid containing a stop codon in all three frames of ORF M6 at *Hind III* site in MHV-68 genome (nucleotide position 26711)
pST 76 K-SR M6 Point	a pUC 19 derived plasmid containing a point mutation (ATG to ACG) in the start codon of ORF M6 in MHV-68 genome (nucleotide position 26555)
pST 76K-SR M6 Stop+ Point	a pUC 19 derived plasmid containing a point mutation in the start codon (nucleotide position 26555) as well as stop codon in all the three possible frames of ORF M6 in MHV-68 genome (nucleotide position 26711)

pUC19 derived plasmids

pUC 19 (*Hind III* negative)	a pUC 19 derivative plasmid without the restriction enzyme *Hind III* site in its multiple cloning site.
pUC 19 M6	a pUC 19 derived plasmid containing 5.237 kbps *Kpn I- Nde I* (containing the 40 bp internal repeat) fragment from pST76K-SR M6.

pUC19 M6 Stop	a pUC 19 derived plasmid containing a stop codon in all three frames of ORF M6 at *Hind III* site in MHV-68 genome (nucleotide position 26711)
pUC 19 M6 Point	a pUC 19 derived plasmid containing a point mutation (ATG to ACG) in the start codon of ORF M6 in MHV-68 genome (nucleotide position 26555)
pUC19 M6 Stop + Point	a pUC 19 derived plasmid containing a point mutation in the start codon as well as stop codon in all the three possible frames of ORF M6 in MHV-68 genome (nucleotide position 26711)

MHV-68 BAC derived plasmid

40 bp mutant BAC plasmid	a MHV-68 derived BAC plasmid without the 40 bp internal repeats (nucleotide position 26778-28191)
M6 Stop BAC mutant	a pST 76K-SR derived BAC plasmid containing a stop codon in all three frames of ORF M6 at *Hind* III site in MHV-68 genome (nucleotide position 26711)
M6 Point BAC mutant	a pST 76K-SR derived BAC plasmid containing a point mutation (ATG to ACG) in the start codon of ORF M6 in MHV-68 genome (nucleotide position 26555)
M6 Stop+ Point BAC mutant	a pST 76K-SR derived BAC plasmid containing a point mutation in the start codon as well as stop codons in all three possible reading frames of ORF M6 in MHV-68 genome

2.7. Oligonucleotides

All oligonucleotides were manufactured by Metabion GmbH, Germany

2.7.1. Primers used for estimation of genomic load in splenocytes

ORF 50 (MHV-68 open reading frame 50)

LDAPCRin1	5`- CCC CAA TGG TTC ATA AGT GG -´3
LDAPCRin2	5'- ATC AGC ACG CCA TCA ACA TC -`3

rpl8 (ribosomal protein l8)

rpl Forward	5'- CAG TGA ATA TCG GCA ATG TTT TG -`3
rpl Reverse	5'- TTC ACT CGA GTC TTC TTG GTC TC -`3

K3 (MHV-68 K3 gene)

K3 for	5'- GAG AGT TCT GTT GGA TCT GC -3'
K3 Rev	5'- GTC GCG ATC GCC TCA TCA ATG -3'

eF1β (elongation factor 1β)

eF1 β for	5'- TTA CCT GGC GGA CAA GAG CT-3'
eF1 β rev	5'- CCA ATT TAG AGG AGC CCC ACA-3'

GAPDH (Glyceraldehyde Phosphate Dehydrogenase)

GAPDH for	5'- CTC ACT CAA GAT TGT CAG CAA TG-3'
GAPDH rev	5'- GAG GGA GAT GCT CAG TGT TGG-3'

2.7.2. Primers used for the generation of mutations in the ORF M6 region of pUC19 M6

pUC19 M6 Stop

M6 Stop for	5'- AAT ATA AAC TCG AGG CGG CAA GG-3`
M6 Stop rev	5'- TAT AGA AAG CTT CTA GTT AAC TAG TTA TAT CAG AAT AAA AC-3'

pUC19 M6 Point

M6 point for1	5'-AAT ATA AAC TCG AGG CGG CAA GG-3'
M6 point rev1	5' TGC CGT CCT GTA TAC CCA TTT TG-3'
M6 Point for 2	5'CAG GAC GGC ACA CAC CAA C-3'

M6 Point rev2	5’ AAG CTC GTG AAA GCT TTT ATA TCA GAA TAA-3’

2.7.3. Primers used for ET cloning to generate 40 bp BAC mutant

40 bp mut for	5’- TCT GGG TCA TTT TGG TTT TGT TCC TCG ACC TGT GCG GAT CGT TTT GGG TTC CAG GGT TTT CCC AGT CAC GAC GT-3’
40 bp mut rev	5’- TGA GAT GAC TTT GTG ACA GGT CCC AGC TCG GGC GAG GGC CGG GAA GGT GGC ACA GGA AAC AGC TAT GAC CAT GA -3’

2.7.4. Primers used for verification of mutations in the ORF M6 region

M6 seq. for	5’-ATC GAT ATC TCC CCT GAA GC-3’
M6 seq. rev	5’- ACA GGT CGA GGA ACA AAA C-3’
B5 NTBAC	5’-TCG ACC TGT GCGGATCG-3’

2.8. Antibodies

Mouse anti-DIG antibody, AP conjugated (Roche Diagnostics GmbH, Germany)

The following antibodies were purchased from Pharmingen, USA

FITC anti mouse CD3

FITC anti mouse CD4

FITC anti mouse CD45R/B220

FITC anti mouse CD8a (Ly-2)

FITC mouse $IgG2_b$

PE anti mouse Vβ4 T-cell Receptor

PE mouse $IgG2_b$

2.9. Enzymes

Alkaline shrimp phosphatase	Roche Diagnostics GmbH,Germany
DNA endonucleases	New England Biolabs, USA;
Klenow fragment	Roche Diagnostics GmbH, German
Proteinase K	Sigma-Aldrich GmbH, Germany
Pwo DNA polymerase	Roche Diagnostics GmbH, Germany
RNAse A	Sigma-Aldrich GmbH, Germany
T4-DNA ligase	New England Biolabs, USA
Taq DNA polymerase	PeqLab, Germany

2.10. Commercial kits

DIG DNA Labeling kit	Roche Diagnostics GmbH, Germany
LightCycler-FastStart	Roche Diagnostics GmbH, Germany
Gel Purification kit	Amersham Biosciences, Germany

2.11. Materials or reagents used for molecular biology techniques

Agarose	Invitrogen, Germany
Bovine serum albumin	New England Biolabs, USA
DIG DNA labelling mix	Roche Diagnostics GmbH, Germany
DNA ladders	Invitrogen, Germany
dNTP	Roche Diagnostics GmbH, Germany
Ethidium-Bromide	Merck KG, Germany

2.12. Plates, flasks and tubes for cell culture

Falcon tubes (15 and 50 ml)	Becton Dickinson
Tissue culture flask (25, 75 and 150 cm^2)	TPP, Switzerland
Tissue culture plates	

(6, 24 and 96 well plates)	TPP, Switzerland
Pippetes (25, 10 and 5 ml)	Greiner bio-one, Switzerland

2.13. Other materials

Gel-Blotting paper	Schleicher & Schuell, Germany
Cell culture plates and flasks	Nunc, Germany
Electroporation cuvettes (2,4 mm)	Bio-Rad Laboratories, UK
Filter , 0.45 μm, 0.2 μm	Sartorius, Germany
Filter, 0.8 μm	Renner GmbH, Germany
Hybond N+ PVDF membrane	Amersham Bioscience, Sweden
Uni-Filter 96 GF/C	Packard Biosciences Germany
X-ray film (Biomax MS)	Eastman Kodak Co, USA

2.14. Laboratory equipment

Bacterial incubator	Heraeus Instruments, Germany
Bacterial shaker C25	New Brunswick Scientific, USA
Centrifuge 2K15	Sigma Centrifuges GmbH, Germany
Centrifuge 35R	Hettich, Germany
Centrifuge 5415D	Eppendorf, Germany
Centrifuge, 5417R	Eppendorf, Germany
Centrifuge 6K10	Sigma Centrifuges GmbH, Germany
Centrifuge EBA12	Hettich, Germany
Centrifuge HSP 9	Heraeus, Germany
Centrifuge RC5C	Sorvall Instruments, USA
Electroporator	Bio-Rad Laboratories, USA
Electroporator	PEQLABBiotechnologieGmbH, Germany
Freezer -20 ^{o}C	Liebherr, Germany
Freezer -80 ^{o}C	Thermo-Lifesciences, Germany
Freezer 4 ^{o}C	Bosch, Germany
Flow cytometer	Beckton-Dickinson GmbH, Germany
Flow hood work bench	Heraeus Instruments, Germany

Gel electrophoresis power supply	Biometra, Germany
Gel electrophoresis units	Amersham Bioscence, Sweden
Gel electrophoresis unit	Biometra, Germany
Hybaid hybridization oven	MWG Biotech, Germany
Light Microscope	Zeiss, Germany
LightCycler	Roche Diognostics GmbH, Germany
Mammalian cell incubator	Heraeus Instruments, Germany
PCR System	Eppendorf, Germany
Power X-ray film developer	Eastman Kodak Company, USA
Spectrophotometer	Biometra GmbH, Germany
Speed-vac, concentrator SVC100	Savant, USA
Thermomixer 5436	Eppendorf, Germany
Thermomixer comfort	Eppendorf, Germany
Ultracentrifuge L7-55, L8-70M	Beckman, USA
UV transilluminator	PeqLab, Germany
Water purification system	Millipore GmbH, Germany
Water Bath	GFL, Germany

3.0 Methods

The standard molecular biology techniques used in this work were performed according to the protocols described (Sambrook and Russell 2001).

3.1. Bacterial cell culture

3.1.1. Maintenance and propagation of bacteria

LB agar plates and LB liquid medium were used to cultivate bacteria. To isolate single colonies of bacteria of interest, bacteria were cultured overnight on LB plates with or without antibiotics. For propagation of single clones, single colonies were cultured in appropriate volume snapcaps or culture flasks in LB medium in a bacterial shaker at 37°C for approximately 16-18 hours. Appropriate antibiotics were added to the culture medium as indicated. The final concentrations of antibiotics were: ampicillin; 100 μg/ml, kanamycin; 25 μg/ml, chloramphenicol; 30 μg/ml. For long-term storage, dense bacterial pellets were stored frozen in deep freeze vials at -80°C in growth media with 15% glycerol.

LB-medium:	20 mM $MgSO_4$; 10 mM KCl; 1% (w/v) Bacto-Tryptone; 0.5% (w/v) Bacto-yeast extract; 0.5% (w/v) NaCl
LB-agar:	LB-medium, 1.5% (w/v) Bacto-agar

3.1.2. Preparation of competent bacteria

3.1.2.1. Preparation of competent bacteria for electroporation

Relevant bacteria were plated out on LB plates to obtain single colonies. A single colony from the culture plate was inoculated in 5 ml of LB media with or without antibiotics and cultured overnight in a shaking incubator. 2 ml from this overnight culture were used for inoculation of 500 ml LB medium with or without antibiotics and cultured at 37°C. Bacterial cultures were grown to an optical density of 0.5 to 0.7 and then kept on ice for 10-15 min. Bacteria were then pelleted at 4200 rpm for 20 min at 4°C and resuspended in 250 ml of chilled H_2O. After a second centrifugation with similar conditions, the bacterial pellet was resuspended in ice cold 200 ml of 0.2 mM Hepes (pH 7.5) solution. A third round of centrifugation was performed, and the pelleted bacteria were resuspended in 10 ml of 15% cold glycerol. After a final centrifugation step, the bacterial pellet was resuspended in 4-5 ml of 10% cold glycerol. This suspension was frozen down to -80°C in 50 µl aliquots for use in electroporation.

3.1.2.2. Preparation of competent bacteria for heat shock transformation

Competent bacteria were prepared following the protocol of Sambrook, Fritsch and Maniatis (page 1.82). Briefly, a single colony from a LB-plate was used to inoculate 5 ml of LB-medium and cultured overnight in a shaking incubator. 1 ml of this overnight culture was used to inoculate 200 ml of LB-medium. Bacteria were grown in a 1 l flask, with shaking, at 37°C until the absorbance at 600 nm reached 0.5 to 0.7. After harvesting, bacteria were kept on ice for 10 min and pelleted at 3500 rpm, 20 minutes at 4°C. The medium was discarded and the bacteria resuspended in 20 ml of ice-cold 0.1 M $CaCl_2$. The cells were then pelleted at 3500 rpm for 20 minutes at 4°C and resuspended in 4 ml of ice-cold 0.1 M $CaCl_2$. Another round of centrifugation was done and finally the cells were resuspended in 2 ml of 10% cold glycerol and frozen immediately at -80°C in 50 µl aliquots.

3.1.3. Transformation of bacteria

3.1.3.1. Heat shock transformation

50 µl competent bacteria were thawed on ice, mixed with 10-100 ng of plasmid DNA or 10 µl of ligation mixture and incubated on ice for 30 min. The bacteria-DNA mix was then subjected to heat shock at 42°C for 2 min and transferred to ice for 5 minutes. 500 µl LB-medium was supplied and the sample incubated at 37°C for 1h. Different amounts of the sample (20-300 µl) were plated onto LB-agar plates containing the appropriate antibiotic. The LB-agar plates were incubated at 30 or 37°C for 16-18 hours after which single colonies growing out could be observed.

3.1.3.2. Transformation using electroporation

50 µl of electro-competent bacteria were mixed with 10-100 ng of plasmid DNA and incubated for 5-10 minutes on ice. The cells were subjected to electric shock in a BioRad electroporator at 2500 V and 335 Ω in a 2 mm cuvette and transferred to ice for 1 minute. 500 µl of LB-medium was then added to the sample and incubated at 30-37°C for 1 hour. Different amounts of bacterial suspension were plated onto LB-agar plates containing appropriate antibiotics. The LB-agar plates were incubated at 30-37°C for 16-18 hours.

3.1.4. Isolation of plasmid

3.1.4.1. Small-scale plasmid DNA isolation (minipreps)

Bacteria carrying the correctly cloned constructs were identified after small scale plasmid DNA isolation. For this purpose, 1 ml overnight single clone bacterial cultures were centrifuged at 14000 rpm for 1 minute in 1.5 ml eppendorf microcentrifuge tubes. The supernatant was discarded and the pellet resuspended in 200 µl of solution I to

obtain a homogenous suspension. Bacteria were lysed by the slow addition of 200 µl of solution II with gentle mixing by inversion of the tube for a few times. Following 5 minutes incubation at room temperature, 400 µl of solution III was added and the tubes were incubated on ice after gentle swirling to obtain a gelatinous mass of precipitate. The samples were then centrifuged at 14000 rpm for 10 minutes to pellet down the debris and the supernatant was collected in a fresh tube. Absolute ethanol was added to this supernatant to a final concentration of 60% and the DNA was precipitated by centrifugation at 14000 rpm for 10 minutes at 4°C. The supernatant was carefully discarded so as to retain the pellet which was then washed with 70% ethanol by resuspension of the pellet and centrifugation for 5 minutes at conditions as for the previous step. The pellet finally obtained by discarding the supernatant was allowed to air dry and resuspended in 50 µl of 1 X TE.

Solution I:	50 mM glucose; 10 mM EDTA; 25 mM Tris-HCl pH 8.0
Solution II:	0.2 M NaOH; 1% SDS
Solution III:	3.1 M K-acetate (pH 5.5)
1 X TE:	10 mM Tris-HCl-1 mM EDTA (pH 7.5)

3.1.4.2. Small-scale preparation of highly pure plasmid DNA

To obtain highly pure plasmid DNA for sequencing, restriction enzyme analysis and transformation a small scale preparation of plasmids was carried out using the GFX^{TM} Micro Plasmid Prep Kit (Amersham Biosciences, Sweden). Briefly, 1.5 ml of overnight bacterial culture was spun down and resuspended in solution I followed by lysis with solution II and neutralised by solution III. The cell debris was pelleted down by centrifugation at 14000 rpm for 5 minutes, and the plasmid-containing supernatant was loaded onto a GFX column. After loading the plasmid containing solution onto the

column, one round of washing with wash buffer was done and finally the plasmid DNA was eluted in 100 µl of 1 X TE.

3.1.4.3. Large-scale preparation of plasmid DNA

Large scale plasmid preparation was carried out using the Qiagen Maxi Kit 25. Briefly, a 300 ml overnight culture was spun down and the pellet was resuspended in 10 ml of solution P1. SDS alkaline lysis of the bacteria was done with 10 ml of solution P2 and the lysate was neutralised with 10 ml of neutralisation solution P3. The neutralised lysate was centrifuged at 20,000 x g for 30 minutes at 4°C. Then, the clear supernatant was transferred onto equilibrated silica columns optimised for plasmid DNA binding and washed with buffer QC. Bound plasmid DNA was eluted with the elution solution (QF) and the DNA in the eluate was acquired by precipitation with l0.5 ml of isopropanol. The supernatant was carefully discarded so as to retain the pellet and the pellet was washed with 70% ethanol and resuspended in 1 X TE. Purity of the plasmid DNA was assessed by measuring the 260 nm/280 nm absorption ratio. The plasmid was analysed by multiple restriction enzyme digestions and sequenced where indicated.

3.1.4.4. Small-scale BAC plasmid isolation

For the screening of BAC clones, a small scale BAC plasmid preparation was completed. 10 ml overnight bacterial cultures were spun down by centrifugation at 3500 rpm for 10 minutes and the pellet was resuspended in 200 µl of solution I (Qiagen Maxi Kit 25). 200 µl of solution II was then slowly added to the suspension and the samples were gently mixed by inverting a few times. Following 5 minute incubation at room temperature, 200 µl of solution III was added and the tubes were incubated on ice for 10 minutes after gentle swirling to obtain a gelatinous mass of precipitate. The samples were then centrifuged at 14000 rpm for 10 minutes at 4˚C to pellet down the debris and the supernatant was collected by transferring to a fresh tube. An equal volume of phenol: chloroform: isoamylalcohol (IAA) (25:24:1) was added to the samples and mixed well by inverting for 10 minutes. The aqueous phase containing the plasmid was separated from the phenol chloroform phase by centrifugation at 14000 rpm for 10 min.

The aqueous phase was transferred to a new eppendorf tube. 1 ml of isopropanol was added and the plasmid DNA was precipitated by centrifugation at 14000 rpm for 15 minutes at 4˚C. The supernatant was carefully discarded so as to retain the pellet and the pellet was washed with 1 ml of 70% ethanol by resuspending the pellet and centrifugation for 5 minutes at 14000 rpm. The pellet was finally obtained by discarding the 70% ethanol, air drying and resuspension in 50 µl of 1 X TE.

3.1.4.5. Large-scale BAC plasmid isolation

To obtain highly pure BAC plasmid for sequencing, restriction enzyme analysis and transformation a large scale preparation of BAC plasmids was carried out using Nucleobond PC Kits AX500. Briefly, 500 ml of a bacterial culture was spun down and resuspended in 24 ml solution S1. This was followed by lysis with 24 ml of solution S2 and neutralization by 24 ml of solution S3. The cell debris was removed by filtering the bacterial lysate through filter paper, and the clear supernatant was transferred onto equilibrated silica columns optimised for plasmid DNA binding and washed with buffer N3. Bound plasmid DNA was eluted with prewarmed 14 ml of elution solution N5 and precipitated with l0.5 ml of isopropanol. The supernatant was carefully discarded so as to retain the pellet and the pellet was washed with 70% ethanol. The pellet was finally resuspended in Tris-EDTA pH 8.0.

3.1.5. BAC plasmid mutagenesis

The mutation of BAC plasmids was carried out either by ET cloning or shuttle mutagenesis.

3.1.5.1. ET Cloning

The 40 bp mutant BAC plasmid was constructed by ET cloning (Fig 3.1) (Zhang *et al.* 1998; Adler *et al.* 2000; 2003). This method uses the recombinant proteins *rec*E and *rec*T of *E. coli* strain JC8679 for carrying out the homologous recombination between the linear and circular DNA and is therefore called ET cloning. For carrying out ET cloning, a linear PCR product was generated using a primer pair that contained 24 nucleotides for the amplification of a tetracycline resistance gene from vector pCP 16 flanked by FRT (FLP recognition target sites) and an additional 50 nucleotides homologous to the sequences flanking the MHV-68 40 bp internal repeat corresponding to nucleotide positions 26778–28191 in the MHV genome. PCR products were purified by electrophoresis in a 1% low-melting-point agarose gel, extracted with phenol-chloroform, and resuspended in 25 µl of Tris-EDTA pH 8.0 buffer. 10 µl of the linear, PCR-generated fragments were electroporated into *E. coli* JC8679 (*recBC sbcA*) containing the MHV-68 BAC plasmid. Bacteria were incubated at 37°C for 1 hour and plated onto agar plates containing chloramphenicol (17 µg/ml) and tetracycline (10 µg/ml). Plasmid DNA was isolated and analyzed by restriction enzyme digestion and the correct 40 bp mutant BAC plasmid was identified and used further. To remove the tetracycline resistance gene from the 40 bp mutant BAC plasmid, the 40 bp BAC plasmid was retransformed into *E. coli* DH10B. The Flp expression plasmid pCP20 was electroporated into *E. coli* DH10B containing the 40 bp mutant BAC plasmid. The bacteria were plated onto chloramphenicol-ampicillin (100 µg/ml) LB agar plates and grown overnight at 30°C. Colonies were replated on chloramphenicol-containing plates and grown overnight at 43°C. Colonies were again replated on both chloramphenicol- and tetracycline-containing plates and grown overnight at 37°C. BAC plasmids from chloramphenicol-positive, tetracycline-negative colonies were analyzed by restriction enzyme analysis for the loss of the tetracycline resistance gene.

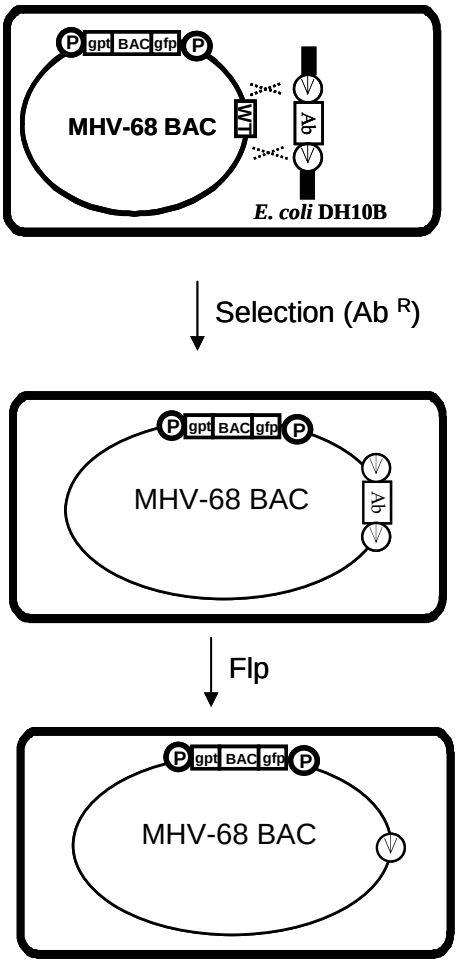


Fig 3.1. ET cloning for the mutagenesis of the BAC cloned MHV-68 genome in *E. coli*. The procedure can be performed in *E. coli* strains such as JC8679 which constitutively express *rec* E and *rec* T. A selectable marker gene (tetracycline gene Ⓥ), flanked by FRT sites (Ab), is created by PCR using primers which contain homologies of about 50 nucleotides according to the desired integration site at their 5'-ends, the mutation and the priming regions specific for the selectable marker gene. The generated linear DNA fragment is transferred to the MHV-68 BAC by a double crossing-over event. The tetracycline cassette can be excised by recombinase FLP, leaving behind one FRT site.

