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Investigation of murine cytomegalovirus US22 gene family members m139 and m142

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To my parents.

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A. INTRODUCTION

1 Cytomegalovirus as a herpesvirus

1.1 The group of herpesviruses

Cytomegalovirus (CMV) is a member of the herpesvirus family which consists of more than 100 members. Eight of these infect humans: herpes simplex virus I and II, varicella-zoster virus, cytomegalovirus, human herpesvirus 6 and 7, Epstein-Barr virus, and human herpesvirus 8. The name “herpes” is derived from the Greek word “herpein” that means “to creep like a snake”. This term describes the capacity of eczemas to slowly spread on the skin of individuals infected with the herpes simplex or varicella-zoster virus.

Herpesviruses share biological features and particle morphology. They have double-stranded DNA genomes with a high coding capacity and all herpesviruses can establish latent infection. Herpesviruses can be subdivided into α-, β- und γ-herpesviruses depending on host spectrum, replication time and cell tropism. CMV is the principal member of the β-herpesvirus subgroup. It exhibits a number of biological characteristics, common to all β-herpesviruses, including salivary gland tropism, strict species specificity, and slow growth in cultured cells (Ho et al., 1991; Roizman et al., 1981). Human herpesvirus 6 and 7 are also members of the β-herpesviruses. γ- and β-herpesviruses display strict species specificity, but the length of the replication cycle varies between the different members of the two families. The γ-species infects B- and T-cells of the lymphoid system. Prominent members of this family are the Epstein-Barr virus and the human herpesvirus 8. α-herpesviruses have a broad host spectrum and short replication cycle in contrast to γ- and β-herpesviruses. They have the capacity to establish latency in ganglia and contiguous neural tissue. Herpes simplex virus I and II and the varicella-zoster virus belong to this family.

HCMV is an important human pathogen that causes disease in the immunologically immature or the immunocompromised host, such as premature infants, newborns, transplant recipients and individuals infected with human immunodeficiency virus.

1.2 Cytomegalovirus

The term cytomegalia was first used in 1921 and describes the prominent cell enlargement induced by the cytomegalovirus (Goodpasture and Talbot, 1921). Cells infected with CMV show virus-induced cytopathic effects (CPE) with characteristic nuclear and cytoplasmic inclusions and cell rounding.

1.2.1 Structure of the cytomegalovirus virion

CMVs resemble morphologically other members of the herpesvirus family. Mature virions have a diameter of 150 to 200 nm. The CMV virion (see Fig. A 1) consists of an icosahedral capsid encasing a double-stranded linear DNA genome with a core surrounded by a tegument (a fairly unstructured matrix of viral proteins) and is enveloped in a lipid bilayer, which carries a large number of virus-encoded glycoproteins (Britt and Mach, 1996). Purified virus particles of HCMV have been estimated to contain 30 to 40 polypeptides with molecular weights ranging from 20 to over 300 kDa in size (Baldick and Shenk, 1995). The capsid is composed of seven proteins, all of which are homologous to those identified in HSV and at least 25 proteins are located in the tegu-