(modified from Adler *et al.* 2003)

3.1.5.2. Two step replacement procedure (Shuttle mutagenesis)

The 40 bp revertant BAC plasmid, M6 Stop mutant BAC plasmid, M6 Point mutant BAC plasmid and M6 Stop + Point mutant BAC plasmid were constructed by shuttle mutagenesis as described (Adler *et al.* 2000; 2003; Messerle *et al.* 1997) (Fig 3.2). For

carrying out the two step replacement procedure, first a DNA fragment of interest containing either a mutant or the wild type fragment (to generate a revertant virus) was cloned into the shuttle plasmid pST76K-SR which contains the *rec*A gene. The *rec*A protein facilitates homologous recombination leading to the formation of a cointegrate; i.e., BAC plasmid with shuttle plasmid sequences. The shuttle plasmid pST76K-SR was electroporated into *E. coli* DH10B bacteria that already contained either wild type MHV-68 BAC plasmid or mutant MHV-68 BAC plasmid. Transformants were selected at 30°C on LB agar plates containing chloramphenicol and kanamycin. Clones containing cointegrates were identified by streaking the bacteria onto new LB plates with chloramphenicol and kanamycin and incubating them at 43°C. To allow resolution of the cointegrates, clones were streaked onto LB plates containing chloramphenicol and incubated at 30°C. To select for clones that had resolved the cointegrate and that contained the mutant BAC plasmid, bacteria were restreaked onto LB plates containing chloramphenicol and 5% sucrose. Resolution of the cointegrate was confirmed by testing for the loss of the kanamycin resistance cassette encoded by the shuttle plasmid. BAC plasmid DNA was isolated from 10 ml overnight cultures by the alkaline lysis procedure and characterized by restriction enzyme analysis.

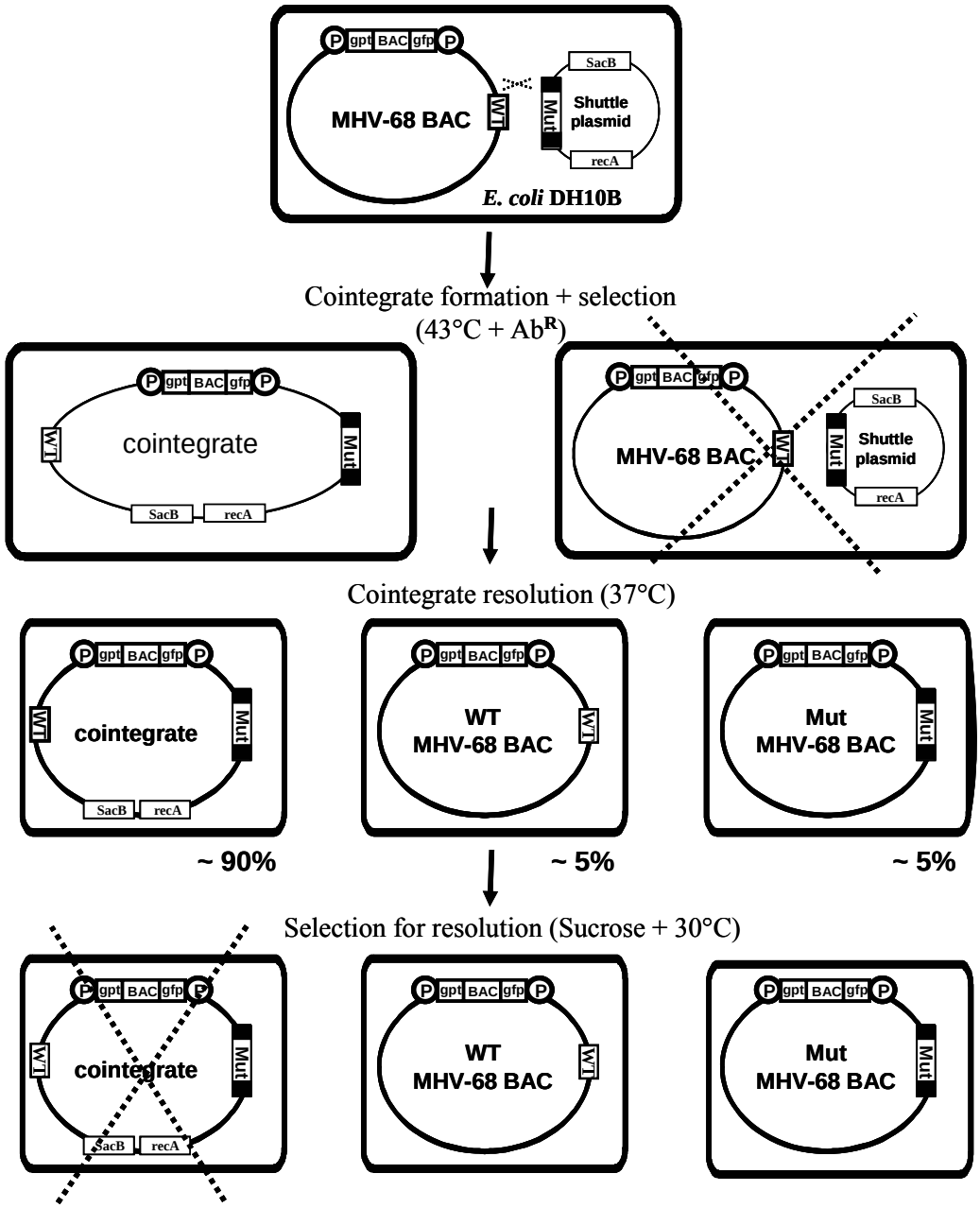


Fig 3.2. Two step replacement procedure (shuttle mutagenesis) for mutagenesis of the BAC cloned MHV-68 in *E. coli*. The mutant allele (Mut), flanked by the sequences homologous to the desired integration site, is provided by a shuttle plasmid with a temperature-sensitive replication mode and the negative selection marker SacB which allows bacteria harbouring a resolved cointegrate to be selected. The shuttle plasmid also contains a kanamycin resistance gene (not shown), while the BAC plasmid contains a chloramphenicol resistance gene (not shown). For mutagenesis in *E. coli* DH10B, the shuttle plasmid also provides the *rec*A gene for homologous recombination. In the first step, the shuttle plasmid carrying the desired mutation plus flanking homologies (black boxes) is transformed into bacteria already containing the BAC plasmid. Through the homologous recombination via one of the two homologies, the shuttle plasmid is completely integrated into the viral BAC genome, leading to a cointegrate. Bacteria containing the cointegrate are selected by incubation at 43°C plus both antibiotics. Resolution of the cointegrate via one of the two homologies leads to either wild type (WT) or mutant (Mut) BAC plasmid.

Bacteria harbouring a resolved cointegrate are selected at 30°C on agar plates containing 5% sucrose (counter-selection against SacB) together with the antibiotic whose resistance gene is encoded on the BAC.

(modified from Adler *et al.* 2003)

3.2. Molecular biology techniques

3.2.1. Strategies for cloning

All the cloning work was performed according to the protocols described (Sambrook and Russell 2001). Plasmid DNA was digested as necessary with 1 or 2 restriction enzymes and purified by agarose gel electrophoresis. Depending upon the requirements, the linearised plasmid DNA or the insert DNA fragment were treated with either alkaline phosphatase to remove 5'-phosphate groups or DNA polymerase I Klenow to repair 3' or 5' overhanging ends to generate blunt ends. The linearised plasmid vector was ligated with the insert DNA fragment obtained either by PCR reaction or restriction enzyme reaction of plasmid DNA, in a volume of 15 µl. The molar ratio of vector to insert was 1:3. Ligation was performed at 16°C overnight in the presence of 40 U of T4 DNA ligase. 50 µl of competent bacteria were transformed with the total ligation reaction.

3.2.1.1. Generation of pUC 19 M6 Stop mutant

Construction of the recombinant plasmid pUC 19 M6 Stop was completed by generating a 838 bp *Xho I- Hind III* fragment by PCR on pST76K-SR M6 using primers M6 Stop forward and reverse (see materials 2.7.2.). The M6 Stop reverse primer contains stop codons in all three frames thus adding stop codons to the 3' end of the PCR product. Further, the addition of the stop codons generates a recognition site for the restriction enzyme *Hpa I*. The PCR product was digested with *Xho I-Hind III and* cloned into pUC 19 using the *Xho I-Hind III* sites. The resulting plasmid was named pUC 19 M6 Stop.

3.2.1.2. Generation of pUC 19 M6 Point mutant

The pUC 19 M6 Point mutant plasmid was generated by a 2 step PCR mutagenesis replacing the start codon of M6 (ATG) by ACG. For this purpose, in the first round 2 PCR products were generated by PCRs on pST76K-SR M6, the first using primers (see materials 2.7.2) M6 Point forward I and M6 Point reverse 2 and the second using primers M6 Point forward 2 and M6 Point reverse 2. Using a mixture of these two PCR products as template and the primers M6 Point forward 1 and M6 Point reverse 2, an 822 bp fragment was generated. Following digestion with *Xho I* and *Hind III*, this new PCR product was cloned into pUC 19 M6 using *Xho I and Hind III*.

3.2.1.3. Generation of pUC 19 M6 Stop + Point mutant

Although the ORF M6 Point mutant contains a point mutation in the start codon, the proposed ORF M6 sequence contains another ATG downstream at the 5' end of the *Hind III* restriction site, where transcription could potentially start. Although, the downstream ATG is not in the same frame as ORF M6, it can regain the same frame as ORF M6 due to presence of 40 bp repeats. Thus, in order to make sure that all possible ORF M6 gene products are disrupted, we decided to construct a double mutant, essentially containing the mutation in the start codon as the Point mutant and also the features of the Stop mutant. For this purpose, a PCR was performed on pUC 19 M6 Point mutant using primers used for construction of the pUC 19 M6 Stop plasmid. This PCR product containing the double mutation was cloned into the pUC 19 plasmid using *Xho I* and *Hind III*. The ligated product was named pUC 19 M6 Stop + Point mutant.

3.2.1.4. Generation of pST76K-SR M6 mutants

After pUC 19 M6 Stop, pUC 19 M6 Point mutant and pUC 19 M6 Stop + Point mutant were confirmed by sequencing and restriction enzyme analysis, a 5.237 or 5.253 kbp *Kpn I – Nde I* fragment from pUC 19 M6 plasmids were cloned into pST76K-SR

M6 using *Kpn I* and *Nde I*, and named according to the mutants of pUC19 M6, i.e., pST76K-SR M6 Stop, pST76K-SR M6 Point and pST76K-SR M6 Stop + Point.

3.2.1.5. Generation of ORF M6 BAC mutants

The BAC plasmid mutants were generated by a two-step replacement procedure as described in methods (3.1.5.2). For that purpose, the shuttle plasmid pST76K-SR containing the appropriate mutations in a 5.916 kbp fragment was electroporated into *E. coli* strain DH10B, which already contained the MHV-68 BAC plasmid. The homologous recombination between shuttle plasmid pST76K-SR M6 mutant and MHV-68 BAC plasmid led to the formation of ORF M6 BAC plasmid mutants. They were named according to the mutation they were carrying, i.e., M6 Stop mutant, M6 Point mutant and M6 Stop + Point mutant.

3.2.2. Circular viral DNA isolation

Circular viral DNA was isolated from infected NIH 3T3 cells by the method described by Hirt (Hirt 1967; Messerle *et al.* 1997). Briefly, 2-10 x 10^7cells were trypsinized and harvested. After harvesting the cells, they were washed with phosphate-buffered saline and resuspended in 500 µl of Tris-EDTA (100 mM Tris-HCl, 20 mM EDTA, pH 8.0). Resuspended cells were treated with 500 µl of 1.2% sodium dodecyl sulphate. The cellular genomic DNA and proteins were precipitated by addition of 660 µl 5 M NaCl overnight at 4^{o}C. Following the addition of NaCl, the samples were centrifuged at 14000 rpm for 30 minutes. The solution free of cell debris was extracted and treated with an equal volume of phenol: chloroform: isoamylalcohol (25:24:1). The samples were mixed by inverting the tubes for 10 minutes and centrifuged at 14000 rpm for 10 minutes at 4^{o}C. After the centrifugation the aqueous phase was transferred to a fresh tube. The viral DNA was precipitated with an equal volume of absolute ethanol, washed with 70% ethanol, and finally resuspended in TE buffer (10 mM Tris-HCl-1 mM EDTA, pH 7.5).

3.2.3. RNA isolation

After 24 hours of infection, total RNA from infected or uninfected cells (NIH 3T3 or Ag8) was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA). Briefly, 5-10 x 10^6 cells were washed with PBS and 1 ml of TRIZOL was added to lyse the cells. 0.2 ml of chloroform was added to the cell lysate for dissociation of nucleoprotein complexes and after the centrifugation, the aqueous phase was transferred to a fresh tube. Total RNA was precipitated with 0.5 ml of isopropanol and the pellet was washed with 70% ethanol. The air dried RNA pellet was dissolved in 40-50 µl RNase-free water.

3.2.4. Isolation of total DNA

Genomic DNA from Ag8 cells or splenocytes was isolated using the QIAamp DNA Mini Kit (Qiagen). Briefly, 5 x 10^6 cells were spun down by centrifugation for 5 min at 2500 rpm, and the pellet was resuspended in 200 µl of PBS. 20 µl of proteinase K (Qiagen) was added to the cells along with 200 µl of buffer AL to lyse and mix the cells by pulse vortexing for 15 seconds. After lysis of the cells, 200 µl of ethanol (100%) was added to the sample and mixed again by pulse vortexing for 15 seconds. The cell lysate was then loaded on the QIAamp spin column and washed with buffer AW1 and AW2. Finally, the genomic DNA was eluted with 200 µl of buffer AE.

3.2.5. Reverse transcription PCR

DNase treated RNA (2 µg) was reverse transcribed by using a 1st strand cDNA Synthesis Kit for RT-PCR (AMV)$^+$ from Roche Diagnostics Corp. in 20 µl system. A reverse transcriptase control (without reverse transcriptase) was included in the experiments. The primer sequences for the ORF K3 gene were 5'-GAGAGTTCTGTTGGATCTGC-3' and 5'-GTCGCGATCGCCTCATCAATG-3'. The conditions for PCR were as follows: initial denaturation at 94^{o}C for 3 min followed by

30 cycles of 94°C denaturation for 30 sec, 55°C annealing for 30 sec and 72°C synthesis for 30 sec. The products were analysed on 1% agarose gels. Also, the house keeping gene eF1β, GAPDH was amplified by PCR from the cDNA samples, to check the input of cDNA.

3.2.6. Non-radioactive Southern blot analysis

5-10 µg viral DNA were digested with restriction enzymes and separated on a 0.8% agarose gel in 1 X TBE buffer at 60-70 V overnight. DNA bands were visualised under UV light after staining with ethidium bromide (50 ng/ml in 1 X TBE). After visualisation, the agarose gel was submerged into depurination solution for 10-20 min, and subsequently into denaturation buffer for 30-60 min and finally to neutralisation buffer for 30-60 min with agitation. After setting up the capillary blot, the DNA was transferred onto a nylon membrane (Hybond N, Amersham) overnight in 20 X SSC. The DNA was cross-linked to the membrane by UV cross-linking at 1200 J and baking at 80°C for 2 hours. After cross-linking, the membrane was soaked in 2 X SSC, and prehybridised with prehyb-mix for 2 hours at 68°C. For hybridisation, the DIG-labelled probe (10 ng/ml in prehyb-mix) added to freshly replaced prehyb-mix on the membrane and hybridisation was carried out at 68°C overnight. To prepare DIG-labelled probe, a PCR product (100 ng to 2 µg) was denatured and immediately cooled down on ice and finally labelled as per directions in DIG-labelling manual. After hybridisation, the membrane was washed 2 times for 5 min each in wash buffer I at room temperature. After the first round of washing, the membrane was washed again with wash buffer II twice 15 minutes each at 68°C. After this step, all the subsequent incubations and washing were done at room temperature. The membrane was incubated in 30 ml of DIG I solution for 5 min followed by incubation with DIG II solution for 30 min. 1 µl of α-DIG-AP conjugate (Roche) was added to fresh DIG II solution and incubated on the membrane for 30 min. After washing 2 x 15 min in DIG I solution containing 0.3% Tween 20, the membrane was first equilibrated in DIG III and then incubated for 5min. Then, 1.5 ml of DIG III containing 15 µl of substrate solution (CDP-Star-ready-to-use substrate, Roche, Germany) was added onto the membrane and incubated for 5 minutes.

After the incubation, the membrane was exposed to X-ray film for 10 minutes to 2 hours depending upon the intensity of signal.

Southern blot analysis for characterization of viral mutants

The probe for southern blot analysis was prepared by PCR amplification with primers M6 point for1 5'-AAT ATA AAC TCG AGG CGG CAA GG-3' and M6 Point rev2 5' AAGCTCGTGAAAGCTTTTATATCAGAATAA-3' using MHV-68 BAC plasmid as template.

1 X TBE:	40 mM Tris-Cl (pH 8.0); 5 mM Na-acetate; 1mM EDTA
Depurination solution:	0.25 N HCl
Denaturation buffer:	0.75 M NaCl; 0.25 N NaOH
Neutralisation buffer:	0.25 M Tris-Cl, pH 7.2; 0.75 M NaCl
20xSSC:	3 M NaCl; 300 mM Na_3- citrate. $2H_2O$, pH 7.0
Prehyb-mix:	5% SSC; 1% blocking reagent, 0.02% SDS; 0.1% Lauroylsarcosin solution
10xblocking reagent:	10% (w/v) blocking powder (Roche, Germany); 100 mM Tris/HCl pH 7.5; 150 mM NaCl
Wash buffer 1:	2% SSC; 0.1% SDS
Wash buffer 2:	0.1% SSC; 0.1% SDS
DIG I:	100 mM Tris/HCl pH 7.5; 150 mM NaCl
DIG II:	100 mM Tris/HCl pH 7.5; 150 mM NaCl; 1% Blocking reagent
DIG III:	0.1 M Tris/HCl pH 9.5; 0.1 M NaCl

3.2.7. Real Time PCR

To determine the genomic load of latent virus in splenocytes, real time PCR was performed. It is based on the measurement of the increasing incorporation of the fluorescent SYBR Green I dye into dsDNA. Real-time PCR was done in 20 µl volumes containing the following reagents: 12.6 µl water, 2.4 µl $MgCl_2$, 1 µl of each primer (10 mM), 2 µl of SYBR Green PCR Master Mix, and 2 µl of DNA template. The reaction mix was added to a capillary and sealed. The cycling parameters were hot start at 95^{o}C for 10 minutes, denaturation at 94^{o}C for 10 seconds, annealing at 60^{o}C for 20 seconds and extension at 72^{o}C for 30 seconds. Melting curve analysis was carried out to confirm the specificity of the products between 65-95^{o}C with 0.2^{o}C increments. The PCR products obtained from real time PCR were also analyzed by agarose gel electrophoresis. A portion (262 bases) of the MHV-68 ORF 50 gene was amplified with the forward primer 5`- CCCCAATGGTTCATAAGTGG -´3 and reverse primer 5'-ATCAGCACGCCATCAACATC -`3. The MHV-68 genome copy number was estimated against a standard curve constructed by serial dilution of plasmid pUC19 ORF50 (*Eco RI – Ava I* fragment 65707-69550 nucleotide position of MHV-68). In each reaction, equal amounts of spectrophotometrically estimated DNA were added. The amount of DNA was also confirmed by the estimation of the murine ribosomal protein L8 (rpl8) gene (Genbank accession number AF091511) by real time PCR. The standard curve for rpl8 was constructed by serial dilution of a plasmid containing a 163 bp fragment of rpl8 (pCR2.1/rpl8). Amplification of pCR2.1/rpl8 was carried out using forward primer 5'-CAGTGAATATCGGCAATGTTTTG -`3 and reverse primer 5'-TTCACTCGAGTCTTCTTGGTCTC -´3. Each sample was tested in triplicates.

Real-time PCR were also used for quantification of mRNA in a sample. For this purpose cDNA from 2 µg RNA sample were prepared and expression of MK3 and murine ribosomal protein L8 (rpl8) were checked in similar condition as for estimation of genomic load with the specific primers for MK3 and L8 gene.

Light Cycler master mix: 12.6 µl water; 2.54 µl $MgCl_2$ (25 mM); 1 µl of each primer (10 mM); 2 µl Master SYBR Green

3.3. Eukaryotic cell culture and virological methods

3.3.1. Cultivation of eukaryotic cells

All the eukaryotic cells were grown at 37°C in a 5% CO_2 humidified incubator. Cells in suspension were usually split every third day 1:10 with fresh medium. Living cells were distinguished from dead cells by tryphan-blue (0.4%) staining. Ag8 cells were maintained in RPMI 1640 medium (Invitrogen, Germany) supplemented with 10% newborn calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and 2 mM L-glutamine. The adherent fibroblast cells NIH 3T3 and 293 T cells were cultured in DMEM high glucose media (Invitrogen, Germany) with 10% newborn calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and 2 mM L-glutamine. The fibroblast cells were split twice a week. Confluent cultures were washed with PBS and cells detached by addition of trypsin-EDTA solution (GIBCO). The BHK-21 cells were maintained in Glasgow's modified Eagle's medium (GIBCO) supplemented with 5% newborn calf serum, 5% tryptose-phosphate broth, penicillin (100 U/ml), streptomycin (100 µg/ml) and 2 mM L-glutamine.

3.3.2. Generation of recombinant viruses

A total of 5 µg MHV-68 BAC plasmid was mixed with 10 µl of superfect reagent (Qiagen) and incubated at 37^{o}C for 10 minutes, followed by addition of 1 ml DMEM medium. DMEM medium containing the superfect reagent and the BAC plasmid was added to NIH 3T3 cells, and the cells were incubated for 3-5 hours (Fig. 3.3).

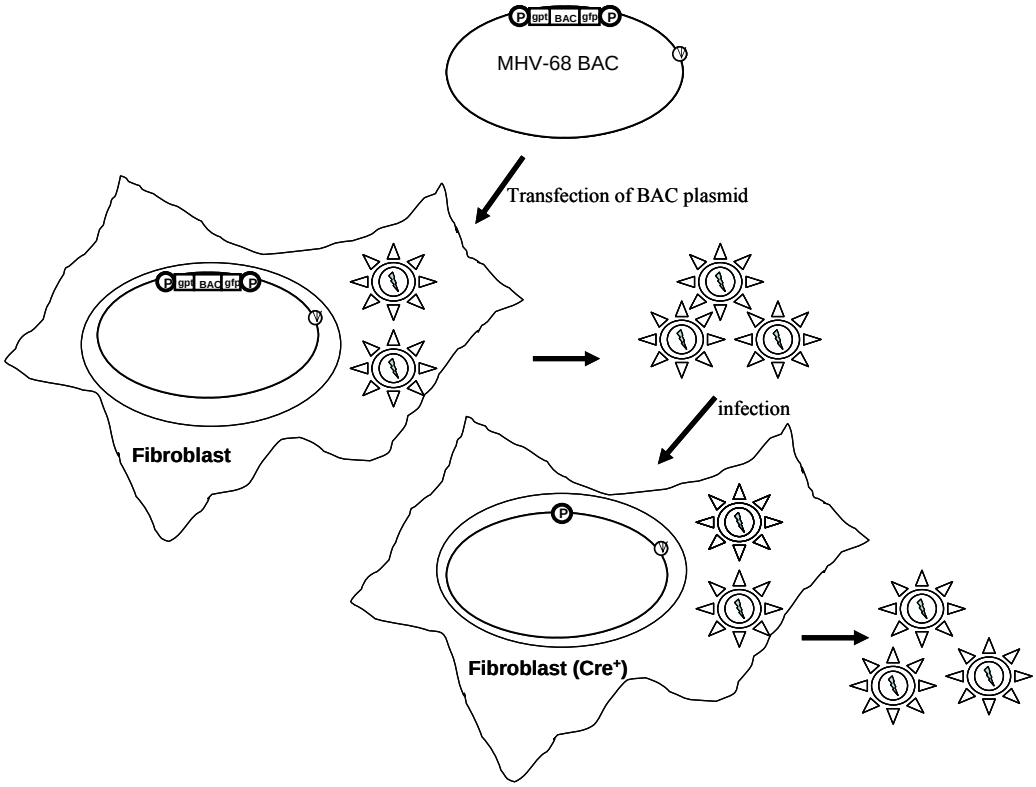


Fig. 3.3. Generation of recombinant viruses. Recombinant viruses were prepared by transfection of BAC plasmids into eukaryotic cells. Propagation of the virus in fibroblasts expressing Cre recombinase results in deletion of DNA sequences of BAC vector. Viral clones with BAC sequences excised were cultured in fibroblast cells to prepare the virus stocks.

(modified from Adler *et al.* 2000)

After incubation, the medium was aspirated and fresh DMEM medium was added, and the cells were cultured for 3-4 days till 70-90% cytopathic effect was observed. The supernatant from these cells containing the virus was collected and stored at -80^{o}C. To remove the BAC vector sequences, rat embryonic fibroblasts expressing Cre recombinase were infected with the virus harvested from NIH 3T3 cells. Viral clones with the BAC vector sequences excised were purified by limiting dilution using loss of GFP expression as a marker. DNA of the mutant, parental and revertant viruses was isolated from infected BHK-21 cells and analyzed by restriction enzyme digestion and southern blot analysis.

3.3.3. Preparation of virus stocks

Virus stocks were prepared on BHK-21 cells. BHK-21 cells were seeded in 75 cm^2 flasks one day prior to the infection. Viral clones obtained from limiting dilution were used for infection of BHK-21 cells at an MOI of 0.1 in 5 ml of complete Glasgow's modified Eagle's medium (GIBCO). Cells were incubated in virus containing media for one hour at 37^oC. Excess virus in culture was removed by aspirating the media and supplementing the culture with fresh complete Glasgow's modified Eagle's medium (GMEM)(GIBCO). The cells were cultured for 4-5 days till complete cytopathic effect was achieved. The cells were harvested by trypsinization and washed with Glasgow's modified Eagle's medium (GIBCO). The cell pellets were resuspended in 1ml of fresh Glasgow's modified Eagle's medium (GIBCO) media, and two rounds of freeze and thaw were performed to release intracellular virus. The cell debris was pelleted down and viruses in the supernatant were stored at -80^oC. The virus titers for the stocks were determined by plaque assay.

3.3.4. Plaque assay

BHK-21 cells were seeded in 24 well plates one day prior to infection to give 60-70% confluence at the time of infection. The virus containing samples were 10-fold serially diluted in Glasgow's modified Eagle's medium (GIBCO) media. The medium from the wells was aspirated, and 900 µl of each dilution was added on top of BHK-21 cells. After 90 minutes of incubation at 37°C, the virus suspension was aspirated and 2 ml of prewarmed overlay medium per well was added. The cells were then cultured for 4-5 days at 37^oC and 5% CO_2. After the formation of plaques, the medium was aspirated and stained with 1 ml of crystal-violet solution for 10 minutes. After the staining, the cells were washed with water and plates were dried. The number of plaques per well were counted and the titer was calculated according the following formula:

PFU/ml = 1/dilution X number of plaques X 1/(ml inoculum per well).

Overlay medium:	Glasgow's modified Eagle's medium (GIBCO) complete media with 0.75% carboxy methylcellulose; 32.7 mM $NaHCO_3$
Crystal-violet stain:	0.1% Crystal-violet in 1% formaldehyde in PBS

3.3.5. *In vitro* growth curves

NIH 3T3 cells were plated at 0.3 x 10^6 cells per well in six well plates 1 day prior to infection. At the time of infection, NIH 3T3 cells were infected with 0.1 PFU per cell (multiplicity of infection = 0.1) of each virus in 1 ml volume with plates rocked every 15 min for 1 h at 37°C. After this hour, virus containing media was aspirated and 2 ml of complete Dulbecco's modified Eagle's medium (DMEM) was added to the monolayer. This time was defined as time zero post infection. At the time of harvesting, cells and supernatant were harvested and stored at –80°C. The amount of infectious virus in culture was estimated by plaque assay on BHK-21 cells. Prior to the plaque assay, samples were subjected to two cycles of freeze and thaw.

3.3.6. *In vitro* reactivation assay

For the *in vitro* reactivation assay, naive mouse splenocytes from C57BL/6 mice were infected overnight with virus at an MOI of 1. The infected splenocytes were harvested and subjected to two washes with RPMI complete medium to get rid of remaining lytic virus present in the medium. Serial threefold dilution (8 dilutions) of single cell suspensions of spleen cells (starting with 10,000 splenocytes) were plated before or after mechanical disruption of viable cells (by two freeze-thaw cycles) on a monolayer of 10^4 low-passage NIH 3T3 cells per well in 96-well tissue culture plates. Twenty four wells were plated per dilutions and NIH 3T3 cells were screened microscopically for viral cytopathic effect for up to 3 weeks. The mechanically disrupted cells allow quantification of preformed lytic virus.

3.3.7. FACS analysis

Single cell suspensions of splenocytes were immunostained with various fluorescence-conjugated antibodies. Staining was performed in FACS-buffer (PBS with 2% FCS) using 1 µg of antibody per 10^6 cells. Samples were incubated at 4^oC for 30 minutes in the dark, washed with FACS-buffer, and resuspended in 0.5 ml of 2% PFA in PBS. Fluorescence was detected using a FACSCalibur flow cytometer and analyzed using the CellQuest software.

3.4. Pathogenesis in mice

The *in vivo* properties of all viruses were studied in female C57BL/6 mice, age 8 to 10 weeks

3.4.1. Maintenance of mice

Mice were housed in individually ventilated cages (ivc) at the mouse facility of the Max von Pettenkofer-Institute, LMU Munich, Germany. Mice were fed with autoclaved chow and supplied with sterile drinking water. All animal experiments were approved by the appropriate local authorities.

3.4.2. Infection of mice

Mice were infected intranasally with a dose of 5 x 10^4 PFU of the indicated viral strain in 40 µl of phosphate-buffered saline. Prior to infection, mice were anesthetised using 70 µl of ketamine/xylazine by i.p. injection. In experiments where $CD8^{-/-}$ or β2

microglobulin$^{-/-}$ mice were used, parental mice obtained from the same laboratory were used in parallel as controls.

3.4.3. Determination of viral titers in the lungs of infected mice

At days 3, 6, 10 and 13 post infections, mice were sacrificed and lungs harvested. Harvested lung tissue was stored in 1 ml of Glasgow's modified Eagle's media at -80°C for future use. Thawed lung tissue was homogenized by douncing and subjected to two rounds of freezing and thawing to disrupt the tissue structure. The resulting suspension was vortexed and used directly in plaque assays on BHK-21 cells to determine the viral titer as described above (3.3.4). To determine the limit of detection of the assay, known amounts of virus were added to uninfected lung tissue, homogenized, and after two rounds of freezing and thawing, the virus titers were estimated by plaque assay. It was found that 50 PFU was the limit of detection.

3.4.4. Preparation of mouse splenocytes

Spleens harvested from mice were immersed in 15 ml Falcon tubes in 3 ml complete DMEM. Single cell suspensions were obtained by macerating the spleens between the frosted surfaces of two glass slides. Single cell suspensions thus obtained were vortexed, centrifuged at 1200 rpm for 10 minutes at 4^{o}C and the supernatant was discarded by inversion of the tube. The cells were resuspended in the residual supernatant. 5 ml of erythrocyte lysis buffer were added to the suspensions and incubated at room temperature for 5 minutes to lyse the erythrocytes. 8 ml of medium (DMEM) was then added to the cells to dilute the lysis buffer. Cell pellets were then obtained by centrifugation. Splenocytes were resuspended in 4-8 ml of fresh medium as necessary and used for further purposes. Where indicated, such single cell suspensions were frozen down at -80°C for future use.

Erythrocyte lysis buffer: NH_4Cl 0.15 M; $KHCO_3$ 1.0 mM; Na_2EDTA 0.1 mM; (pH 7.2-7.4)

3.4.5. *Ex-vivo* reactivation assay

To determine the load of preexisting lytic infectious virus and the extent of reactivation of latent virus in the splenocyte preparations from infected mice, an *ex vivo* limiting-dilution reactivation assay was carried out. For this purpose, serial threefold dilution (8 dilutions) of single cell suspensions of spleen cells were plated before or after mechanical disruption of viable cells (by two freeze-thaw cycles) on a monolayer of 10^4 low-passage NIH 3T3 cells per well in 96-well tissue culture plates. Twenty-four wells were plated per dilution (starting with 1.5 x 10^5 splenocytes). The NIH 3T3 cells were screened microscopically for viral cytopathic effects for up to 3 weeks. At day 13, 17 and 23 post infections, the splenocytes from wild type C57BL/6 mice carried only latent virus and no lytic virus was detected in the assay. However, in $CD8^{-/-}$ and β-2 microglobulin knockout mice, the first dilution of single cell suspension of spleen cells showed the presence of a small amount of preformed lytic virus.

3.5. Statistical analysis

Data was evaluated by using the Student's *t* test for dependent or independent samples (Microsoft EXCEL). Differences with *P* values < 0.05 were considered statistically significant. The data from the reactivation assays were statistically analyzed using the Graph Pad Prism software (GraphPad Software, San Diego, California). The frequency of reactivating splenocytes was calculated from the number at which 63% of the wells scored positive for reactivating virus based on the Poisson distribution, and then evaluated by using the *t* test.

4.0 Results

4.1. Generation and characterization of the 40 bp internal repeat and/or ORF M6 mutants

ORF M6 including the 40 bp internal repeats are located at the left side of the MHV-68 genome (Fig. 4.1).

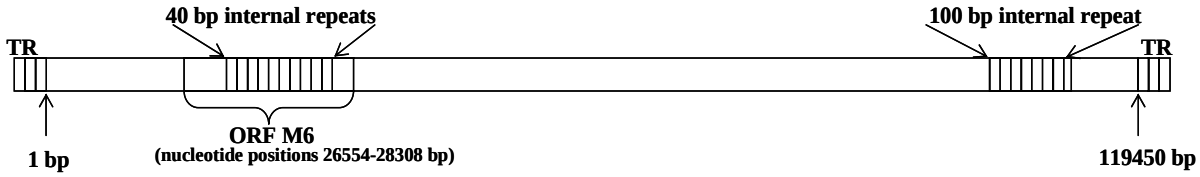


Fig. 4.1. Schematic presentation of the MHV-68 genome. The MHV-68 genome consists of a 119450 bp unique DNA sequence which is flanked by multiple copies of terminal repeats (TR). The unique DNA sequence is also characterized by the presence of two internal DNA repeat sequences, i.e., the 40 bp internal repeat and the 100 bp internal repeat. The 40 bp internal repeat is located at the left side of the genome and is part of a hypothetical ORF M6.

To determine the role of the 40 bp internal repeat and/or ORF M6, we constructed six independent viral mutants by different methods using MHV-68 cloned as a bacterial artificial chromosome (BAC). BACs allow the maintenance of large viral genomes as a plasmid in *E. coli* and the reconstitution of viral progeny by transfection of BAC plasmid DNA into eukaryotic cells. Many herpesvirus genomes have been successfully cloned as BAC including HCMV, MCMV, HSV, pseudorabies virus (PRV), EBV, HHV-8 and MHV-68 (Adler *et al.* 2000; Borst *et al.* 1999; Delecluse *et al.* 1998; Messerle *et al.* 1997; Saeki *et al.* 1998; Smith and Enquist 1999; Zhou *et al.* 2002). The low and stringently controlled copy number of BAC has made them superior to cosmids and yeast artificial chromosomes (Kim *et al.* 1992; Messerle *et al.* 1997; Shizuya *et al.* 1992). Maintenance and manipulation of large viral genomes in *E. coli* is especially interesting for those viruses that are difficult to grow in cell culture systems and that are cumbersome to manipulate and to analyze. BACs provide an efficient, fast and valuable tool for mutagenesis using the bacterial recombination machinery. Construction of the mutant genome is completely independent of the biological fitness of the mutant virus,

and the recombinant genome can be characterized and controlled prior to reconstitution of viral progeny. MHV-68 cloned as BAC has the genes for gpt (guanosine phosphoribosyl transferase) and *gfp* (green fluorescent protein) as selection and screening markers and the chloramphenicol resistance gene for maintenance of BAC plasmids in *E. coli* (Adler *et al.* 2000)

A schematic presentation of all the mutants is depicted in figure (4.2). In three of six mutants, the 40 bp repeat was either completely or partially deleted. In three further mutants, the N-terminus of ORF M6 was targeted leaving the repeat intact, except the loss of some of the repeat units during shuttle mutagenesis (see below).

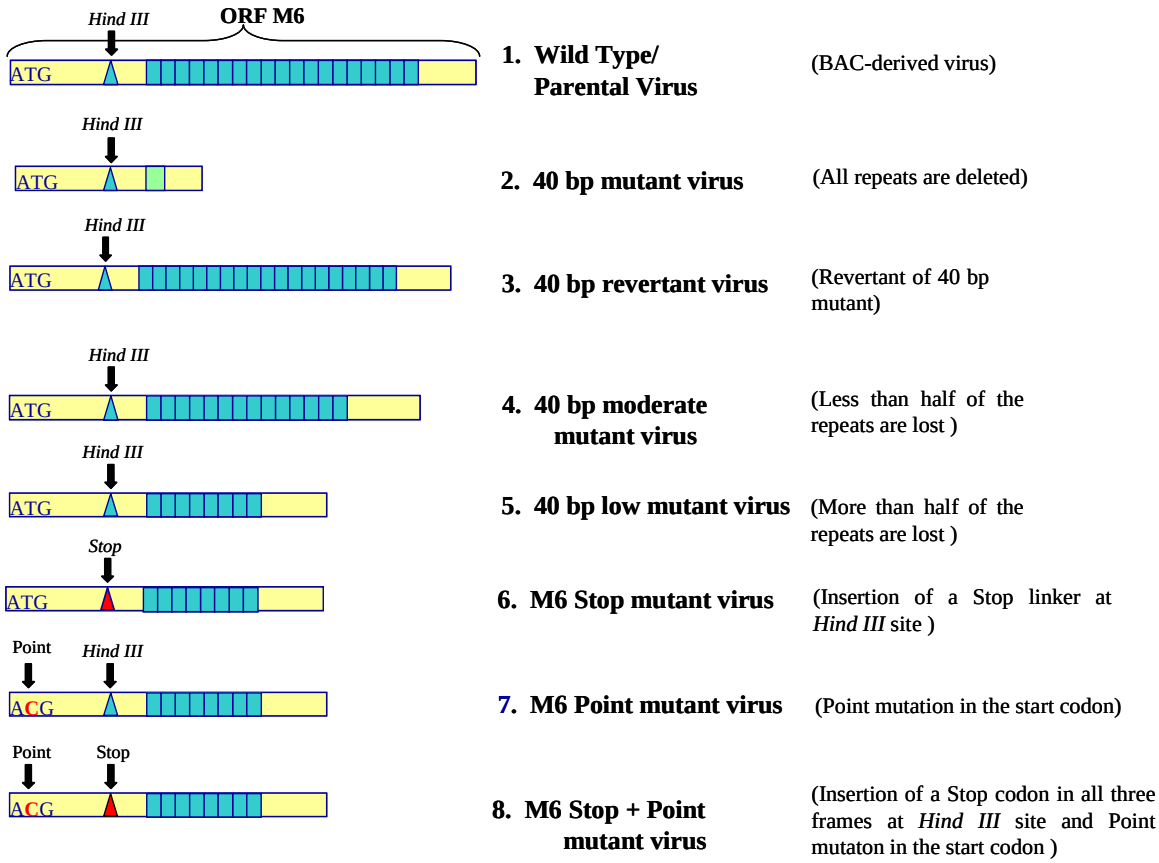
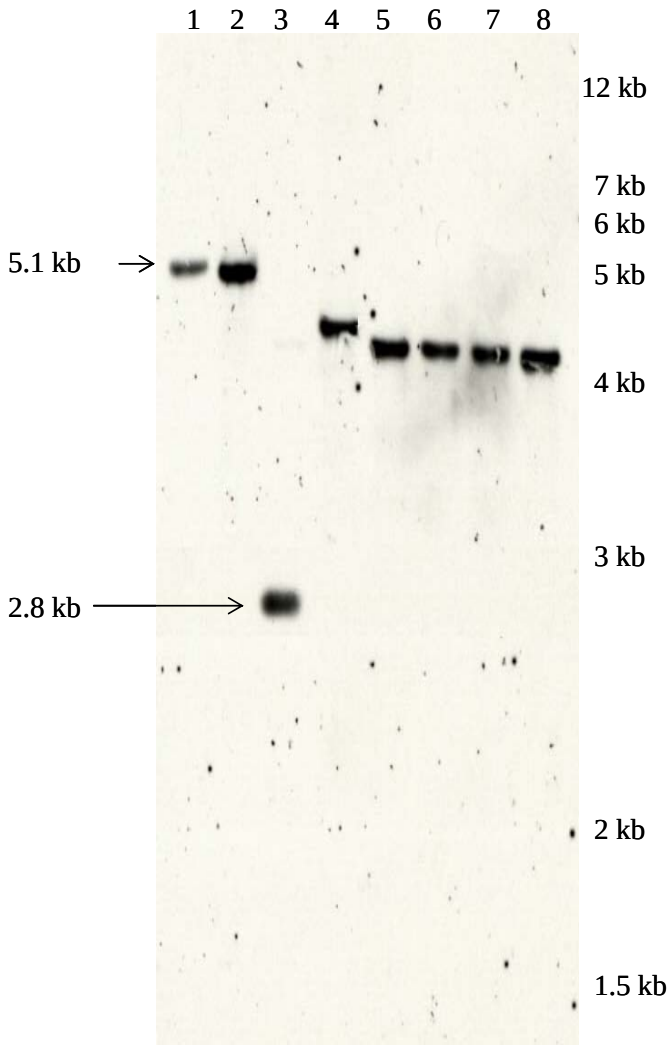


Fig. 4.2. Schematic presentation of the generated mutants. The hypothetical ORF M6 as proposed by Virgin *et al.* 1997 is approximately 1.8 kb long and is located at the left side of the genome (nucleotide positions 26554-28308) (Virgin *et al.* 1997). Approximately 36 copies of the 40 bp direct repeats are present in ORF M6. The 40 bp mutant virus was made by ET cloning and all the other mutants as well as the revertant were generated by the two step replacement procedure.

4.1.1. Construction of the 40 bp mutant virus

A deletion mutant (40 bp mutant), completely lacking the 40 bp internal repeat of MHV-68, was constructed by ET cloning as described in methods (3.1.5.1). This method is called ET cloning since it utilizes the recombinant proteins *rec*E and *rec*T of *E. coli* strain JC8679. For this purpose, a linear PCR product was generated using the primers containing 24 nucleotides for the amplification of a tetracycline resistance gene flanked by FRT (FLP recognition target) sites and an additional 50 nucleotides homologous to the sequences flanking the MHV-68 40 bp internal repeat. Fragment was created by PCR from the plasmid pCP16. The PCR-generated fragments were electroporated into *E. coli* JC8679 (*recBC sbcA*) containing the MHV-68 BAC plasmid. Homologous recombination resulted in the generation of a BAC mutant completely lacking the 40 bp repeats. Thus, in the new BAC plasmid, the 40 bp repeat region was replaced by a tetracycline resistance cassette. The cloning of mutants was confirmed by restriction enzyme analysis and Southern blot analysis. The 40 bp mutant BAC plasmid was then transformed into *E. coli* DH10B containing the Flp expression plasmid pCP20. Chloramphenicol-positive, tetracycline-negative clones were then selected. After removal of the tetracycline resistance gene by FLP-mediated recombination, a small residual insert consisting of the FRT site and some vector sequence (161 bp) with an *Eco RI* restriction site was left in the disrupted 40 bp repeat sequence. This was confirmed by restriction fragment analysis with different enzymes and by sequencing. The 40 bp mutant virus was transfected into NIH 3T3 cells and virus reconstituted in baby hamster kidney (BHK-21) cells as described (3.2.2) and viral DNA from Hirt extracts of cells infected with recombinant virus was analyzed by restriction enzyme analysis and Southern blot analysis (Fig. 4.3 and 4).



1. Parental virus
2. 40 bp revertant virus
3. 40 bp mutant virus
4. 40 bp moderate mutant virus
5. 40 bp low mutant virus
6. M6 Point mutant virus
7. M6 Stop mutant virus
8. M6 Stop + Point mutant virus

Fig. 4.3. Construction of mutants and structural analysis of the genomes of reconstituted viruses Southern blot analysis of viral DNA isolated from the infected NIH 3T3 cells by the method of Hirt (Hirt 1967) and digested with *EcoR I.* Viral DNA was hybridized with a 822 bp DNA probe (nucleotide positions 25889-26711). The 822 bp probe used for the Southern blot analysis binds at N-terminal region of ORF M6 and 670 bp DNA sequence present upstream of the 5' end of ORF M6 (Fig. 4.4).

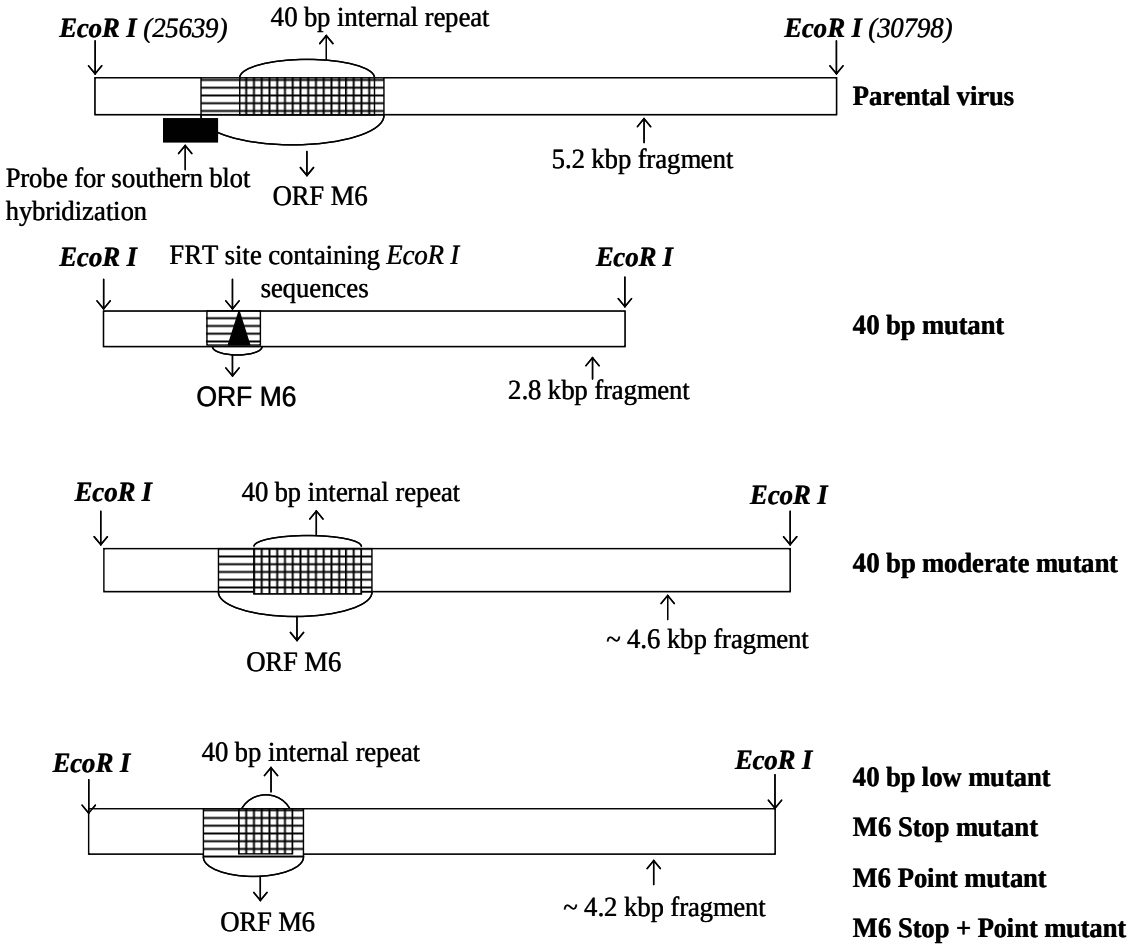


Fig. 4.4. Schematic presentation of the *EcoR I* fragment containing ORF M6 and the 40 bp repeat region of MHV-68. Digestion of MHV-68 genomic DNA with *EcoR I* generates 14 fragments of different lengths. The ORF M6 and/or 40 bp repeat region fragment of parental virus is 5.1 kbp long. Due to loss of repeats and insertion of a FRT site which also contains an *EcoR I* site, the 40 bp mutant fragment is 2.8 kbp. The other mutants show different lengths of this fragment due to the loss of repeats.