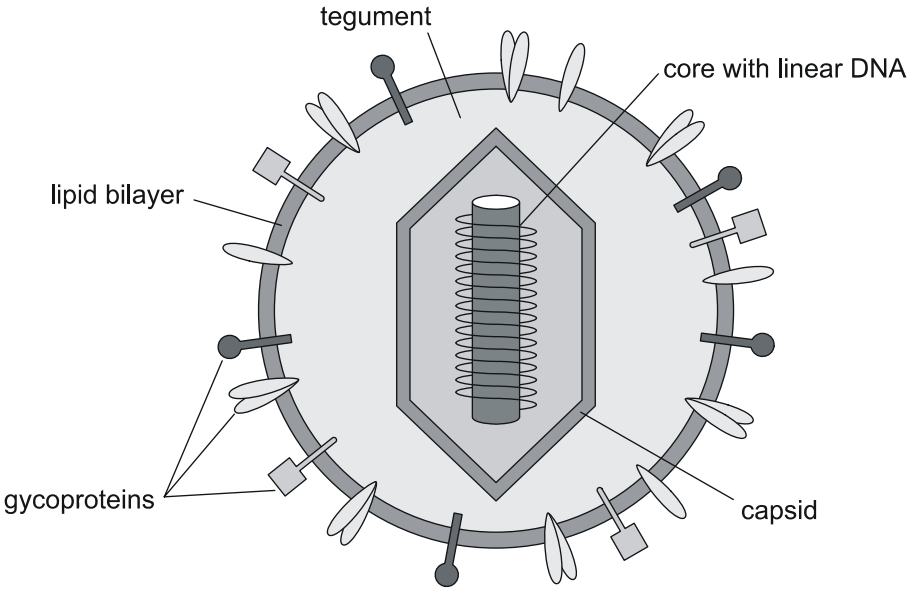

Fig. A 1 The CMV virion. The nucleocapsid containing the core, the linear DNA and the icosahedral capsid, is surrounded by tegument. The viral envelope is composed of a lipid bilayer with viral glycoproteins.

ment between the virion capsid and the envelope (Gibson 1996). The virion envelope carries two prominent herpesvirus-conserved glycoprotein complexes (one composed of dimers of glycoprotein B (gB) and the other of gH, gL, and gO) embedded in a host lipid bilayer derived from intracellular membranes (Britt and Mach, 1996).

Apart from infectious virions, non-infectious enveloped particles and dense bodies are produced in HCMV-infected cells (Irmiere and Gibson, 1983). Dense bodies do not contain viral DNA or capsid. They mainly constitute of the tegument protein pp65 (Craighead et al., 1972), while the less abundant non-infectious particles do have a capsid but lack DNA. In murine CMV infected cells dense bodies are not produced, but virions that contain several capsids have been observed (Chong and Mims, 1981).

1.2.2 Organization of the cytomegalovirus genome

The linear molecules of HCMV and MCMV are significantly larger than those of other herpesviruses and are among the largest and most complex virus genomes (Fig. A 2) (Chee et al., 1990; Rawlinson et al., 1996).

The HCMV genome has two unique components, the unique long (U_L) and unique short (U_S) sequences, flanked by inverted repeats *b* (TR_L/IR_L) and *c* (IR_S/TR_S) (Fig. A 2). Different orientations of the inverted repeats can establish four isomers of HCMV. The HCMV open reading frames (ORFs) are numbered sequentially and designated by their location within the unique and repeated regions of the viral genome (Chee et al., 1990).

The MCMV genome comprises of a single unique sequence (Ebeling et al., 1983). Direct terminal repeats are present at the ends of the MCMV genome and multiple other direct and inverted repeats of different lengths are found (in a higher quantity than in other herpesviruses) at different positions of the genome (Rawlinson et al., 1996). MCMV ORFs that show sequence similarity to HCMV ORFs are designated with upper-case letters (e.g. M36), whereas those not conserved in HCMV are represented by lower-case letters (e.g. m152). Families of ORFs that result from gene duplication events are present in HCMV and MCMV (Chee et al., 1990; Rawlinson et al., 1996). These also show sequence homology to other herpesviruses. Before DNA replication is initiated, the linear genomes of HCMV and MCMV both circularize in the nucleus of infected cells.