4.1.2. Construction of the 40 bp revertant virus

The 40 bp mutant virus contains a deletion of ~ 1.2 kbp (nucleotide positions 26778–28191). To ensure that the phenotype of the mutant virus is not due to rearrangements and/or mutations outside the mutated region, a revertant virus was generated by replacing the 40 bp mutant fragment with a wild type genomic fragment (nucleotide positions 24918-30834) by a two step replacement procedure.

For this purpose, a 5.916 kbp *Sma I* fragment of MHV-68 (nucleotide positions 24918-30834) containing the 40 bp internal repeat region was cloned into the shuttle plasmid pST76K-SR, resulting in the plasmid pST76K-SR M6. This new plasmid pST76K-SR M6 was electroporated into *E.coli* DH10B containing the 40 bp BAC mutant plasmid. Homologous recombination between the shuttle plasmid pST76K-SR M6 and the 40 bp mutant BAC plasmid in the bacteria led to the formation of the revertant 40 bp BAC plasmid, which contains nearly all the repeats as well as an intact ORF M6 (Fig. 4.5.). It was not possible to restore the repeats completely, since few repeat units are always lost during the cloning and mutagenesis procedures. This has been described before by others (Virgin *et al.* 1997; Adler *et al.* 2000; 2003).

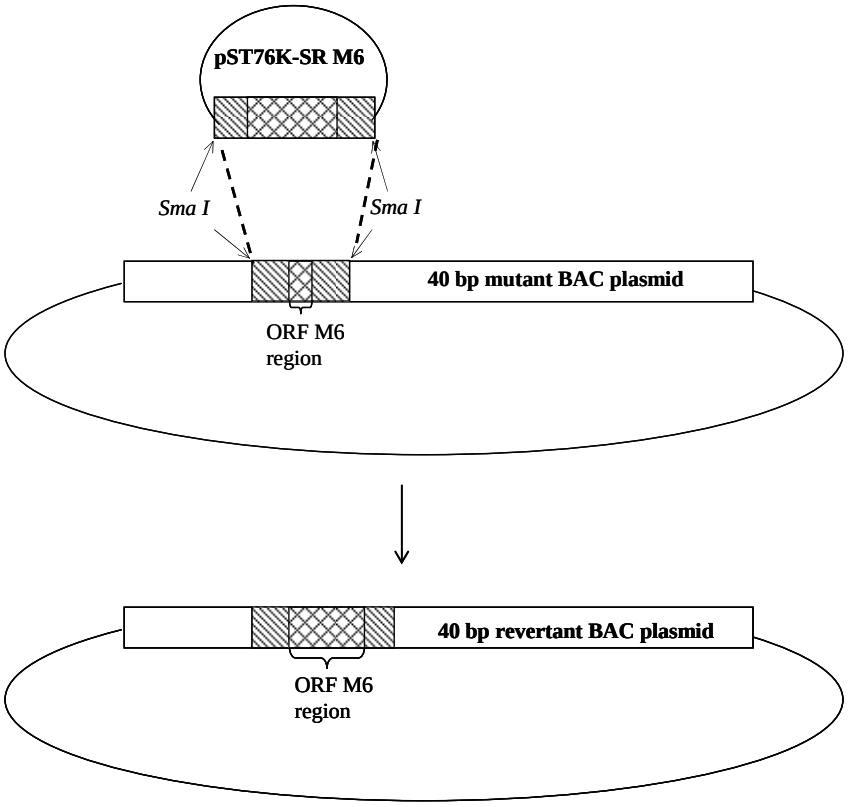


Fig. 4.5. The 40 bp revertant BAC plasmid prepared by homologous recombination between pST76K-SR M6 and the 40 bp mutant BAC plasmid.

Bacterial clones positive for the revertant 40 bp BAC plasmid were analyzed and confirmed by restriction enzyme analysis. Using a positive bacterial clone, the recombinant 40 bp revertant virus was reconstituted as before.

4.1.3. Construction of the 40 bp moderate mutant and the 40 bp low mutant

Restriction enzyme digestions and sequencing analyses showed that during shuttle mutagenesis, many clones lost a number of their 40 bp internal repeat units, due to recombination between the direct repeats. Two clones, i.e., the 40 bp low mutant which had lost more than half of the 40 bp repeat units, and the 40 bp moderate mutant which had lost less than half of the repeat units, were selected for further use. These two clones were used for the generation of mutant viruses lacking different numbers of 40 bp repeat units. They should allow determining the significance of the number of repeat units for *in vitro* replication and/or pathogenesis. Sequencing analysis showed that the 40 bp low mutant contained 15 repeat units. Probably due to the formation of secondary structures in the 40 bp moderate mutant, sequencing of the 40 bp moderate mutant was not possible. Restriction enzyme analysis of the 40 bp moderate mutant showed the loss of a considerable number of 40 bp repeat units, with approximately 20-25 copies of 40 bp repeat units left.

4.1.4. Construction of the ORF M6 mutants

Three additional mutants, i.e., ORF M6 Stop, ORF M6 Stop + Point, and ORF M6 Point, were generated by targeting the N-terminus of ORF M6 using a two-step replacement procedure. For this purpose, a 5.237 kbp *Kpn I – Nde I* M6 fragment from pSTK-SR M6 plasmid was cloned into pUC 19 (Fig. 4.6). Prior to the cloning of the 5.237 kbp fragment in pUC 19, the *Hind III* site from the multiple cloning site was disrupted. This construct pUC 19 M6 was used for PCR mutagenesis to generate the ORF M6 mutants. After PCR mutagenesis, the 5.237 kbp *Kpn I – Nde I* fragment was

cloned back into pST76K-SR M6 to generate pS76TK-SR M6 mutants. The mutant pST76K-SR M6 plasmids then underwent homologous recombination with MHV-68 BAC plasmids generating ORF M6 mutant BAC plasmids. These ORF M6 BAC plasmid mutants were named according to the mutation they carried, i.e., M6 Stop mutant, M6 Point mutant and M6 Stop + Point mutant. Recombinant mutant viruses could be successfully reconstituted.

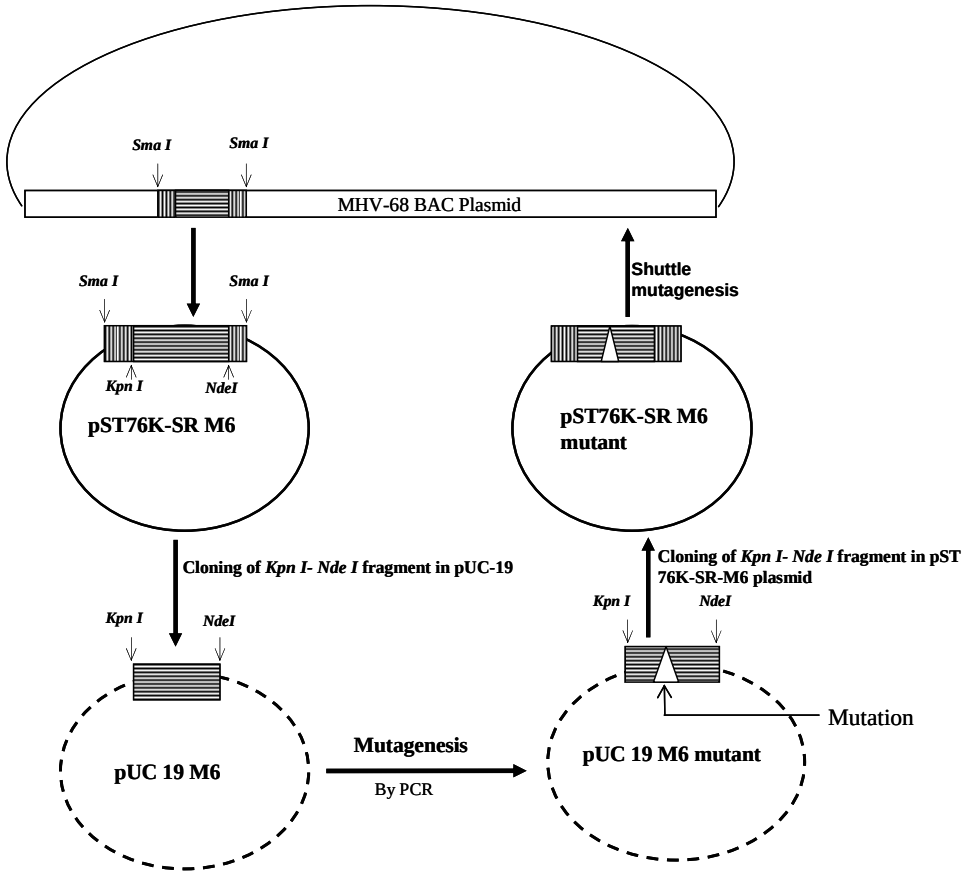


Fig. 4.6. Schematic presentation of construction of ORF M6 mutants. For the construction of ORF M6 BAC mutants, a 5.916 kbp *Sma I* fragment of MHV-68 (nucleotide positions 24918 to 30834) was cloned into the shuttle plasmid pST76K-SR. From pST76K-SR M6, a 5.237 kbp *Kpn I – Nde I* fragment was cloned into pUC 19, which was used for PCR mutagenesis to generate ORF M6 mutants. After the mutation in pUC 19 M6 plasmid, the 5.237 kbp *Kpn I – Nde I* fragment was cloned back into pST76K-SR M6 to generate the pSTK-SR M6 mutant. These pST76K-SR M6 mutant plasmids were used to generate ORF M6 BAC mutants by shuttle mutagenesis.

The circular viral DNA from Hirt extracts of cells infected with recombinant virus was analyzed by restriction enzyme analysis and southern blot analysis. It was observed that

the ORF M6 mutant viruses, i.e., M6 Stop mutant virus, M6 Point mutant virus, and M6 Stop + Point mutant virus had lost a considerable number of repeat units (as determined by restriction enzyme analysis), comparable to the 40 bp low mutant (Fig. 4.3 and 4). Therefore, in further studies, we compared the ORF M6 mutant viruses with the 40 bp low mutant virus and not with the parental virus.

Summary of chapter 4.1

1) *All the mutant BAC plasmids for either the 40 bp repeat region or ORF M6 and the 40 bp revertant BAC plasmid were constructed successfully by either ET cloning or shuttle mutagenesis.*
2) *Recombinant viruses were reconstituted by transfection of all the BAC plasmids for either the 40 bp repeat region or ORF M6 and the 40 bp revertant BAC plasmid in fibroblasts.*

4.2. The 40 bp internal repeat and/or ORF M6 of MHV-68 are dispensable for *in vitro* replication.

Transfection of baby hamster kidney cells (BHK-21) with DNA of the parental virus or the BAC mutant for the 40 bp repeat or ORF M6 resulted in the reconstitution of infectious virus, indicating that the 40 bp internal repeat and/or ORF M6 are not essential for lytic replication. To check if the 40 bp internal repeat and/or ORF M6 have a role in *in vitro replication*, multi-step growth curve experiments were performed in NIH 3T3 cells (Fig. 4.7). NIH 3T3 cells, a mouse fibroblast cell line, efficiently support the lytic replication of MHV-68.

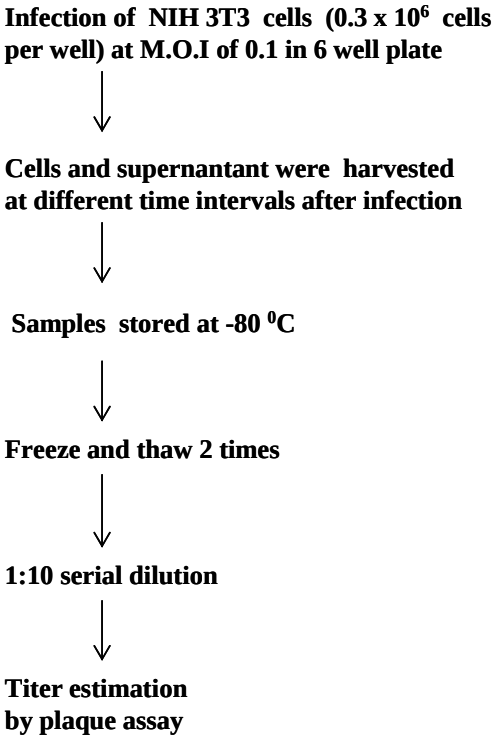


Fig. 4.7. Schematic presentation of *in vitro* multi-step growth curve. NIH 3T3 cells were infected with 0.1 PFU per cell in 1 ml volume for one hour. The medium containing virus was aspirated, and 2 ml of fresh media were added. At definite time intervals (i.e., 24, 48, 72 and 96 hours post infection), cells and supernatant were harvested and two rounds of freezing and thawing were performed. The virus titer was determined by plaque assay on BHK-21 cells.

For this purpose, NIH 3T3 cells were infected with different mutant viruses as well as parental virus at a M.O.I of 0.1 to determine the replication efficiency of all the mutant viruses compared to the parental virus. At definite time intervals (24, 48, 72 and 96 hours post infection) the complete cultures (containing intracellular as well as extracellular virus) were harvested and viral titers were determined on BHK-21 cells by plaque assay.

All mutant viruses, i.e., 40 bp moderate mutant virus, 40 bp low mutant virus, M6 stop mutant virus, M6 Stop + Point mutant virus, M6 Point mutant virus and 40 bp mutant virus replicated efficiently with similar kinetics and attained similar titers as both the parental virus and the 40 bp revertant virus (Fig. 4.8). At 24 and 48 hours post infection, the 40 bp mutant virus replicated less efficiently and the virus titers were 10 to 5 fold less than the parental virus. However, at 72 and 96 hours post infection, the 40 bp mutant virus attained similar titers as the parental virus. It was also observed that infection of NIH 3T3 cells or BHK cells with the parental virus or the mutant viruses either for the 40 bp internal repeat or ORF M6 resulted in the formation of plaques of similar size (data not shown). Thus, it can be concluded that the 40 bp internal repeats of MHV-68 and/or ORF M6 are dispensable for lytic replication *in vitro*.

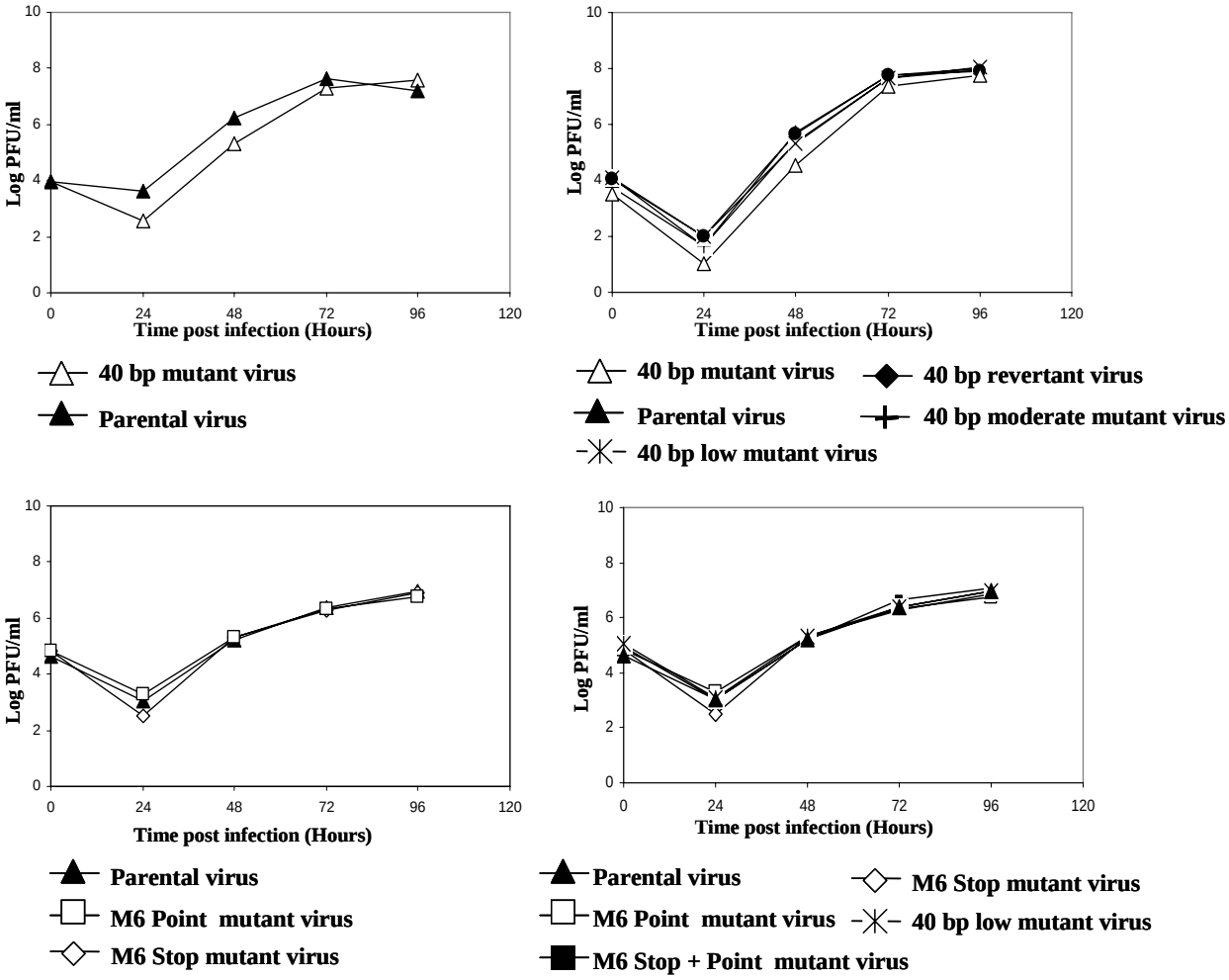


Fig. 4.8. ***In vitro*** **multi-step growth curves comparing the replication of mutant viruses of either the 40 bp internal repeat or ORF M6 to the parental virus in NIH3T3 cells.** Data shown are from representative, independent experiments. All together, 2-3 independent *in vitro* growth curves were performed with each mutant virus and compared to either parental or revertant virus.

Summary for chapter 4.2

The 40 bp internal repeat and/or ORF M6 of MHV-68 are dispensable for in vitro lytic replication.

4.3. The 40 bp internal repeats and/or ORF M6 of MHV-68 are dispensable for *in vivo* lytic replication

To check the efficiency of acute lytic replication of the mutant viruses *in vivo*, C57BL/6 mice were infected intranasally with 5 x 10^4 PFU of virus. Lungs of the mice were harvested at 3, 6, 10 and 13 days post infection and viral titers were determined on BHK-21 cells by plaque assay (Fig. 4.9).

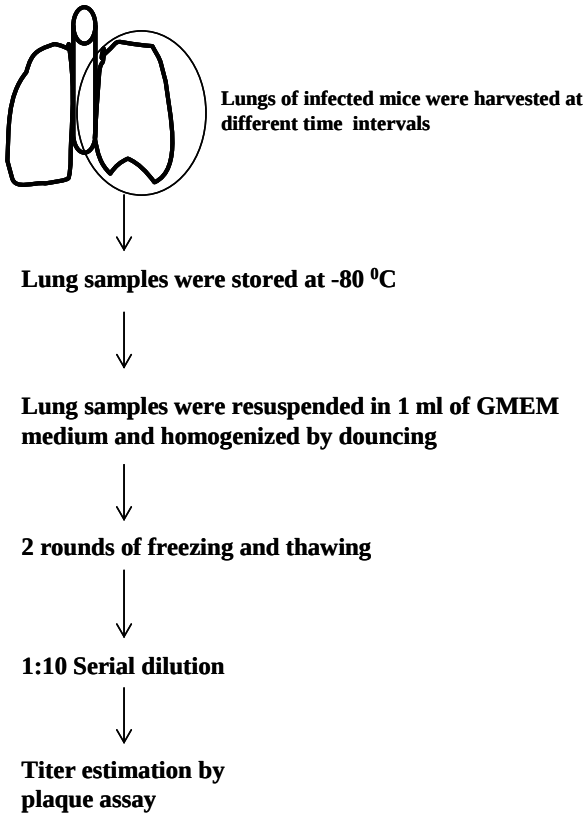


Fig. 4.9. Experimental design to monitor lytic replication in the lung of infected mice. The details are mentioned in methods (3.4.3.)

The 40 bp mutant efficiently replicated *in vivo* and attained similar titers compared to parental MHV-68 at day 3 and 6 post infection (day 3: P = 0.1 and day 6: P = 0.996). Both viruses were cleared from the lungs at day 10-12 post infection (Fig. 4.10).

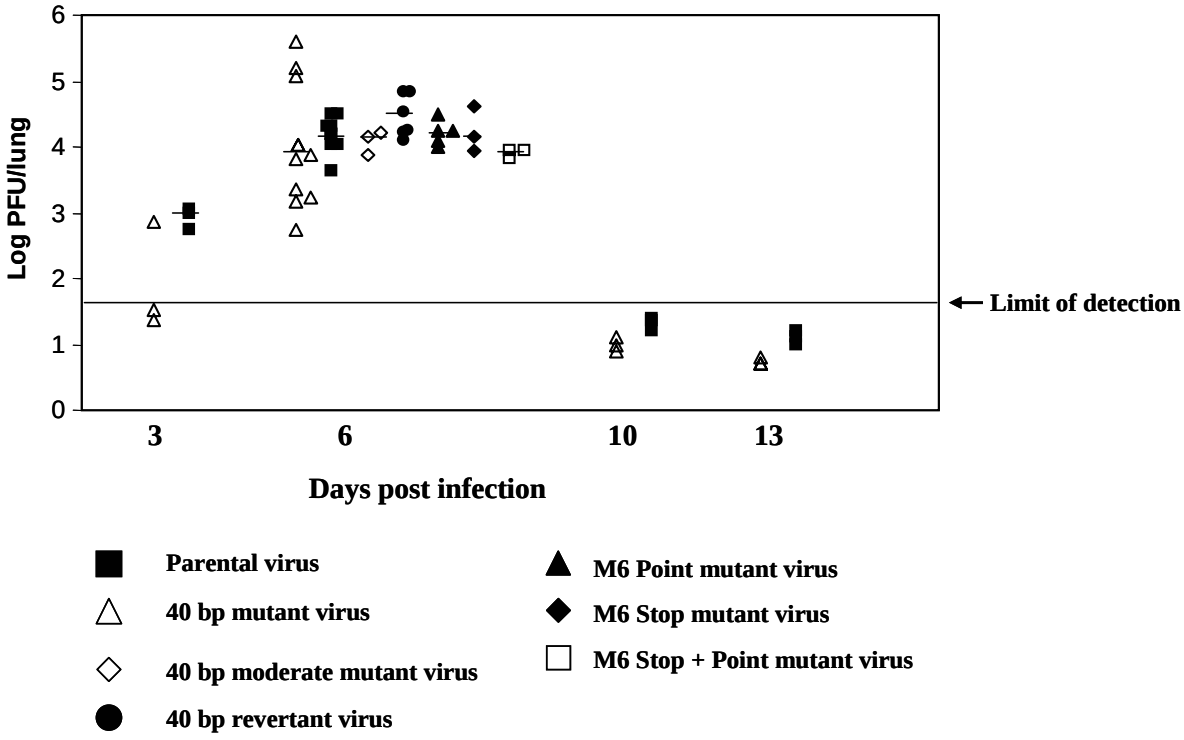


Fig. 4.10. The 40 bp internal repeat and/or ORF M6 of MHV-68 are dispensable for lytic replication in the lungs: C57BL/6 mice were infected with 5 x 10^4 PFU intranasally. At different days (day 3, 6, 10 and 13), lungs were harvested and the virus titers from lung homogenates were determined by plaque assay on BHK-21 cells. Each symbol represents a single mouse. The limit of detection is 50 PFU and was determined by spiking uninfected lung tissue with known amount of virus (see 5.4.3.). For each group of mice the median value is indicated by a horizontal bar (-) and the P-values were determined by Student's t-test.