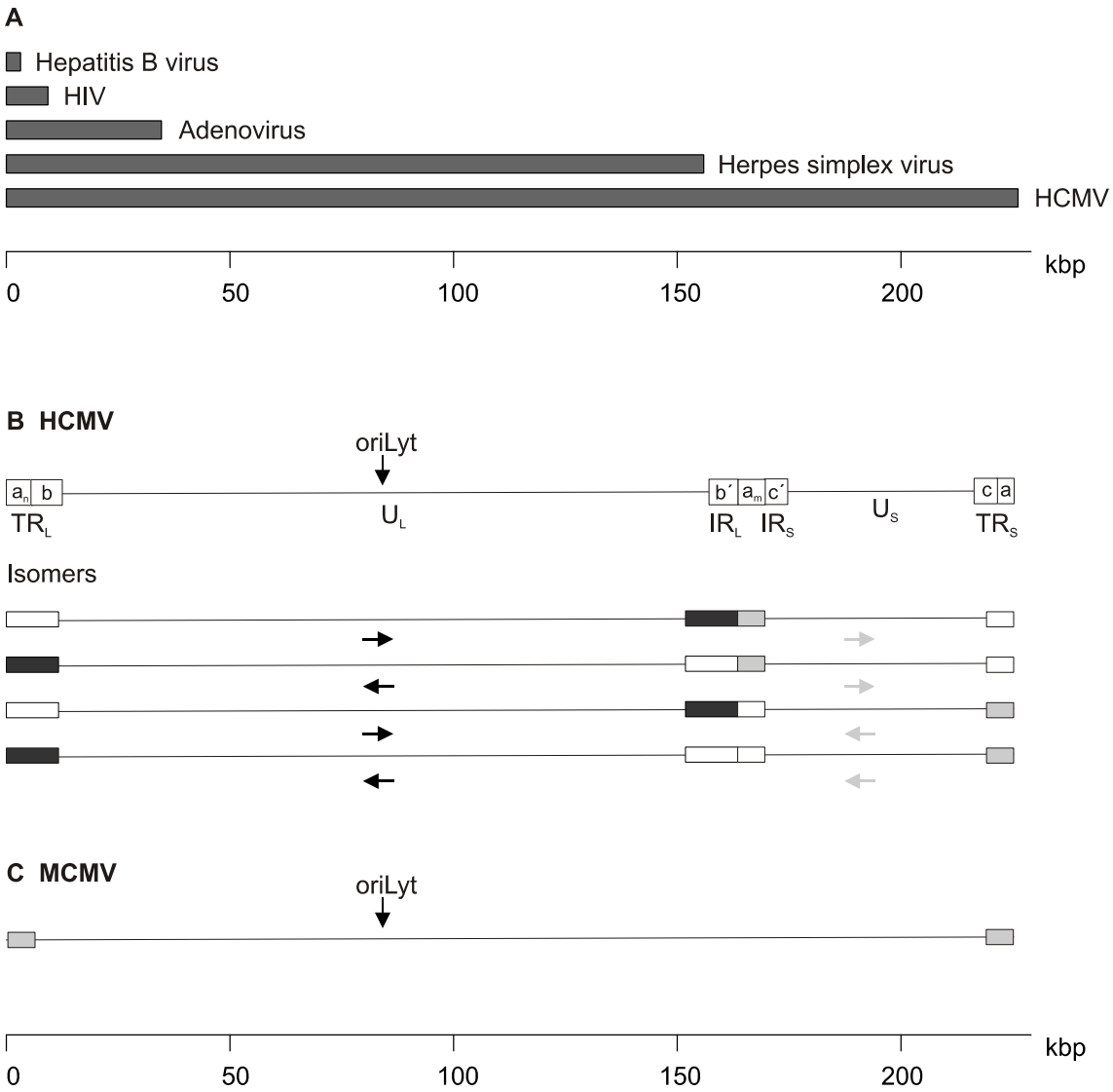

Fig. A 2 CMV genome structure. (A) Representation of lengths of different virus genomes. The line below shows a size scale in kbp. **(B)** HCMV genome structure. The first line depicts the two unique sequences of HCMV, the unique long (U_L) and unique short (U_S) sequences, flanked by inverted repeats b (TR_L/IR_L) and c (IR_S/TR_S). The sequences a, b and c represent terminal and internal repeats. HCMV has four isomers depending on the orientation of the internal and terminal repeats. The oriLyt depicts the origin of replication. **(C)** MCMV genome structure. The MCMV genome is flanked by terminal repeats. The line below depicts the size in kbp.

1.2.3 Gene expression and replication of cytomegalovirus

The CMV replication cycle and that of all other herpesviruses is stringently regulated and follows a downwelling organization. HCMV penetrates cells by fusion between the virus envelope and the host cell plasma membrane (Compton et al., 1992). Attachment and penetration at the cell surface is rapid and efficient in permissive as well as in non-permissive cell types, suggesting that cellular receptors for CMV are widely distributed. To date, molecular details underlying the mechanism of CMV entry into host cells remain elusive. The major cell surface proteoglycan heparan sulfate has been implicated in the initial interaction between the virus and the cell (Compton et al., 1993), and coiled-coil domains in glycoproteins B and H, and the epidermal growth factor receptor play a role in membrane fusion (Kinzler and Commpton 2005; Lopper and Compton, 2004; Wang et al., 2003). After penetration and uncoating, viral nucleocapsids migrate to the nucleus where the linear genome circularizes as an episome. Gene expression and replication of the CMV genome both take place in the nucleus.

Viral gene expression follows different kinetic classes depending on the time of expression: immediate early (IE), early (E), and late (L). The complete replication cycle is long compared to other herpesviruses: 48 to 72 hours for HCMV and 24 hours for MCMV. During the IE-phase, regulatory proteins are expressed which induce, as transcriptional activators, the expression of E- and L-proteins as well as cellular proteins (Kowalik et al., 1993; Nicholas, 1996). During the E-phase, proteins which are necessary for the viral genome replication are expressed, and during the late phase mostly structural proteins or proteins involved in the assembly of the virus are expressed. The DNA replication starts during the E-phase and is probably enabled by viral proteins only.

After circularization of the parental genome, DNA synthesis takes place according to the “rolling-circle” mechanism and results in the formation of a single double-stranded DNA molecule, which contains several copies of the viral genome (concatemers). The concatemers are cleaved between the a-sequences at the cleavage-sites and packaged into the capsid. Capsids and tegument proteins associate at the inner nuclear membrane. Egression of the capsid from the nucleus and gain of the envelope occurs according to the “double envelope theory”: by leaving the nucleoplasm, the viral capsid obtains a first envelope, which is lost by fusion with the outer nuclear membrane. The

final envelope is acquired during migration through the endoplasmic reticulum and the Golgi apparatus (Mettenleiter 2002; Radsak et al., 1996).

1.2.4 Pathogenesis of cytomegalovirus infection: mechanisms of dissemination and tissue tropism

The cellular pathway of host entry by CMV has not been fully delineated. Epidemiologic evidence suggests that the entry of CMV follows contact between infectious virus and mucosal surface. The site of entry seems to be the epithilium of the genitourinary tract, the upper alimentary tract, or the respiratory tract (Sinzger and Jahn, 1996). However, CMV infection can also be established by transplanted organs, blood transfusion or by the hematogenous route during fetal development. Although the key pathways for spread of human CMV from the initial site of replication have not been clearly proven, circulating virus is usually found in leukocytes (Dankner et al., 1990; Saltzmann et al, 1988). The virus may be carried by peripheral monocytes and circulating endothelial cells to reach different organs (Gerna et al., 2000). A wide range of tissues and often multiple cell types within an organ are involved in CMV infection (Plachter et al., 1996). Cells with cytopathology typical of CMV have been described in the epithelium of salivary gland, bile duct epithelium, bronchial epithelium, islet cells, renal tubular epithelium, capillary endothelium, astrocytes, neurons, and epithial cells of the inner ear (Ho, 1995). Not only epithelial and endothelial cells of different organs, but also fibroblasts, macrophages, and smooth muscle cells have been shown to support productive replication of CMV (Sinzger et al., 1995).

1.2.5 Immune response and latency in cytomegalovirus infection

Despite the response of the innate and adaptive immune system the CMV-infected host does not achieve viral clearance. In most cases CMV establishes latent infection by evading the immune system. There are multiple mechanisms by which CMV can achieve this and apparently during latency CMV transcripts can be detected in the blood of infected individuals (Kondo et al., 1994). CMV can compromise antiviral host defence mechanisms by expressing a diversity of proteins focused on mimicking and altering the major histocompatibility complex protein function (Ahn et al., 1996; Gewurz et al., 2001; Kavanagh et al., 2001a; Kavanagh et al., 2001a; Tomaszin et al, 1999), leuko-

cyte migration (Penfold et al., 1999; Saederup et al., 2002), activation and cytokine responses (Akdis and Blaser, 2001; Kotenko et al., 2000), and host cell susceptibility to apoptosis (Zhu et al., 1995; Brune et al., 2001: Menard et al., 2003). The evidence for molecular mechanisms by which CMVs avoid detection and elimination is rapidly growing.