At day 6 post infection, all the other mutant viruses efficiently replicated in the lungs of C57BL/6 mice and their titers were not significantly different from the titers of the parental virus, i.e., M6 Stop + Point mutant virus (P = 0.59), M6 Point mutant virus (P = 0.53), M6 Stop mutant virus (P = 0.63), 40 bp moderate mutant virus (P = 0.62). Thus, these data suggest that the 40 bp internal repeat of MHV-68 and/or ORF M6 are dispensable for acute, lytic replication in the lungs.

Summary for chapter 4.3

ORF M6 and/or 40 bp internal repeat of MHV-68 are dispensable for lytic replication in lungs.

4.4. The role of the 40 bp internal repeat of MHV-68 during the latent phase of infection in mice

The role of the 40 bp internal repeat of MHV-68 in latency was investigated by measuring the extent of splenomegaly, *ex vivo* reactivation and genomic load of latent virus in the spleen. For this purpose, C57BL/6 mice were infected with 5 x 10^4 PFU of virus and the spleen was harvested at specific days post infection.

4.4.1. The 40 bp internal repeat of MHV-68 is important for the virus-induced transient splenomegaly

The extent of splenomegaly was determined by measuring the spleen weight. At day 10, when establishment of latency starts after intranasal infection, the spleen weight of C57BL/6 mice infected with the 40 bp mutant was not significantly different to the spleen weight of mice infected with the parental virus (P = 0.623) (Fig. 4.11). However, during amplification of the latency stage, when massive proliferation of splenocytes takes place (splenic mononucleosis), i.e., day 13 and 17 post infection, the spleen weight of mice infected with the 40 bp mutant virus was significantly lower than the spleen weight of mice infected with the parental virus (day 13: P = 0.04 and day 17 P = 8 x 10^{-7}) or the revertant virus. At day 13 and 17 post infection, the weight of the spleen from mice infected with the 40 bp mutant virus was approximately 2 fold lower compared to the spleen weight of mice infected with parental virus. At days 23 and 42 post infection, when splenic mononucleosis decreases to a baseline, the extent of splenomegaly for the 40 bp mutant virus and the parental virus was not significantly different (day 23: P = 0.52 and day 42: P = 0.37). This data suggests that the 40 bp internal repeat and/or ORF M6 might have a role in latency and in the EBV-like infectious mononucleosis syndrome seen in mice after MHV-68 infection.

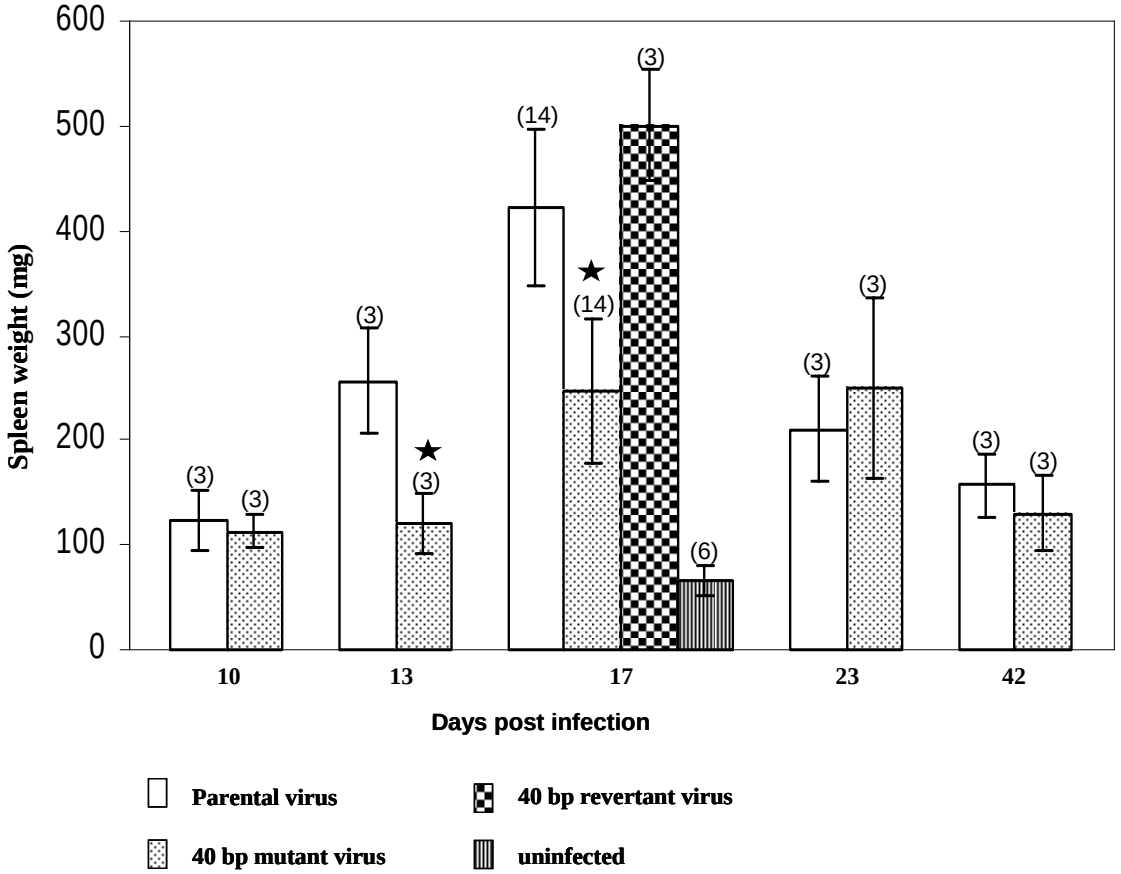


Fig. 4.11. The 40 bp internal repeat of MHV-68 is important for virus-induced splenomegaly. C57BL/6 mice were infected with 5 x 10^4 PFU intranasally. The extent of splenomegaly was measured by taking the spleen weight (mean values ± SD) of each mouse at different post infection days (day 10, 13, 17, 23 and 42). Numbers in brackets represent the number of mice per group. ★ The asterisk indicates a significant decrease in the spleen weight of C57BL/6 mice infected with the 40 bp mutant virus compared to the infection with the parental virus (Student's t-test).

4.4.2. The 40 bp internal repeat of MHV-68 is important for viral reactivation from latently infected splenocytes

To determine the level of viral reactivation from latently infected splenocytes at specific days after infection (day 10, 13, 17, 23 and 42), an *ex vivo* limiting dilution reactivation assay was carried out (Fig. 4.12). The limiting dilution reactivation assay provides an assay for simultaneously quantifying both preformed infectious virus and cells that reactivate latent MHV-68 (Weck *et al.* 1996, 1999 a & b). In this assay, latent cells are

serially diluted onto permissive monolayers and the presence of infectious virus is detected by cytopathic effect over 2-4 weeks in culture, allowing prolonged time for reactivation. To distinguish preformed infectious virus from latent virus, cells are mechanically destroyed (latent virus cannot reactivate from killed cells) and diluted in parallel with live cells (i.e. not mechanically destroyed) onto permissive monolayer.

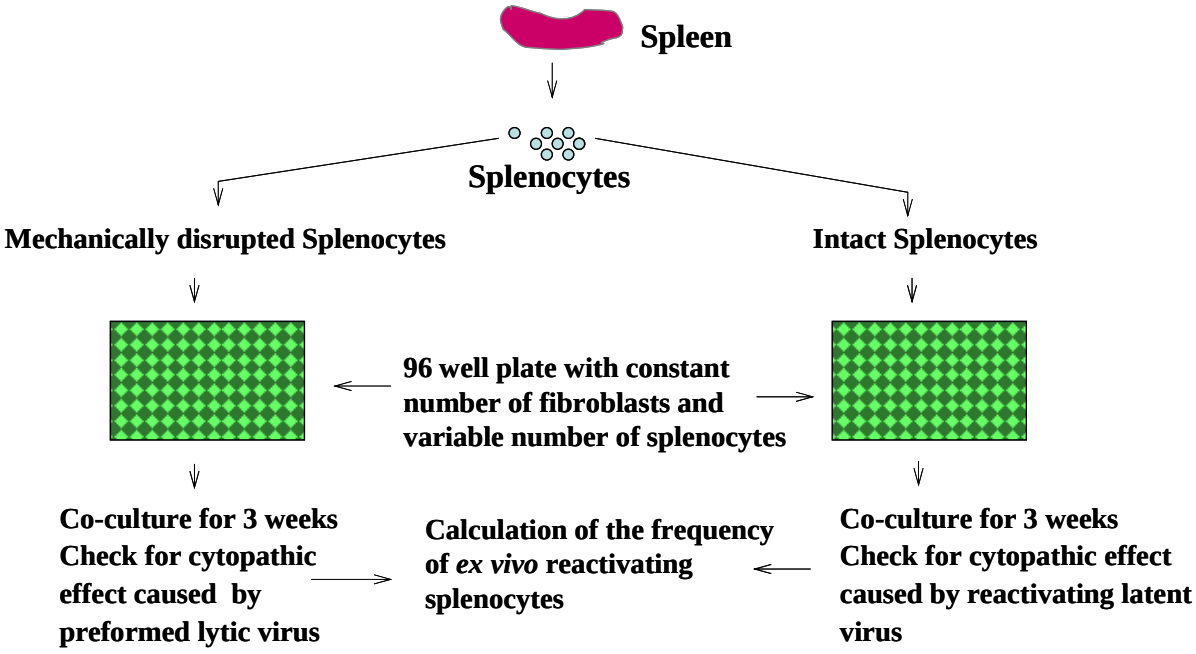


Fig. 4.12. *Ex vivo* reactivation assay for splenocytes: The details for the *ex vivo* reactivation assay are mentioned in methods (3.4.5.).

At day 10 post infection, latent virus in the splenocytes of mice infected with the 40 bp mutant virus showed a similar extent of reactivation when compared to splenocytes from mice infected with parental virus (P = 0.47), i.e., the number of latently infected splenocytes which reactivated and entered the lytic phase of replication was similar (Fig. 4.13). However, during the stage of amplification of latency (day 13 and 17 post infection), there was a significant decrease in the number of latently infected splenocytes which reactivated from mice infected with the 40 bp mutant virus (day 13: P = 0.05 and day 17: P = 0.025) (Fig 4.13.). At day 23 post infection, the extent of reactivation was again similar for both viruses (P = 0.99).

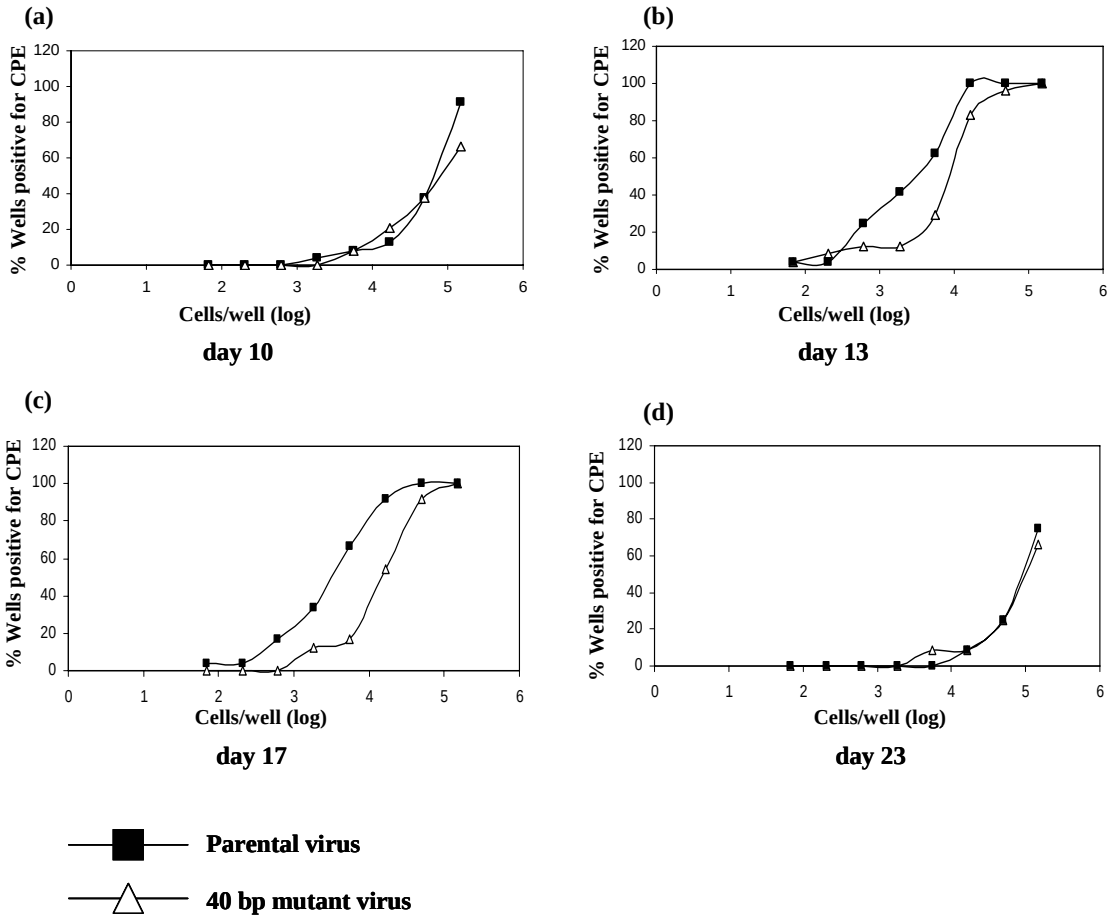


Fig. 4.13. The extent of *ex vivo reactivation* after infection of mice with the 40 bp mutant virus compared to the parental virus. Splenocytes from mice infected with either the 40 bp mutant virus or parental virus were used for the *ex vivo* reactivation assay at day 10 (a), 13 (b), 17(c) and 23(d) post infection. Splenocytes from 3 mice per group were pooled and serial dilutions were made, which were co-cultured with NIH 3T3 cells for up to three weeks and checked for cytopathic effects in each well. For each dilution, cytopathic effect was monitored in 24 wells. Data shown are from single experiments at each time point, using pooled cells from 3 mice per group. The experiment at day 17 was repeated 3 times with similar results.

The frequency of *ex vivo* reactivating splenocytes was determined by using the Poisson distribution, assuming that the cell number at which 63.2% of the wells scored positive for reactivation represents a single event. The frequencies of *ex vivo* reactivating splenocytes are represented as the reciprocal frequency of *ex vivo* reactivating splenocytes. For example, 1000 means 1 latently infected splenocyte is reactivating from 1000 splenocytes. The reciprocal frequency of reactivating splenocytes from

C57BL/6 mice was 2-2.5 fold less for the 40 bp mutant virus (1 in 15135) when compared to the parental virus (1 in 6875) at day 17 post infection (P = 0.04) (Fig. 4.14).

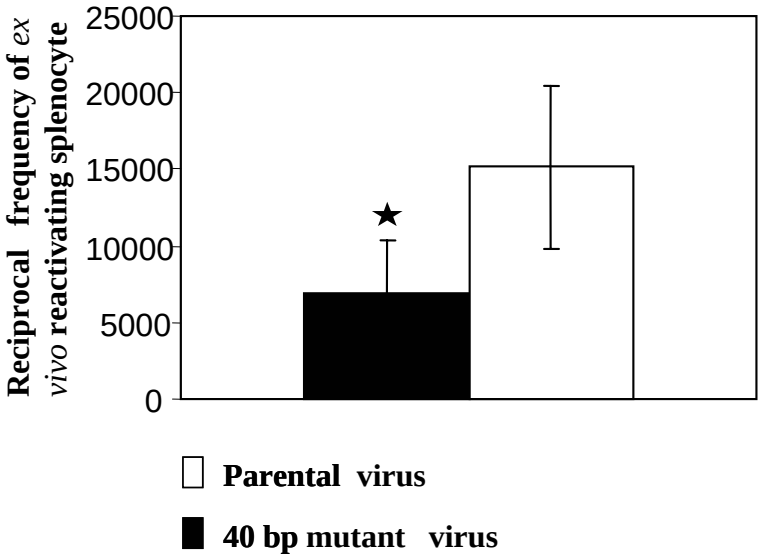


Fig. 4.14. Reciprocal frequency of *ex vivo* reactivating splenocytes at day 17 post infection. To calculate the frequency of cells reactivating in the *ex vivo* reactivation assay, data were subjected to nonlinear regression to generate a curve fit based on a sigmoidal dose response curve. Frequencies of reactivation events were determined by using the Poisson distribution, assuming that the cell number at which 63.2% of the wells scored positive for reactivation represents a single event. ★ The asterisk indicates a statistically significant decrease in the reciprocal frequency of *ex vivo* reactivating splenocytes after the infection of mice with the 40 bp mutant virus compared to the infection with parental virus (P = 0.04) (Student's t-test). Data (mean values ± SD) are compiled from four independent experiments.

4.4.3. The viral genomic load in the spleen of mice infected with the 40 bp mutant is similar to the genomic load after infection with parental virus

A significant decrease in spleen weight (day 17 $P = 8 \times 10^{-7}$) and a reduction in the extent of reactivation of latently infected splenocytes (2-2.5 fold) were observed in

C57BL/6 mice after infection with the 40 bp mutant virus as compared to infection with the parental virus. The difference in the extent of reactivation might be due to either a defect in the reactivation process *per se* or to a decrease in the genomic load of the 40 bp mutant virus in the spleen. To test whether the phenotype is a result of a decrease in the latent viral genome load per spleen and not to a defect in the reactivation process, real time PCR was performed. DNA was extracted from splenocytes of mice 17 and 42 days after infection with parental virus or the 40 bp mutant.

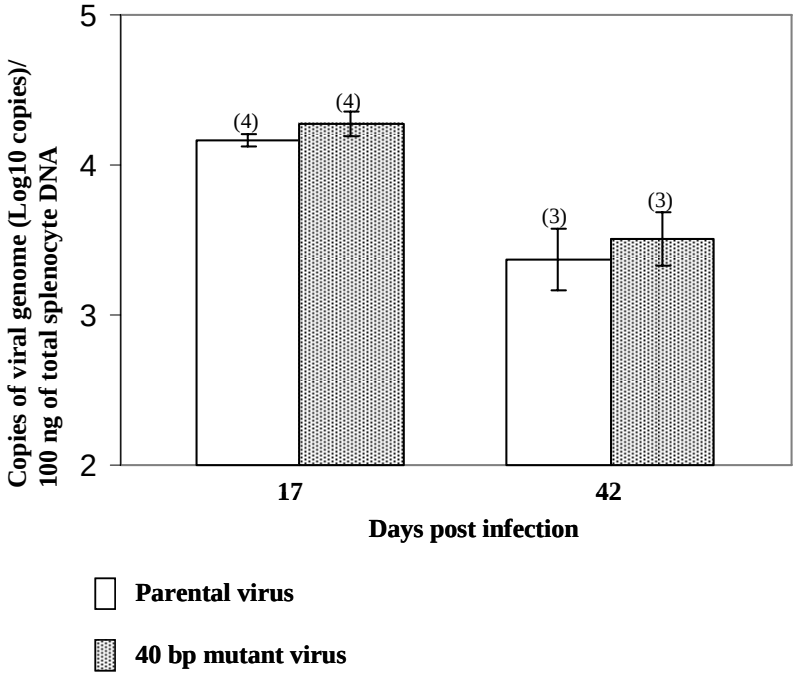


Fig. 4.15. The latent viral genome load in the splenocytes from mice infected with either the parental virus or the 40 bp mutant virus was not significantly different. C57BL/6 mice were infected with 5 x 10^4 PFU, and total DNA was extracted from splenocytes harvested at day 17 and 42 post infection. Real time PCR was performed with 100 ng of total splenocyte DNA using primers specific for the MHV-68 ORF 50 gene, to estimate the latent viral genome load in the spleen. Numbers in brackets represent the number of mice. Data (mean values ± SD) are compiled from two independent experiments.

At day 17 as well as at day 42 post infection, the latent viral copy number per 100 ng of splenic DNA from mice infected with the mutant did not significantly differ from the copy number of the parental virus (Fig. 4.15). At day 42 post infection, the viral genome load was around 10 fold lower than that at 17 days post infection in both infection groups.

Summary for chapter 4.4

1) *The 40 bp internal repeat of MHV-68 is important for the amplification of latently infected cells.*
2) *At day 17 post infection, the extent of reactivation for the 40 bp mutant virus was 2-2.5 fold less than for the parental virus.*
3) *At day 17 and 42 post infection, the latent viral copy number per 100 ng of splenic DNA from mice infected with either the 40 bp mutant virus or the parental virus was not significantly different.*

4.5. The 40 bp internal repeat of MHV-68 is not important for *in vitro* reactivation

During the amplification stage of latency, splenocytes from mice infected with the 40 bp mutant virus show a decrease in the extent of reactivation compared to splenocytes from mice infected with parental virus. However, the viral genome load in the splenocytes did not significantly differ. Therefore, we checked whether the reduction in the extent of reactivation was due to a defect in the reactivation process *per se* by performing an *in vitro* reactivation assay. The *in vitro* reactivation assay was carried out as described in methods (3.2.6). The *in vitro* reactivation assay was similar to the *ex vivo* reactivation assay except for the source of latently infected cells. In case of *ex vivo* reactivation assay the splenocytes were derived from infected mice and in *in vitro* reactivation assay the splenocytes were derived from a naïve mouse. Naive mouse splenocytes were infected with virus at an M.O.I of 1.0 overnight and serial three-fold dilutions of viable splenocytes (starting with 10,000 splenocytes) were plated on monolayers of 10^4 low-passage NIH 3T3 cells per well in 96-well tissue culture plates and the frequency of reactivating cells was calculated from Poisson distribution.

The 40 bp mutant virus established latency in the splenocytes and reactivated from the latently infected splenocytes to the same extent as did the parental virus ($P = 0.98$) (Fig 4.16). In case of the 40 bp mutant virus, 1 in 48 splenocytes reactivated, and 1 in 57 in case of the parental virus. Also, the 40 bp revertant virus reactivated to a similar extent (1 in 61 splenocytes). Latent and persistent productive infection can coexist in the same culture. Therefore, we checked for the presence of preformed lytic viruses by co-culturing the fibroblast cells with infected splenocytes which had been disrupted by two rounds of freezing and thawing. Only the first dilutions of splenocytes showed the presence of few lytic viruses. This data suggests that the 40 bp internal repeat of MHV-68 might have no role in the reactivation process *per se*. The difference in the results of the *ex vivo* reactivation assay and the *in vitro* reactivation assay might be due to the difference in the source of splenocytes. The host immune response and several other *in vivo* factors could affect the *ex vivo* reactivation (Weck, Barkon *et al.* 1996; Weck, Kim *et al.* 1999), whereas in the *in vitro* reactivation assay, no host immune response is involved.

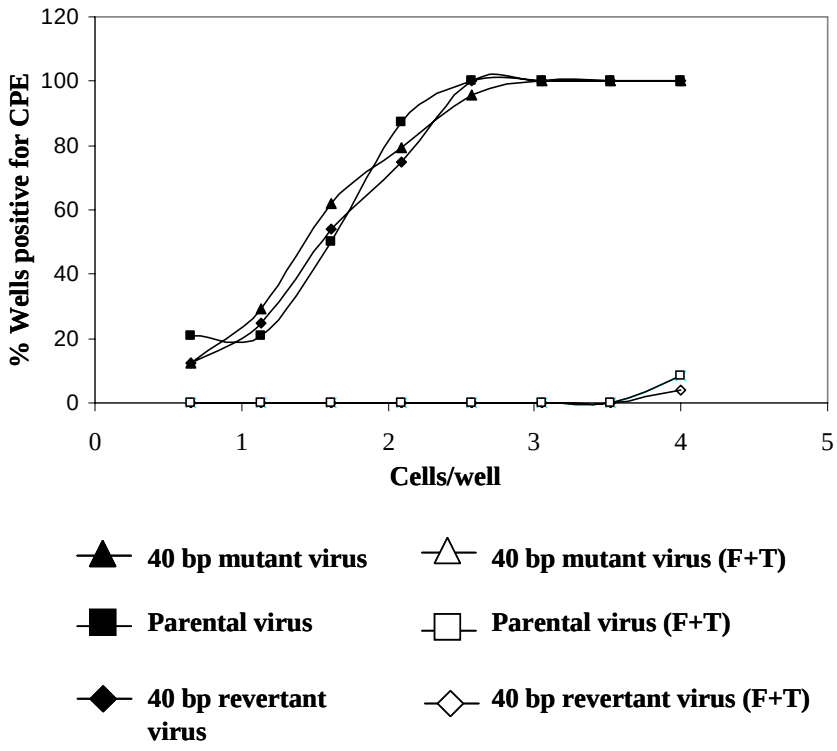


Fig. 4.16. The *in vitro* reactivation of the 40 bp mutant virus and the parental virus is not significantly different. Splenocytes from naïve mice were infected with different viruses at an M.O.I of 1 overnight. The splenocytes were washed 2 times with DMEM and then three-fold dilutions of splenocytes were co-cultured with NIH3T3 cells for three weeks (closed symbols). Additionally, splenocytes disrupted by 2 rounds of freezing and thawing (F+T) were co-cultured for three weeks (open symbols) to detect the presence of preformed lytic viruses. The frequencies of cells reactivating in the *in vitro* reactivation assay were calculated as described in (4.4.2).

Summary for chapter 4.5

The 40 bp repeat of MHV-68 seems to have no role in the reactivation process per se.

4.6. Splenomegaly and the extent of reactivation of latently infected splenocytes are independent of a putative ORF M6 protein expression

4.6.1. The extent of splenomegaly depends on the number of 40 bp internal repeat units present in the MHV-68 genome but is independent of ORF M6 protein expression

To determine whether ORF M6 protein expression is necessary for the EBV-like infectious mononucleosis syndrome in mice and/or viral reactivation from latently infected splenocytes, C57BL/6 mice were intranasally infected with 5 x 10^4 PFU of two selected ORF M6 mutant viruses, mutant viruses for 40 bp internal repeat and parental virus. Spleens were harvested at day 17 post infection. The spleen weights were determined and found to be highest in mice infected with parental virus, followed by the 40 bp moderate mutant, the 40 bp low mutant and finally the 40 bp mutant virus (Fig 4.17). Consistent with our previous results, the decrease in spleen weight of mice infected with the 40 bp mutant was significant (P = 0.0001), when compared to the spleen weight of mice infected with the parental virus. The decrease in spleen weight after infection with the 40 bp low mutant was still significant (P = 0.011), when compared to the spleen weight of mice infected with the parental virus, whereas the 40 bp moderate mutant showed no significant decrease (P = 0.51). This suggests that the number of 40 bp repeat units determines the extent of splenomegaly.

If the phenotype observed after infection with the 40 bp mutant would be due to disruption of ORF M6, then mutations in the N-terminus of ORF M6 (which prevent the expression of the putative M6 protein) should display the same phenotype. However, after infection with the ORF M6 mutant viruses, no significant difference in the spleen weight, when compared to the parental virus, was observed (M6 Stop mutant virus: P = 0.105 and M6 Stop + Point mutant virus: P = 0.1).

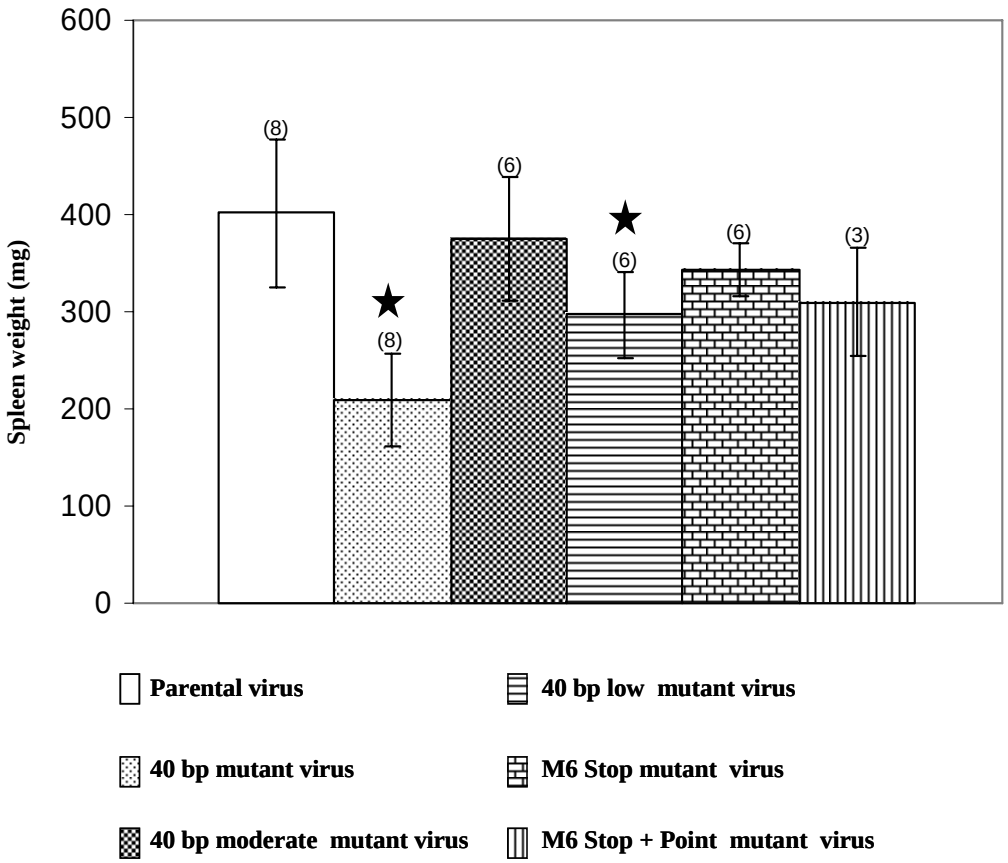


Fig. 4.17. The extent of splenomegaly is dependent on the number of 40 bp units present in viral genome. C57BL/6 mice were infected with 5 x 10^4 PFU intranasally. The extent of splenomegaly was measured by taking the spleen weight of each mouse at day 17 post infection. The asterisk indicates a statistically significant decrease in the spleen weight of mice (Student's t-test). Numbers in brackets represent the number of mice. Data (mean values ± SD) from two independent experiments are shown and in each experiment, 3-4 mice per group were used.

In contrast, when compared to the spleen weight of mice infected with the 40 bp mutant virus, the difference was significant (M6 Stop mutant virus: $P = 0.0003$ and M6 Stop + Point mutant virus: $P = 0.049$). The ORF M6 mutant viruses caused a similar extent of splenomegaly as the 40 bp low mutant which has a similar number of repeat units (M6 Stop mutant virus: $P = 0.06$ and M6 Stop + Point mutant virus: $P = 0.7$). Thus, the extent of splenomegaly was dependent on the number of 40 bp repeat units present in the viral genome and not on the expression of a putative M6 protein.

4.6.2. The 40 bp internal repeat of MHV-68, but not the ORF M6, is important for viral reactivation from latently infected splenocytes

To analyze the role of ORF M6 in *ex vivo* reactivation, mice were infected with 5 x 10^4 PFU intranasally, spleens were harvested on day 17 post infection, and an *ex vivo* reactivation assay was performed. From the panel of our ORF M6 mutants, we selected the ORF M6 Stop mutant and compared it to the different 40 bp mutants as well as to the parental virus. Splenocytes from mice infected with the parental virus or the different mutants reactivated to a similar extent but consistent with our previous results (Fig. 4.18), a reduction in reactivation was observed for the 40 bp mutant.

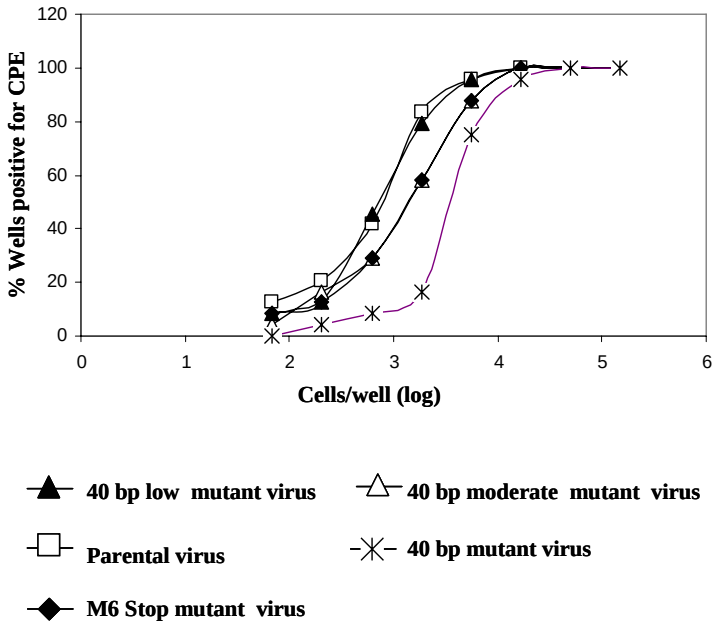


Fig. 4.18. The extent of *ex vivo* reactivation after infection of mice with an ORF M6 mutant. At day 17 post infection, single cell suspensions of splenocytes from C57BL/6 mice infected with parental or different mutant viruses (40 bp moderate mutant virus, 40 bp low mutant virus, M6 Stop mutant virus, and 40 bp mutant virus) were used for an *ex vivo* reactivation assay. Splenocytes from 3 mice per group were pooled and then three-fold serial dilutions were made. The different dilutions were co-cultured with NIH 3T3 cells for three weeks and checked for cytopathic effects microscopically. The frequencies of cells which are reactivating from the latently infected splenocytes and entering the lytic phase of infection were calculated as described in 4.4.2.

Using splenocytes from infections with the 40 bp mutant virus, 1 in 4490 splenocytes reactivated ($P = 0.04$). The 40 bp moderate mutant showed a reactivation of 1 in 2057 ($P = 0.05$), the 40 bp low mutant of 1 in 1052 ($P = 0.25$), the M6 Stop mutant of 1 in 2077 ($P = 0.05$) and the parental virus 1 in 1045. Thus, ORF M6 does not seem to be important for *ex vivo* reactivation from latently infected splenocytes.

4.6.3. Determination of subsets of cells in the spleen of mice by FACS analysis

As described above, mice infected with the 40 bp mutant virus displayed a significant decrease in the extent of splenomegaly compared to infection with parental virus. This reduction in the spleen weight could be due to a decrease in the activation and proliferation of cells in the spleen or lymphocyte recruitment to the spleen. Therefore, we analyzed the subsets of cells in the spleen by flow cytometry. We found that the relative proportion of $CD4^+$, $CD3^+$ and $CD8V\beta4^+$ T cells was not significantly different in C57BL/6 mice infected with either parental virus or mutant viruses, i.e., 40 bp mutant virus, M6 Stop mutant virus and 40 bp low mutant virus (Fig. 4.19). This suggests that the decrease in the spleen weight of C57BL/6 mice infected with the 40 bp mutant virus is not due to a decrease in a particular cell population, but might be due to an overall decrease in the proliferation of cells in the spleen or in the recruitment of lymphocytes to the spleen.

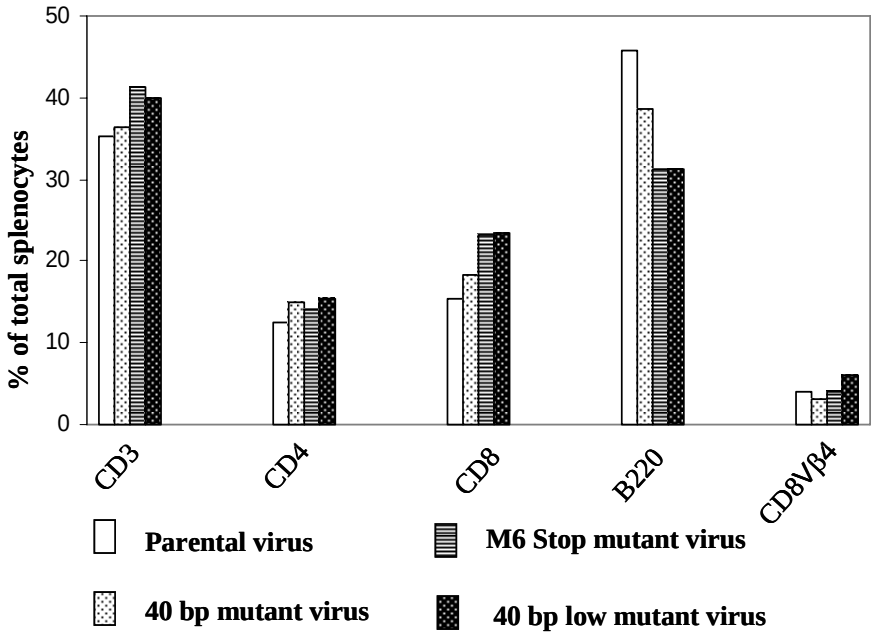


Fig. 4.19 Lymphocyte subsets in the spleens of C57BL/6 mice infected with parental virus or mutant viruses (40 bp mutant virus, M6 Stop mutant, and 40 bp low mutant). Single cell suspensions from spleens were prepared and stained with monoclonal antibodies (MAbs) against cell type specific markers and fixed in 1% paraformaldehyde after staining. Splenocytes were stained for B cells (FITC anti mouse CD45R/B220), total T cells (FITC anti mouse CD3), cytotoxic T lymphocytes (FITC anti mouse CD8a), $CD4^+$ T cells (FITC anti mouse CD4), and CD8Vβ4 T cells (PE anti mouse Vβ4 T-cell Receptor). Splenocytes from 3 mice per group were pooled and analyzed. Data shown are from a representative experiment which was repeated once with similar results.

<u>*Summary for chapter 4.6*</u>

1) The extent of splenomegaly depends on the number of 40 bp internal repeat units present in the MHV-68 genome.

2) ORF M6 of MHV-68 has apparently no role in lytic replication in lungs or latent infection in the spleen.

3) The reduction in the spleen weight of C57BL/6 mice infected with the 40 bp mutant virus is due to an overall decrease in all cell subsets contributing to the cellularity of the spleen.

4.7. In the absence of $CD8^+$ T cells, the extent of splenomegaly induced by the 40 bp mutant virus is not significantly different from the parental virus

It has been shown that during MHV-68 infection, $CD8^+$ T cells are important for both the clearance of acute infection from the lungs and the long term control of persistent infection. Infectious virus is cleared from the lungs by $CD8^+$ T cells and $CD4^+$ T cells within 10-12 days post infection, however, the T cell response is apparently ineffective in preventing massive amplification of latently infected cells at germinal centres. We had observed that infection with the 40 bp mutant virus causes a reduced EBV-like infectious mononucleosis syndrome in mice, similar to what has been reported after infection of mice with ORF MK3 mutants and M3 mutants. Both the MK3 and the M3 genes are involved in evasion from $CD8^+$ T cell immunity (Bridgeman, Stevenson *et al.* 2001; Stevenson, May *et al.* 2002). Additionally, the decreased reactivation from latently infected splenocytes in the case of the 40 bp mutant virus infection suggests an involvement of virus specific immune responses. Therefore, we investigated whether the 40 bp internal repeat might play a role in escape from the $CD8^+$ T cell immune response. For this purpose, $CD8^{-/-}$ mice, β2 microglobulin knockout mice as well as C57BL/6 control mice were intranasally infected with 5×10^4 PFU of either parental or 40 bp mutant virus intranasally.

The spleen weights of $CD8^{-/-}$ mice infected either with the parental virus or with the 40 bp mutant virus were not significantly different ($P = 0.117$) (Fig. 4.20). In contrast, consistent with our previous results, there was a significant difference in the spleen weights of wild type mice infected with the 40 bp mutant virus ($P = 0.0022$) compared to those infected with the parental virus. Thus, in the absence of $CD8^+$ T cells, the infection of mice with the 40 bp mutant led to a normal splenomegaly. However, the extent of splenomegaly in $CD8^{-/-}$ mice infected with parental virus was lower compared to C57BL/6 mice infected with parental virus ($P = 0.038$). There are two possible explanations for this observation: either, the absence of $CD8^+$ T cells rescued the phenotype of the 40 bp mutant, or, infection with the parental virus lead to a reduced splenomegaly in $CD8^{-/-}$ mice compared to C57BL/6 wild type mice. To assess this further, similar experiments were performed in β2 microglobulin knockout mice which also lack $CD8^+$ T cells.

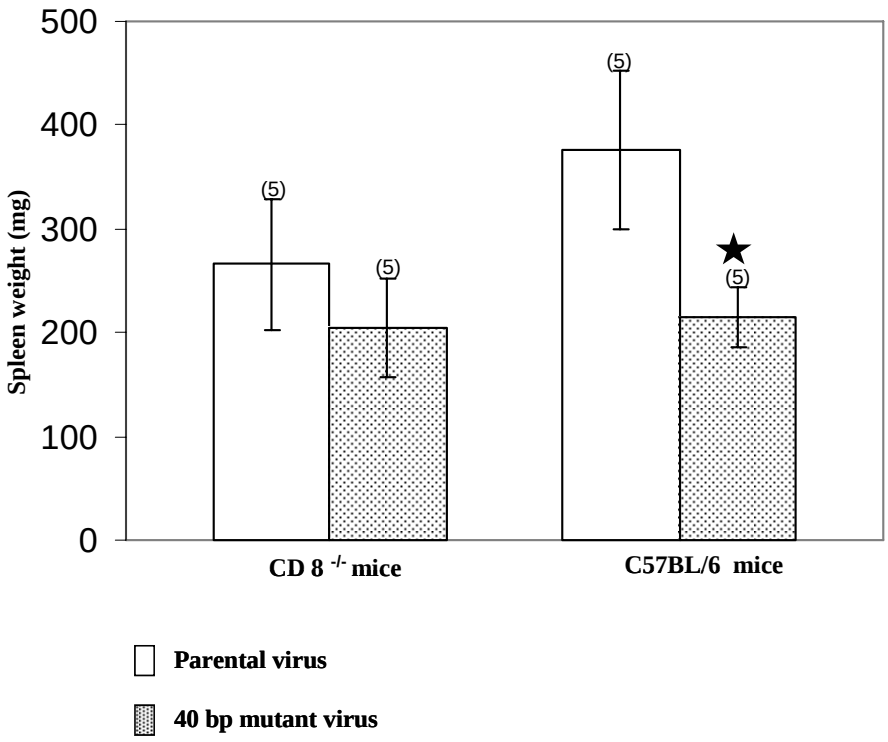


Fig. 4.20. The absence of CD8$^+$ T cells in mice abolishes the difference in splenomegaly after infection with parental virus or 40 bp mutant virus. C57BL/6 and CD8 T cell knockout mice (5 mice per group) were infected with 5 x 10^4 PFU intranasally. Spleens were harvested at day 17. Numbers in brackets represent the number of mice. Data (mean value ± SD) are compiled from two independent experiments and in each experiment 2-3 mice were used per group. The asterisk indicates a statistically significant decrease in the spleen weight of C57BL/6 mice infected with the 40 bp mutant virus compared to the infection of C57BL/6 mice with the parental virus (P = 0.0022) (Student's t-test). However, no significant difference in the spleen weight of CD8 T cell knockout mice was observed after the infection with the parental virus or the 40 bp mutant virus (P = 0.117).

In β2 microglobulin knockout mice, the absence of CD8$^+$ T cells again abolished the difference in splenomegaly between parental and 40 bp mutant (P = 0.34) (Fig. 4.21). In contrast to the CD8$^{-/-}$ mice, there was no reduced splenomegaly when compared to wild type mice (P = 0.689). This provides further evidence that in the absence of CD 8$^+$ T cells, the extent of splenomegaly following infection with the 40 bp mutant virus and parental virus were not significantly different.

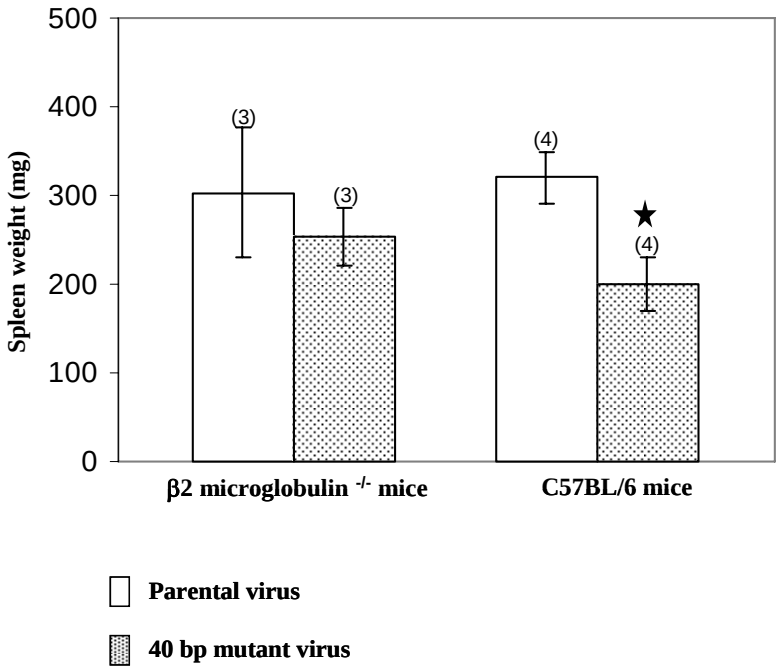


Fig. 4.21. The absence of CD8$^+$ T cells in β2 microglobulin knockout mice leads to a reversal in the phenotype after infection with the 40 bp mutant virus. C57BL/6 mice (4 per group) and β 2 microglobulin knockout mice (3 per group) were infected with 5 x 10^4 PFU intranasally. The spleen weight was taken at day 17 post infection. Numbers in brackets represent the number of mice. Data (mean valuse ± SD) are compiled from two independent experiments. The asterisk indicates statistically significant decrease in the spleen weight of C57BL/6 mice infected with the 40 bp mutant virus compared to the infection of C57BL/6 mice with the parental virus (P = 0.001). However, no significant difference in the spleen weight of β 2 microglobulin knockout mice was observed after the infection with the parental virus or the 40 bp mutant virus (P = 0.34).

Summary for chapter 4.7

In the absence of CD8$^+$ T cells, the spleen weight of mice infected with the 40 bp mutant virus is comparable to the spleen weight of mice infected with the parental virus and therefore, the 40 bp internal repeat of MHV-68 might have role in escaping the immune response mediated by CD8$^+$ T cells.

4.8. The 40 bp internal repeat of MHV-68 is involved in the regulation of the expression of the MK3 gene

Compared to infection with the parental virus, infection of C57BL/6 mice with the 40 bp mutant virus caused both a decrease in the extent of splenomegaly as well as a 2-2.5 fold decrease in the extent of reactivation of latent virus. However, this phenotype was reversed in CD8 T cell knockout mice (4.20 & 21). Similar observations have been reported after the infection of mice with MK3-deficient MHV-68 (Stevenson, May 2002). MK3 plays an important role in evasion from $CD8^+$ T cell responses against MHV-68. The 40 bp internal repeat is located 1067 bp upstream of the 5' end of the ORF MK3 start codon (nucleotide position 25335) (Fig. 4.22). Therefore, we hypothesized that the 40 bp internal repeat might potentially influence the expression of MK3. To investigate this hypothesis, we analysed the expression of MK3 by RT-PCR in fibroblasts (NIH 3T3), where the virus undergoes lytic replication, and in the B cell line Ag8, where MHV-68 establishes a latent infection.