Specific cellular and humoral immunity all play important roles in the control of CMV disease. Shellam et al. (1981) showed that mice devoid of NK cells are more susceptible to MCMV infection. Indirect evidence for the importance of NK cells in defense of CMV infection also comes from the observation that the viral gene products that appear to be conserved can protect against NK cell-mediated lysis (Tomasec et al., 2000). Nearly all immune human sera have antibody to envelope glycoproteins gB (UL55) and gH (UL75), to the tegument phosphoprotein pp150 (UL32) and to a non-structural DNA binding phosphoprotein pp 52 (UL44) (Greijer et al., 1999; Schoppel et al., 1997). Neutralizing antibody is mainly directed against envelope proteins gB and gH (Britt, 1991).

CMV disease is seen almost exclusively in patients with profoundly impaired cell-mediated immunity, such as patients undergoing bone marrow transplantation or patients with AIDS. The severity of disease due to CMV infection parallels the degree of impairment of T-lymphocyte response. The suppression of T-lymphocyte function led to reactivation and dissemination of the natural infection in a murine model. An important role for CD4+ T-lymphocytes in γ-interferon production has also been suggested (Hengel et al., 1994). Although CD4+ T-lymphocyte response to envelope glycoproteins gB and gH, to lower matrix protein pp65, and to immediate-early proteins, are most commonly detected, responses to other proteins have been also reported (Alp et al., 1991; Beninga et al., 1995). It is likely that more targets for CTL will be identified among CMV proteins. At present proteins pp65 (UL83) and IE1 (UL123) appear to be major targets.

CMV not only establishes a latent state of infection and restricts the number of viral genes expressed in order to minimize exposure to the immune system, but also uses specific tissues for replication, that have a less stringent immune surveillance. There is growing evidence that cells in bone marrow and peripheral blood are a key reservoir for CMV through latent infection. A small percentage of peripheral blood monocytes from subjects with past infection harbor CMV DNA, but viral gene expression is limited to

early genes (Danker et al., 1990; Ibanez et al. 1991; Taylor-Wiedemann et al., 1991). However, tissue macrophages express early and late CMV antigens (Sinzger et al., 1996). These observations led to propose a model for human CMV persistence and reactivation in which bone marrow precursors are a key site of latency; peripheral blood monocytes provide a means to disseminate virus, and differentiation of latently infected monocytes into macrophages leads to reactivation and productive infection. The experimental evidence supports a model of CMV persistence in which myelomonocytic stem cells resident in bone marrow maintain latent infection, and latently infected CD 14+ monocytes circulate in peripheral blood. Differentiation of latently infected monocytes into tissue macrophages as a result of allogenic stimulation, or triggering of proinflammatory cytokines as occurs with intercurrent infections, may lead to productive CMV infection (Hahn et al., 1998; Ibanez et al., 1991; Soderberg-Naucler et al., 1997).

2 Clinical aspects of cytomegalovirus infection

2.1 Epidemiology and transmission of cytomegalovirus

Human cytomegalovirus has a worldwide distribution and infection occurs from human to human. Depending on the socioeconomic status and living conditions, the rate of seropositive individuals varies between 50% to 100% in the adult population (Mustakangas et al., 2000; Wong et al., 2000). In general, prevalence is higher and CMV is acquired earlier in life in developing countries and in the lower socioeconomic strata of developed countries (Britt and Alford, 1996; Pass, 1985).