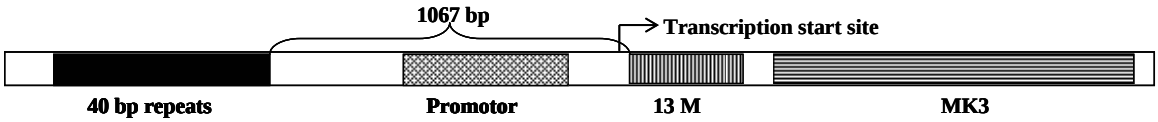


Fig. 4.22. Schematic presentation of the organization and position of ORF 13M/MK3 and the 40 bp internal repeat region of MHV-68. The 40 bp internal repeat of MHV-68 is present at the 5' end of ORF 13M/MK3. The start codon of ORF 13M/MK3 and the 40 bp internal repeats are separated by 1067 bp.

We infected fibroblasts and B cells with either parental virus or the 40 bp mutant virus with an M.O.I of 1. The expression of ORF MK3 was evaluated by isolation of RNA from infected cells and subsequent semiquantitative RT-PCR and real-time RT-PCR. To estimate the genomic load of latent virus, DNA was isolated from the Ag8 B cell line infected with the parental virus and the 40 bp mutant and subjected to real-time PCR analysis.

In the fibroblast cell line, no significant difference in the expression of MK3 was found after infection with either the 40 bp mutant or the parental virus 24 hours post infection

(Fig. 4.23). To exclude viral DNA contamination of the RNA extracted from infected cells, RT-PCR reactions without reverse transcriptase were run as controls. They yielded no PCR products (not shown). The expression of the house keeping gene elongation factor 1 β was used as a control of equal amounts of input cDNAs.

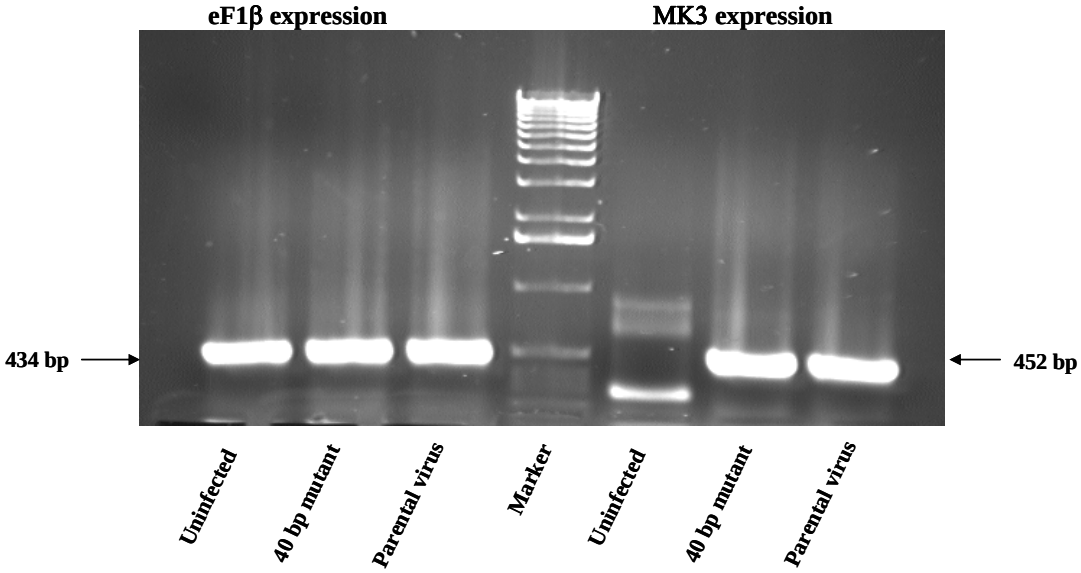


Fig. 4.23. The deletion of the 40 bp internal repeat of MHV-68 has no influence on the expression of the neighboring gene MK3 in fibroblasts. The expression of MK3 was analysed by RT- PCR and visualized on an ethidium bromide stained 1% agarose gel. RNA was prepared from NIH 3T3 cells infected with the parental virus or the 40 bp mutant or from uninfected cells, and cDNA was prepared from 2 μg of DNase treated RNA. The conditions for PCR were as follows: initial denaturation at 94°C for 3 min followed by 30 cycles of 94°C denaturation for 30 sec, 55°C annealing for 30 sec and 72°C synthesis for 30 sec. A house keeping gene elongation factor 1 β was amplified by PCR to check the input of cDNA. Also, control reactions without reverse transcriptase were included to check for the presence of genomic DNA, and yielded no PCR products (not shown). In the uninfected sample, only unspecific PCR products were observed after performing the PCR with primers for MK3 gene.

In contrast to the fibroblasts, MK3 expression in the B cell line Ag8 was substantially higher following infection with either the parental virus or 40 bp revertant virus as compared to infection with the 40 bp mutant. The expression of a house keeping gene (GAPDH) was similar (Fig. 4.24)

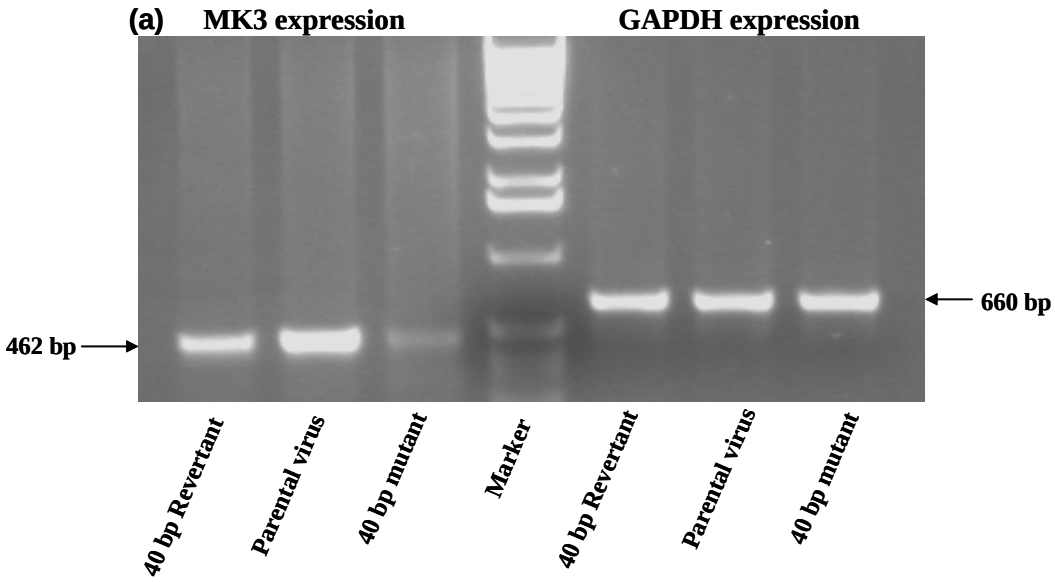


Fig. 4.24. The deletion of the 40 bp internal repeat of MHV-68 leads to a reduced expression of the neighboring gene MK3 in B cells (Ag8). (a) The expression of MK3 was analyzed by RT- PCR and visualized on an ethidium bromide stained 1% agarose gel. RNA was prepared from the B cell line Ag8 infected with the parental virus or the 40 bp mutant or the 40 bp revertant virus. A house keeping gene GAPDH was amplified by PCR to check the input of cDNA. Also, control reactions without reverse transcriptase were included to check for the presence of genomic DNA, and yielded no PCR-products (not shown). The conditions for PCR reaction were similar as implied for checking the expression of ORF M6 in fibroblasts. For the estimation of MK3 expression, the PCR was performed using 2 µl of cDNA. The cDNA was prepared from 2 µg of RNA sample.

These results were confirmed by real time RT-PCR analysis. It was found that in the B cell line Ag8, the expression of MK3 in cells infected with the parental virus is 7-8 fold higher than in those infected with the 40 bp mutant (Fig. 4.25). To exclude that the reduction is not just due to a lower infection rate, we determined the total latent genomic load of parental virus as well as of the 40 bp mutant in the B cell line Ag8.

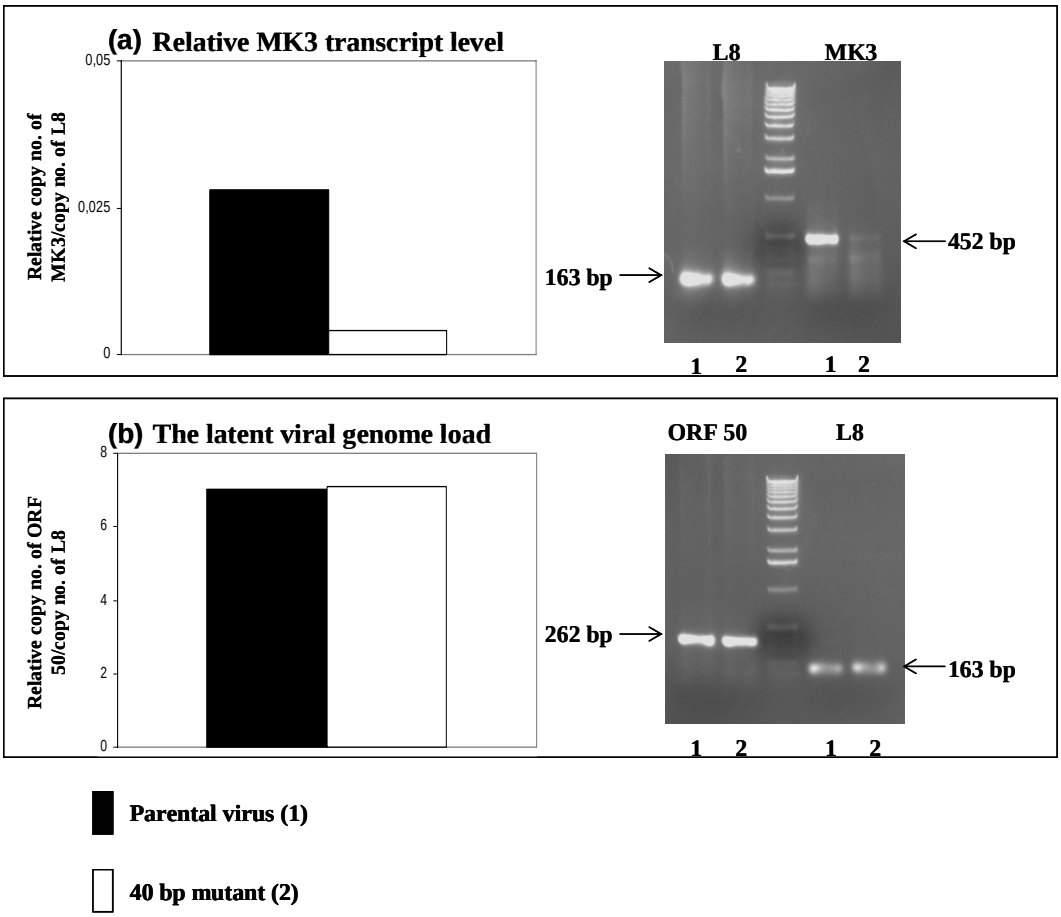


Fig. 4.25. The deletion of the 40 bp internal repeat of MHV-68 leads to a reduced expression of the neighboring gene MK3 in B cells (Ag8). (a) Estimation of the expression of MK3 in Ag8 cell line by real-time PCR. For the estimation of MK3 expression, the PCR was performed using 2 μl of cDNA in a 20 μl system. The cDNA was prepared from 2 μg of RNA from infected B cells. The expression of MK3 was estimated as relative copy number of MK3 cDNA per copy number of rpl8 cDNA. The copy number of MK3 was estimated against a standard curve constructed by serial dilution of MHV-68 BAC plasmid containing the ORF MK3. The amount of cDNA was also confirmed by the estimation of the murine ribosomal protein L8 (rpl8) gene by real time PCR. The standard curve for rpl8 was constructed by serial dilution of a plasmid containing a 163 bp fragment of rpl8 (pCR2.1/rpl8) (Stewart *et al.* 2004). The PCR products from the real time PCR were visualized on an ethidium bromide stained 1% agarose gel. (b) Estimation of the latent genomic load in the Ag8 cell line by real-time PCR. The real time PCR was performed with 200 ng of total Ag8 cell DNA using primers specific for the MHV-68 ORF 50 gene and L8 (cellular) to estimate the latent viral genome load in the Ag8 cells. The MHV-68 genome copy number was estimated against a standard curve constructed by serial dilution of plasmid pUC19 ORF50 (*Eco RI – Ava I* fragment, nucleotide position 65707-69550 of MHV-68). The copy number of rpl8 was estimated against a standard curve constructed by serial dilution of plasmid containing a 163 fragment of rpl8.

Consistent with our *in vivo* genomic load experiments (Fig.4.15), the latent viral genome load was found to be similar after infection with either the 40 bp mutant or the parental virus. We also demonstrated that the amount of template DNA used for the PCR was similar as assayed by amplifying a genomic region within the murine ribosomal protein coding gene L8 (Fig. 4.25). These data indicate that the infectivity of both viruses was similar. This suggests that the expression of MK3 in the B cell line Ag8 is reduced in the presence or absence of the 40 bp internal repeat and may thus explain the phenotype of reduced splenomegaly in CD8 expressing mice. B cells are a major reservoir for MHV-68 latent infection, and the expression of the MK3 protein may protect against $CD8^+$ T cell immunity. Thus, in the absence of the 40 bp internal repeat the expression of MK3 is down-regulated which in turn leads to enhanced antiviral CTL responses, impaired expansion of latently infected cells in lymphoid germinal centers and an attenuation of the MHV-68-induced splenomegaly.

Summary for chapter 4.8

1. *In the fibroblast cells, the expression of ORF MK3 is independent of the presence of the 40 bp internal repeat of MHV-68.*
2. *In the B cell line, the expression of ORF MK3 is dependent on the presence of the 40 bp internal repeat in the genome of MHV-68. Thus, the 40 bp internal repeat is involved in regulation of expression of MK3 in a tissue specific or infection cycle specific fashion.*

5.0. Discussion

One of the common features of gamma herpesviruses is the presence of various kinds of repeats in their genome. Usually, the unique region of the genome which codes for most of the genes is flanked by terminal repeats and interspersed by many internal repeats. In Epstein-Barr virus, there are four different internal repeats termed IR1-4. The IR3 repeat plays a role in the evasion from the immune response (Levitskaya *et al.* 1995; 1997; Sharipo *et al.* 1998), and some repeats (IR2 and IR4) are part of the origins of lytic replication (Hammerschmidt and Sugden 1988). The HHV-8 genome has approximately six internal repeats and two repeats, like in EBV, are part of the origins of lytic replication (AuCoin *et al.* 2002). However, the role of the gamma herpesvirus repeats in pathogenesis is not clear.

MHV-68 which is considered to be a model for gamma herpesvirus pathogenesis has two internal repeats, i.e., the 40 bp repeat and the 100 bp repeat. The 100 bp repeat is located at the right side of the genome (nucleotide positions 98981-101170) and consists of approximately 21 copies of 100 bp repeat units. The 100 bp repeat is part of three overlapping ORFs: ORF M10a, ORF M10b and ORF M10c. It has been shown that the 100 bp repeat contributes to the lytic origin of replication at the right side of the MHV-68 genome (Deng *et al.* 2004).

The 40 bp internal repeat of MHV-68 is located at the left side of the genome (nucleotide positions 26778-28191). It consists of approximately 36 copies of 40 bp repeat units, some of which can vary in their sequence (Virgin *et al.* 1997). The 40 bp internal repeat is also part of a hypothetical ORF M6 (Virgin *et al.* 1997).

In this study, we addressed the role of the 40 bp internal repeat and/or ORF M6 in *in vitro* replication and in pathogenesis.

5.1. Construction of the 40 bp and ORF M6 mutant viruses

To analyze the role of the 40 bp internal repeat and/or ORF M6, six independent mutants were constructed by the different methods using MHV-68 cloned as a BAC. To study the role of the 40 bp internal repeat and to determine the significance of the number of the 40 bp internal repeat units, we constructed three viral mutants, i.e., the 40

bp mutant virus, the 40 bp moderate mutant virus and the 40 bp low mutant virus. The 40 bp mutant virus contains a deletion of all the 40 bp internal repeat units which was confirmed by restriction enzyme analysis and sequencing of the 40 bp internal repeat region. The 40 bp low mutant contains 15 copies of 40 bp internal repeat units as determined by restriction enzyme analysis and sequencing. The 40 bp moderate mutant virus has approximately 20-25 copies of the 40 bp repeat units as determined by restriction enzyme analysis (Fig. 4.3 & 4). Probably due to the formation of secondary structures in the 40 bp repeat region, sequencing of the 40 bp moderate mutant was not possible and thus, the precise number of 40 bp internal repeat units present in the 40 bp internal repeat region could not be determined. Virgin *et al.* proposed that the 40 bp internal repeat contains approximately 36 copies of repeat units, however it was not possible to stably clone and sequence the 40 bp internal repeat region (Virgin *et al.* 1997). To ensure that there are no rearrangements and/or mutations outside the 40 bp internal repeat region created during mutagenesis, a revertant virus was generated by replacing the 40 bp mutant fragment with the wild type genomic fragment.

In addition, we constructed three viral mutants to study the role of ORF M6, i.e., M6 Stop mutant virus, M6 Point mutant virus, and M6 Stop + Point mutant virus. M6 Stop mutant virus contains stop codons in all three possible open reading frames at the *Hind III* site (nucleotide position 26711) which lead to the termination of ORF M6 translation after 31 amino acids. Since the M6 Stop mutant virus can still encode for 31 amino acids present at the N-terminus of ORF M6, we inserted a point mutation in the start codon (ATG to ACG) of ORF M6 leading to the formation of M6 Point mutant virus. Thus, ORF M6 cannot be translated. We also constructed a double mutant virus, i.e., M6 Stop + Point mutant virus, which contains a mutation at the start codon and additionally an insertion of stop codons in all three possible open reading frames. It has been reported that the 40 bp internal repeat is unstable in the MHV-68 BAC and can lead to an unwanted loss of the 40 bp internal repeat in *E. coli* (Adler *et al.* 2000; 2003). Indeed, it was not possible to construct the ORF M6 mutants without losing some of the 40 bp internal repeat units. The three ORF M6 mutant viruses and the 40 bp low mutant virus have a similar number of repeats as determined by restriction enzyme analysis. Therefore, the phenotype of the ORF M6 mutant viruses can be compared to the 40 bp low mutant virus. Transfection of all the mutant MHV-68 BAC plasmids into fibroblasts allowed reconstitution of lytic viruses, which shows that neither the 40 bp repeat nor ORF M6 are essential for virus replication in cell culture.

5.2. The 40 bp internal repeat of MHV-68 is dispensable for lytic replication both *in vitro* and *in vivo*

Infection of NIH 3T3 cells at low MOI (0.1) showed that all mutant viruses efficiently replicated and attained similar titers when compared to the parental virus (Fig. 4.8). The replication efficiency of the 40 bp mutant in the initial phase of infection (24 and 48 hours post infection) appeared to be slightly reduced; however, at later time points (72 and 96 hours post infection) it attained similar titers as the parental virus. It was also observed that the plaque size after infection with the mutant viruses was similar to the parental and revertant viruses. The replication efficiency of the 40 bp mutant or the ORF M6 mutants in the lungs of C57BL/6 mice was not significantly different to the parental and revertant virus (Fig. 4.10), indicating that the 40 bp internal repeats and/or ORF M6 are dispensable for lytic replication in the lungs.

It has been proposed that the ORF M6 region (containing the 40 bp internal repeat) might contain a second MHV-68 lytic origin of replication (Coleman *et al.* 2004). A part of the M6 coding region (nucleotide positions 26232-26374) has more than 70% homology to nucleotide positions 101624 to 101765 of the MHV-68 genome. The latter region neighbouring the 100 bp internal repeat harbors a known origin of lytic replication (Deng *et al.* 2004). Both loci have A/T-rich and G/C-rich regions characteristic of herpesvirus lytic origins of replication, and this function has also been confirmed for the equivalent regions of HHV-8 and RRV (AuCoin *et al.* 2002; Deng *et al.* 2004; Pari *et al.* 2001). In the prototype EBV genome, there are two copies of *ori* Lyt, called DS-L (Dyad symmetry left) and DS-R (right). However, it was shown that one copy is sufficient for lytic-cycle replication of EBV strain B95-8 (Fixman *et al.* 1992; Laux *et al.* 1985). It was proposed that the presence of more than one origin of DNA replication (*ori*) might provide some replicative advantage for large DNA genomes compared to genomes with a single *ori*. Multi-*ori* genomes are less sensitive to mutations in one *ori* and the initiation of replication from more than one genome locus might result in an accelerated speed of replication (Jehle 2002). Preliminary experiments with double knockout mutants, i.e., mutants containing a deletion of the 40 bp internal repeat as well as the 100 bp internal repeat have shown that the double knockout mutant has a very low replication efficiency compared to mutants carrying a deletion in either of the two internal repeats (Adler *et al.*, unpublished results). The 100 bp internal repeat region of MHV-68 acts as an auxiliary element to enhance the replication efficiency of

MHV-68 (Deng *et al.* 2004). It is likely that the 40 bp internal repeat contains sequences involved in lytic replication, but the presence of the other origin of lytic replication in the 100 bp repeat region might compensate for the loss of the sequences in the 40 bp internal repeat region. This is most likely the reason why little to no replication deficiency was observed in the *in vitro* replication of the 40 bp mutant virus.

5.3. The 40 bp internal repeat of MHV-68 is important for the latent phase of infection

MHV-68 establishes a latent infection in lymphocytes and drives the proliferation of infected lymphocytes during the establishment and maintenance of a large reservoir of latent viral genomes. The colonization of lymphoid tissue by MHV-68 is a complex process which involves multiple cell types including B lymphocytes, macrophages, dendritic cells and some epithelial cells with different programs of viral gene expression (Virgin *et al.* 1999). To study the role of the 40 bp internal repeat for the latent phase of infection, we used different methods to measure viral latency: the extent of splenomegaly, the extent of *ex vivo* reactivation as well as the genomic load of latent virus in splenocytes. We found that infection of C57BL/6 mice with the 40 bp mutant during the amplification stage of the latency (day 13 and 17 post infection) caused significantly less splenomegaly and a reduced frequency of *ex vivo* reactivating splenocytes, when compared to the infection with parental virus (Fig. 4.11 and 14). However, this phenotype was not observed during the initial stage of latency establishment (day 10) and the long term latency (day 23 and 42).

During the amplification stage of latency, the splenocytes from mice infected with the 40 bp mutant virus showed a decrease in the extent of reactivation compared to splenocytes from mice infected with parental virus. We checked whether this decrease in the extent of reactivation from splenocytes of mice infected with the 40 bp mutant was due to either a defect in the reactivation process *per se* or a decrease in the number of latently infected cells in the spleen. Using real time PCR, we estimated the latent genomic load in the spleens of mice infected with the 40 bp mutant virus or the parental virus. At day 17 and 42 post infection, real time PCR analysis showed that there was no significant difference in the viral genome copy number per 100 ng of total DNA after

infection with either the parental virus or the 40 bp mutant (Fig. 4.15). However, since the total spleen weights as well as the total number of splenocytes are higher after infection with the parental virus at day 17 post infection, the overall latent genomic load in the spleen of mice infected with the parental virus is higher. At day 42, where no difference in the spleen weight or extent of reactivation was observed, the overall latent genomic load in the spleen of mice infected with either virus is similar. These data suggest that the 40 bp internal repeat of MHV-68 is important for pathogenesis, especially for the amplification stage of the latency but not for long-term persistent infection. The internal repeats of HSV also play an important role in pathogenesis. It has been shown that the deletion of internal repeats from the HSV genome results in a pronounced effect on neurovirulence, neuroinvasivness and in the ability to establish a reactivable latent infection in mice (Jenkins *et al.* 1996).