Despite these high rates of CMV infection, CMV is not highly contagious. Excretion of the virus after primary infection can be detected for months to years and the virus can generally be detected in oropharyngeal secretions, breast milk, feces, urine, cervical and vaginal secretions, semen and blood (Dworsky et al., 1983; Handsfield et al., 1985; Montgomery et al., 1972). Transmission appears to require frequent contact with body fluids from humans excreting the virus and can occur perinatally, intrapartally, through breast milk, between sex partners, between children in day care centers, between preschool-aged children and their care givers, and through organ transplantations and blood transfusions (Pass et al., 1990; Pereira et al., 1990).

CMV infection is the most common congenital viral infection in developed countries (Demmler, 1991), overtaking rubella since the introduction of the rubella immunization. Approximately 3000 infants in Germany (Scholz, 2000) and 40 000 infants in the United States are born with congenital CMV infection annually (Stagno and Whitley, 1985). This corresponds to approximately 1% of newborns that are infected in utero during maternal viremia. The occurrence of congenital infection is unique in CMVs among the human herpesviruses and congenital infection plays an important role in maintaining CMV infection in the population. Mothers are mostly infected by young infants and children shedding the virus after primary infection. Around 5% to 25% of newborns with congenital CMV infection are symptomatic (Fig. A 3). The incidence of perinatal (up to 1 month after birth) and postnatal (up to 12 months after birth) infection is still higher than that of intrauterine infection. Typically, the infant is exposed to virus shed from the cervix, excreted in breast milk, or, less commonly, shed from other infants infected with the virus. Although the incidence of perinatal and postnatal CMV infection is higher, these infants have asymptomatic or self-limited disease but do not have the neurologic sequelae seen in some congenitally infected infants (Demmler, 1991).

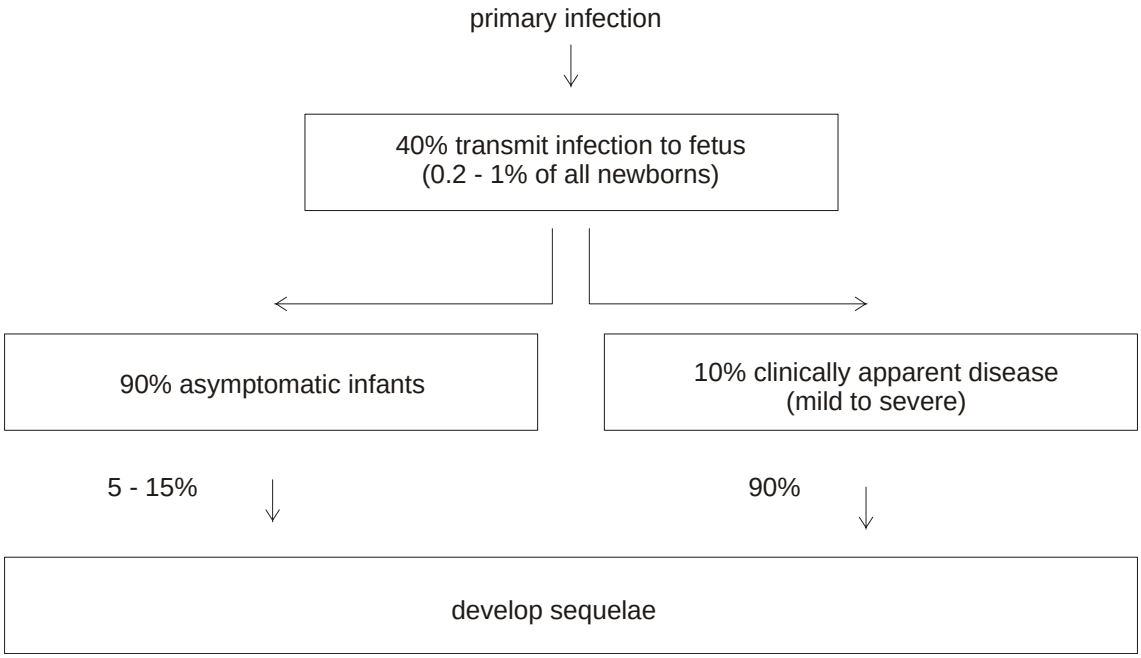

Fig. A 3 Characteristics of cytomegalovirus infection during pregnancy.