We found that the extent of splenomegaly is dependent upon the number of 40 bp internal repeat units present in the MHV-68 genome (Fig. 4.17). Thus, the highest spleen weight was observed in mice infected with the parental virus which contains all the repeat units, followed by the 40 bp moderate mutant virus which has lost a few repeat units, and the 40 bp low mutant which has lost a considerable number of repeat units. The lowest spleen weight was found in mice infected with the 40 bp mutant virus in which all the repeat units were deleted. The extent of splenomegaly was found to be not associated with the expression of the hypothetical ORF M6, as the mice infected with the ORF M6 mutant viruses showed no significant difference in the spleen weight when compared to the parental virus but a significant difference when compared to the 40 bp mutant virus.

5.4. In the absence of CD 8^+ T-cells, the 40 bp mutant causes a similar extent of splenomegaly

$CD8^+$ T cells play a major role in controlling the acute lytic infection that follows primary MHV-68 infection; however, the $CD8^+$ T cell response is ineffective in preventing the massive proliferation of latently infected B cells in germinal centers (Ehtisham *et al.* 1993; Weck *et al.* 1996; Stevenson *et al.* 1999 b; 2002 a). This suggests

that MHV-68 has evolved a mechanism to evade $CD8^+$ T cell immunity by which it can achieve the burst of lymphocyte proliferation that initially establishes the latent pool.

So far, two MHV-68 genes, i.e., M3 and MK3, have been identified which play important roles in the evasion of $CD8^+$ T cell mediated immunity. The mutant viruses for these genes show normal lytic replication in the lungs; however, they have enhanced CTL responses, impaired expansion of latently infected cells in lymphoid germinal centers and an attenuation of the MHV-68-induced infectious mononucleosis. *In vivo,* the defect in the amplification of latency is largely reversible by $CD8^+$ T cell depletion (Bridgeman *et al.* 2001; Stevenson *et al.* 2002 a). Infection of C57BL/6 mice with the 40 bp mutant caused significantly less splenomegaly and a reduced frequency of *ex vivo* reactivating splenocytes, when compared to the infection with parental virus. This phenotype was similar to the phenotype which has been reported after infection of mice with ORF MK3 mutant viruses and M3 mutant viruses. Therefore, we considered the possibility that the 40 bp internal repeat might also play a role in the evasion of $CD8^+$ T cell mediated immunity. To address this possibility, we infected mice lacking $CD8^+$ T cells ($CD8^+$ T cell knockout mice) and parental C57BL/6 mice with either the 40 bp mutant or the parental virus and measured the extent of splenomegaly. $CD8^+$ T cell knockout mice lack mature $CD8^+$ T cells, due to disruption of CD8 genes (Lyt-2 and Lyt-3). It has been shown that *in vivo,* CD8 expression is necessary for the development of MHC class I-restricted cytotoxic T cells and hence, these mice have impaired $CD8^+$ T cell mediated immunity (Fung-Leung *et al.* 1991). These mice have been used to evaluate the role of $CD8^+$ T cells during viral infection (Kimura and Griffin 2000; Liu and Chamber 2001; Nass *et al.* 2001; Xu *et al.* 2004). We found that the spleen weights of $CD8^+$ T cell knockout mice infected with either the parental virus or the 40 bp mutant virus were not significantly different (Fig. 4.20). Yet the weight of the spleen infected with parental virus was lower than in wild type mice. The reason for this difference is not clear. The lower spleen weight of $CD8^+$ T cell knockout mice after infection with parental virus might be due to the absence of $CD8^+$ T cells which also contribute to the splenomegaly. Such a difference has not been reported by other laboratories (Bridgeman *et al.* 2001; Stevenson *et al.* 2002 a). However, in their experiments, $CD8^+$ T cells were depleted from wild type mice by injection of monoclonal antibodies. Since there are no reports in the literature on the extent of splenomegaly in $CD8^+$ T cell knockout mice, we could not really compare our findings to published data. Therefore, to confirm our results from the $CD8^+$ T cell knockout mice experiments, we performed similar

experiments in β2 microglobulin knockout mice. β2 microglobulin knockout mice lack efficient surface major histocompatibility complex class I expression as a result of a null mutation in the β2 microglobulin gene (Koller *et al.* 1990), with consequent deficiency in $CD8^+$ T cells (Doherty *et al.* 1993; Hou *et al.* 1992; Lehmann-Grube *et al.* 1993; Matloubian *et al.* 1994; Muller *et al.* 1992). Apart from classical MHC-I molecules, several other non-classical MHC class I-related molecules (Qa, h2-M3, and Cd1) require β2 microglobulin for their expression and recognition of foreign antigens or altered self antigens (Kronenberg *et al.* 1999). β2 microglobulin knockout mice have been extensively used by others to investigate the role of $CD8^+$ T cells in viral infection (Doherty *et al.* 1993; Lehmann-Grube *et al.* 1993; Muller *et al.* 1992). It has been shown that the spleen weights of β2 microglobulin knockout mice and parental C57BL/6 mice are not significantly different after infection with MHV-68 (Weck *et al.* 1996). Consistent with this report (Weck *et al.* 1996), we found no significant difference in the spleen weight of the two strains of mice (β2 microglobulin knockout mice and parental C57BL/6 mice) (Fig. 4.21). Also, consistent with our $CD8^+$ T cell knockout mice experiments, no significant difference in the spleen weight of β2 microglobulin knockout mice after infection with either the parental virus or the 40 bp mutant virus was observed.

In contrast to the $CD8^+$ T cell knockout mice which lack $CD8^+$ T cells only, β2 microglobulin knockout mice have no MHC class I protein expression on the cell surface. In β2 microglobulin knockout mice, a compensatory increase in $CD4^+$ T cells has been observed (Fung-Leung *et al.* 1991). It is known that after infection of mice with MHV-68, the induction of splenomegaly, B cell proliferation, maturation, and activation are dependent on $CD4^+$ T cells (Cardin *et al.* 1996; Sangster *et al.* 2000; Stevenson and Doherty 1999; Usherwood *et al.* 1996 a). Therefore, it is possible that after MHV-68 infection there is an increase in $CD4^+$ T cells in β2 microglobulin knockout mice. This might induce the proliferation of B cells and thus splenomegaly similar to C57BL/6 mice, even in the absence of $CD8^+$ T cells which also contribute to the transient splenomegaly.

It has been shown that the CD1 molecule requires β2 microglobulin to be expressed (Brutkiewicz *et al.* 1995). CD1 molecules are MHC class I-like transmembrane glycoproteins that are encoded by a family of genes located outside of the classical MHC loci (Porcelli and Modlin 1999). CD1 is required for the development of a subset of specialized T cells, termed NKT cells, which express the semivariant Vα14-Jα281 TCR

(T cell receptor) paired with Vβ8.2-7 or -2 (Bendelac *et al.* 1997). This natural T-cell subset occurs in both mice and humans, and responds rapidly to stimulation by the release of cytokines; as a result, it is thought to represent a bridge between the innate and the acquired immune response (Porcelli and Modlin 1999). CD1 and/or NKT cells have been implicated in immunity to a number of pathogens (Denkers *et al.* 1993; Gonzalez-Aseguinolaza *et al.* 2000; Kakimi *et al.* 2000; Pied *et al.* 2000) and in case of HSV, it has been shown that CD1d and CD1d-restricted NKT cells are important in the control of infection and in clearance of the virus (Ashkar and Rosenthal 2003; Grubor-Bauk *et al.* 2003). Therefore, it is possible that in $CD8^+$ T cell knockout mice after infection with parental virus, CD1-restricted NKT cells clear a small fraction of latently infected cells and thus the mice have less splenomegaly compared to that observed after infection of β2 microglobulin knockout mice with parental virus, as β2 microglobulin is required for the expression of CD1.

Keeping the above considerations in mind, we nevertheless conclude from our experiments that the absence of $CD8^+$ T cells largely reversed the phenotype of the 40 bp mutant virus. In other words, in mice deficient of $CD8^+$ T cells, infection with the 40 bp mutant causes splenomegaly to a similar extent as infection with parental virus. Thus, a major role of the 40 bp internal repeat of MHV-68 *in vivo* seems to be to prevent CTL recognition during the amplification stage of latency (2-3 weeks post infection) which is important for host colonization. Although latency amplification is not essential for persistence and reactivation *per se* (Stewart *et al* 1998), it probably increases viral seeding to systemic sites and makes long-term viral transmission more robust in immunocompetent hosts (Stevenson *et al.* 2002 b).

5.5. The 40 bp internal repeat of MHV-68 is involved in the establishment of latency by regulating the expression of MK3 in a tissue specific/infection cycle specific fashion

The establishment of latency provides unique advantages for herpesviruses to escape host immune surveillance and to establish lifelong persistent infection. Latency of gamma herpesviruses is characterized by silencing of most of their genes present in the genome. One critically important regulatory mechanism utilized by gamma

herpesviruses is DNA methylation. It has been shown that methylation of cytosines within CpG dinucleotides at the promotor regions of EBV are important for gene silencing and genome integrity to maximize persistence and hide from immune detection (Ernberg *et al.* 1989; Minarovits *et al.* 1994; Paulson and Speck 1999; Salamon *et al.* 2001; Tao and Robertson 2003). However, to maintain latency, gamma herpesviruses encode a limited number of genes which can play an important role in immune evasion and maintenance of episomal DNA. It has been shown that three distinct regions of the MHV-68 genome are transcriptionally active during the latent phase of infection. The location of these regions correlates well with regions in HHV-8, EBV and HVS that contain known latency and/or tumor-associated genes, and some of the ORFs in these regions are homologous to known latency- or tumor-associated genes of EBV, HVS, or HHV-8 (Virgin *et al.* 1999). Two of these genes (M3 and MK3) are involved in the evasion of CTLs to achieve the burst of lymphocyte proliferation that initially establishes the latent pool (Bridgeman *et al.* 2001; Stevenson *et al.* 2002 a). The MHV-68 MK3 protein contributes to this process *in vivo* by evading CD 8^+ T cell recognition during latency as well as during lytic infection. A major feature of the MK3-deficient MHV-68 phenotype is a defect in viral latency amplification (Stevenson *et al.* 2002 a). MK3 is transcribed as part of a bicistronic mRNA, downstream of ORF 13M (Fig. 4.22) (Coleman *et al.* 2004) and it is classified as an immediate early gene (Virgin *at al.* 1997; Ahn *et al.* 2002). In functional terms, 13M and MK3 are controlled by the same promoter elements (genomic coordinates 25735 to 26778) which are located at 1 kbp 5' of the transcription start site. It has been demonstrated that the dependence of ORF MK3 expression on the ORF 50 lytic transactivator (Rta) varies, depending upon the cell line in which it is tested (Coleman *et al.* 2004). ORF 50 (Rta) of MHV-68 is homologous to the Rta genes of other gamma herpesviruses, including HHV-8 and EBV. Transfection of HHV-8 and MHV-68 Rta expressing plasmids into latently infected cell lines has been shown to activate expression of early and late viral lytic genes (Lukac *et al.* 1998; Sun *et al.* 1998) and can induce viral lytic DNA replication and virus production in MHV-68 (Wu *et al.* 2000). It has been proposed that during *de novo* infection of permissive cells, Rta can be activated by cooperative action of a virion protein similar to HSV VP16 and cellular transcription factors. In nonpermissive cells, Rta expression is blocked, thus preventing the initiation of the viral lytic cascade, leading to latency (Wu *et al.* 2000).

In our studies, infection of C57BL/6 mice with the 40 bp mutant led to a significant decrease in the extent of splenomegaly, and this phenotype was reverted in mice lacking $CD8^+$ T cells. This phenotype is comparable to the phenotype observed after infection of mice with the MK3 deficient MHV-68. Also, we have shown that the extent of splenomegaly is not dependent on ORF M6 protein expression, but depends on the number of 40 bp internal repeats present in the MHV-68 genome. Therefore we analyzed whether the 40 bp internal repeats of MHV-68 affect the expression of the MK3 gene as they are located at the 5' end of the ORF 13M/MK3 promotor. We found that in fibroblasts (NIH 3T3), expression of the MK3 gene after infection with the 40 bp mutant was unaffected, whereas in the B cell line Ag8, a marked reduction of MK3 expression was observed (Fig. 4.23, 24 & 25). These data indicated that expression of MK3 might be differentially regulated during lytic replication and latent phase of the infection.

It is likely that in fibroblasts, where MHV-68 replicates lytically, Rta cooperates with some transcriptional factors and/or viral genes leading to the expression of 13M/MK3. However, in the latent phase of infection where the expression of Rta has not been detected (Rochford *et al.* 2001; Virgin *et al.* 1999), MHV-68 could use the 40 bp internal repeat to regulate the expression of 13M/MK3 expression by recruiting additional transcription factors. It is quite likely that the 40 bp repeat might act as an enhancer for the expression of ORF MK3 during the latent phase of infection (Fig. 5.1).

Transcriptional enhancers are discrete DNA elements that contain specific sequence motifs with which DNA-binding proteins interact and transmit molecular signals to genes. Enhancer sequences are part of transcriptional control regions that might be present in multiple copies and are often called enhancer modules. Each of these modules is designed to perform a specific function, such as the activation of its cognate gene in a specific cell type or at a particular stage in development (Blackwood and Kadonga 1998).

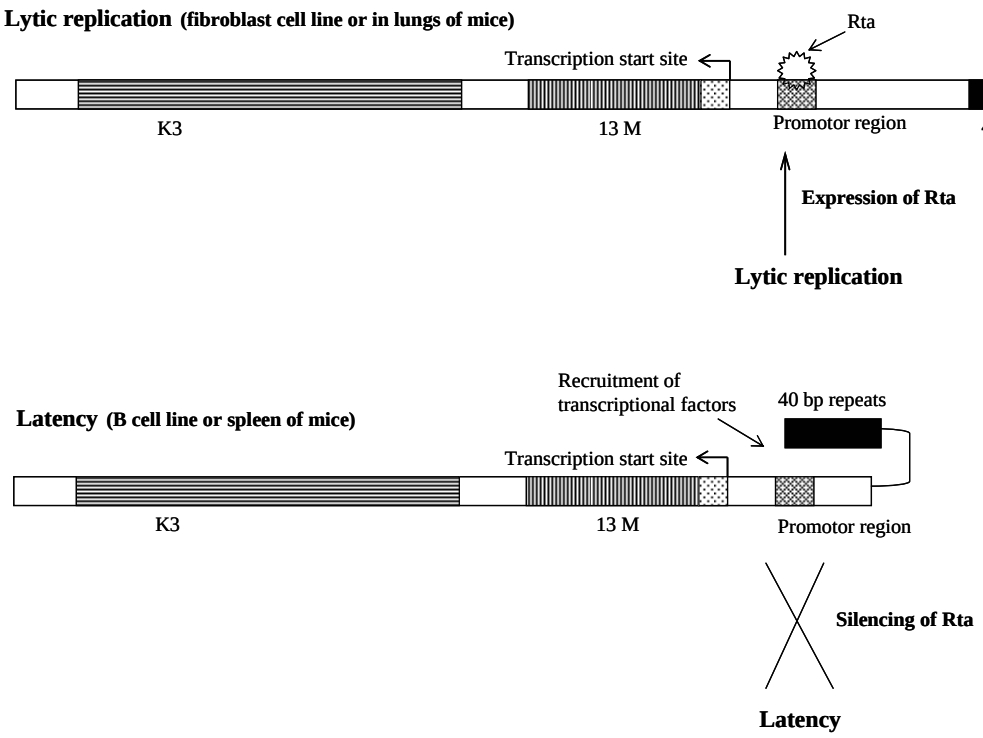


Fig. 5.1. Hypothetical model for the function of the 40 bp internal repeat of MHV-68. 13M/MK3 expression is regulated differentially in fibroblasts and B cells. During lytic replication in fibroblasts, the 13M/MK3 promotor utilizes the aid of Rta complexes for its expression, whereas in B cells, it might utilize the 40 bp internal repeat as an enhancer for its expression.

Similar enhancer elements containing repeat structures or multiple enhancer modules have been reported in many viruses. In murine leukemia virus (MuLV) and other non-acute retroviruses, it has been shown that these enhancer sequences are important determinants of both the pathogenic potential and disease specificity of the respective viruses (Chatis *et al.* 1984; DesGroseillers and Jolicoeur 1984; Golemis *et al.* 1989; Holland *et al.* 1989; Li *et al.* 1987). In case of cytomegalovirus, the major immediate early promotor (MIEP) enhancer is required for optimal infection and plays an important role in determining the cell type tropism (Baskar *et al.* 1996 a and b; Koedood *et al.* 1995). It has been suggested that enhancers affect pathogenicity by modulating the level of transcription of viral and cellular genes in a tissue specific fashion. Transient transfections of a number of different fibroblast and haematopoietic cell lines with MuLV-derived enhancer and promoter regions driving reporter genes have shown that LTR-mediated transcriptional activity correlates with the tissue specificity and disease-inducing potential of the virus (Celander and Haseltine 1984; Nishigaki *et al.* 1997;

Short *et al.* 1987; Speck *et al.* 1990). The enhancer sequences of non-defective Friend and Moloney virus consist of densely packed series of motifs which contain actual or putative binding sites for cellular proteins and transcription factors (Golemis *et al.* 1990) It has been shown that the triplication of viral enhancer elements in Type B leukemogenic virus (TBLV) leads to upregulation of reporter gene activity and its enhancer activity is relatively orientation independent (consistent with the activity of eukaryotic enhancer elements). However, the enhancer activity is dependent on the cell type (Mertz *et al.* 2001). In case of MHV-68, there are approximately 36 copies of the 40 bp internal repeat units, and therefore it is likely that MHV-68 requires many copies of these repeats for efficient transcription of 13M/MK3 during latency amplification. This is consistent with our observation that with the decrease in the number of 40 bp internal repeat units in the MHV-68 genome, the extent of splenomegaly after the infection also decreases (Fig. 4.17). Thus, the 40 bp internal repeat of MHV-68 could act as an enhancer sequence for the expression of MK3 in a dose dependent manner during the amplification stage of the latency.

5.6. Outlook

Infection of mice with murine gamma herpesvirus 68 (MHV-68) presents the opportunity to evaluate the role of specific immune system genes and individual gamma herpesvirus genes in a small animal model amenable to both genetic and pathogenetic studies. The MHV-68 genome contains two internal repeats, i.e., the 40 bp internal repeat and the 100 bp internal repeat. We wanted to study the role of these repeats in gamma herpesviruses pathogenesis and immunity. We have shown that the 40 bp internal repeat is important for the latent phase of infection, especially for the amplification of latency by regulating the expression of MK3. We have shown that in the B cell line Ag8, the expression of MK3 is dependent on the presence of the 40 bp internal repeat in the MHV-68 genome. However, it is not known whether the regulation of the expression of MK3 by the 40 bp internal repeat *in vivo* is dependent on the cell type or on the phase of infection. Studies of the expression of MK3 in various cell types (macrophages and dendritic cells), where MHV-68 can establish latent infection, and in *in vivo* samples could perhaps define the 40 bp internal repeat dependent ORF MK3

expression in a tissue specific/infection cycle specific fashion. The study of DNA-protein interaction, i.e., of proteins or transcription factors binding to the 40 bp internal repeat region, would help to better understand the molecular mechanism by which the expression of ORF MK3 is regulated, interpreting the function of MK3 and relating this to immune evasion by other gamma herpesviruses.

Our initial data also indicate that the 40 bp internal repeat of MHV-68 could be a part of a lytic origin of replication. Identification of a second MHV-68 origin of lytic replication (*ori* Lyt) and mapping the *ori* Ly*t* through deletion analysis will help to understand the biology of MHV-68 replication and also for the development of MHV-68 based amplicon vectors.

6. Summary

Gamma herpesvirus genomes have been observed to contain different kinds of repeat sequences, the functions of which range from immune evasion to being a part of the lytic origin of replication. The MHV 68 genome also contains two such internal repeats. In this study, we addressed the role of the 40 bp internal repeat and the hypothetical ORF M6 (which contains the 40 bp repeat) in *in vitro* replication and pathogenesis in mice.

The 40 bp internal repeat and/or ORF M6 of MHV-68 are dispensable for lytic replication both *in vitro* and *in vivo*, however, it might be possible that the 40 bp internal repeat contains sequences involved in lytic replication, i.e., an origin of lytic replication. The 40 bp internal repeat plays an important role for the amplification of latency, as demonstrated by the reduction in the extent of splenomegaly and reduced reactivation. This phenotype is dependent on the number of 40 bp internal repeat units present in the genome, and is not associated with expression of ORF M6. During latency, the expression of the MHV-68 ORF MK3, known for its role in immune evasion, is reduced in the absence of the 40 bp repeat, suggesting that the 40 bp repeat is involved in the establishment of latency via regulation of the expression of ORF MK3 in a tissue specific/infection cycle specific fashion. For the first time, we have demonstrated the importance of an internal repeat in latency of a gamma herpesvirus and in the pathogenesis. Our data suggest that internal repeats could act as enhancer regions for certain virus specific genes, thus playing a role in affecting the phenotype of virus-induced disease.

7.0. Bibliography

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9.0 Curriculum vitae

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University Education

B.Sc	1997 Baroda, India
M.Sc	1999 Baroda, India

Research Experience

1. Masters dissertation (1997-1999) project "**Studies of polyhydroxybutyric acid production by *Methylobacterium* zp on dual substrates**" at Department of Microbiology and Biotechnology Center, M.S. University, Baroda, India.

2. Research Assistant (1999-2000) "**High scale production of polyglutamic acid from *Bacillus licheniformis* ATCC 9945**" at Department of Chemical Engineering, Korean Advanced Institute of Science and Technology, Taejon, South Korea.

3. Research Assistant (2000-2001) "**The effect of Cecropin on giant liposomes**", at Department of Biochemistry, Hong Kong University of Science and Technology, Hong Kong.

4. Research Assistant (2001-2002) "**Antibiotic resistance mechanism in *Lactococcus* and *Streptomyces* strains**" at department of Biochemistry and Microbiology, Southern Denmark University, Denmark.

Doctoral Thesis

PhD thesis (2002-2005) "**Functional Characterization of the 40 bp internal repeats of murine gamma herpes virus-68**" at Institute for Molecular Immunology, GSF, LMU, Munich, Germany.

Publications

1. Yellore, V., **N. Thakur**, and A. Desai (1999). Enhancement of growth and poly-3-hydroxybutyrate production from *Methylobacterium* sp. ZP24 by formate and other organic acids. *Lett. Appl.Microbiol.* 29: 171-175.

2. **Nagendra N. Thakur**, Do, J.H., Chang, H.N., Lee, S.Y. Efficient polyglutamic acid production by *Bacillus licheniformis.* (Manuscript in preparation).

3. Lee, S.Y., Chang,H.N., **Thakur, N.N.**, Do, J.H. (Korean patent filed 10-2000-0027278).

4. Hueih Min Chen, King Wong Leung, **Nagendra N. Thakur,** Anmin Tan and Ralph W. Jack. (2003) Distinguishing between different pathways of bilayer disruption by the related antimicrobial peptides cecropin B, B1 and B3. *Eur. J. Biochem.* 270; 911-920.

5. Heiko Adler, **Nagendra N. Thakur** and Jürgen Haas. Evidence for more than one lytic origin of replication in murine gammaherpesvirus 68 (MHV-68). (Manuscript in preparation).

6. **Nagendra N.Thakur** and Heiko Adler. The 40 bp internal repeat of murine gamma herpesvirus 68 (MHV-68) is required for virus induced infectious mononucleosis like syndrome. (Manuscript in preparation).

Conferences and Presentations

1. Oral Presentation – Nagendra N. Thakur, Beatrix Steer and Heiko Adler: The 40 bp internal repeat of murine gammavirus 68 (MHV-68) is important for latent

but not the laytic phase of infection. Gesellschaft für Virologie, Jahrestagung, March 2003 Berlin.

2. Poster Presentation - Nagendra N. Thakur and Heiko Adler: The 40 bp internal repeat and/or ORF M6 of murine gammavirus 68 (MHV-68) is required for amplification of latent phase. Gesellschaft für Virologie joint meeting with 'Societa Italiana di Virologia' Jahrestagung, March 2004 Tübingen.