For horizontal spread close or even intimate contact is required. Sexual transmission is common in adolescents and the adult population. Patients with multiple sexual partners are attributed with a higher risk (Jordan et al., 1973). In immunocompromised patients in terms of malignancy, acquired immunosuppression or immunosuppression in transplant recipients, CMV reactivation develops more frequently. The sources of infecting virus in allograft recipients include the transplanted organ and reactivation of endogenous virus of the transplant recipient. CMV infection can also be acquired during the use of blood products containing an increased amount of leukocytes at a frequency of 0.9% (Preiksaitis et al., 1988).

2.2 Clinical manifestations of cytomegalovirus

Most infections with HCMV are clinically inapparent. Symptomatic infections occur more commonly in preterm infants, newborns and immunocompromised patients. In immunocompetent individuals, primary infection is usually without clinical manifestations or can cause symptoms that resemble those of mononucleosis with prolonged high fevers, sometimes accompanied with chills, profound fatigue and malaise.

Congenital CMV infection involves damage to the central nervous system and organs of perception (eyes, ears) and neonatal death in around 10% symptomatic newborns (Boppana et al., 1999). Protracted interstitial pneumonitis has been associated with perinatally acquired CMV infection, particularly in premature infants. Five percent of fetuses develop cytomegalic inclusion disease. Common symptoms of cytomegalic inclusion disease are petechiae, hepatosplenomagaly, jaundice, microcephaly, intrauterine growth retardation, and prematurity (Fig. A 4).

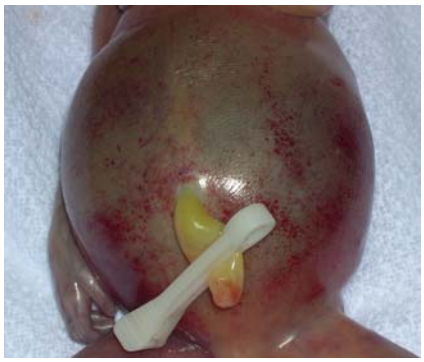

Fig. A 4 Congenital HMCV infection. Premature infant with clinical findings of petechial rash and hepatosplenomegaly (Neonatal Intensive Care Unit, Children´s Hospital Munich).

However, the majority (50% to 100%) of symptomatic newborns will have some combination of deficits, including mental retardation, cerebral palsy, sensorineural loss, and impaired vision (Bale et al., 1990) (Fig. A 3). Approximately 5% to 15% of infants with asymptomatic congenital CMV infection will have CNS sequelae, mainly sensorineural hearing loss (Ivarsson et al., 1997).

Depending on the immune status of the host, reinfection with a novel exogenous strain or reactivation of the latent strain can occur. Patients who are immunosuppressed can develop life threatening disease such as pneumonitis, gastrointestinal ulceration or severe hepatic dysfunction. Primary infection acquired from a transplanted organ is generally more severe than reactivation of the recipient's latent virus and can lead to impaired graft function. In recipients of allogenic bone marrow transplants, pneumonitis is a clinically significant CMV disease (Boeckh and Bowden, 1995). In patients with AIDS, risk of CMV disease is linked closely to the immune impairment as reflected by low CD4(+) T-cell counts.

The major clinical manifestations among AIDS patients with CMV disease are retinitis, esophagitis and colitis, and CMV has also been suspected to be a cofactor in the development of AIDS (Kovacs et al., 1999; Ostrowski et al., 1998). Furthermore, it has been reported that CMV-infected cardiac transplant patients have higher rates of graft rejection, more severe coronary atherosclerosis, and higher death rates (Danesh et al., 1997; Grattam et al., 1989; Zhou et al., 1996). HCMV may play a causal role in restenosis by interacting with the tumor suppressor p53 (Spier et al., 1994). Further investigations will be needed to establish a more comprehensive relationship of CMV infection and atherosclerosis.

2.3 Diagnosis of cytomegalovirus infection

HCMV infection can be diagnosed by serological tests and direct detection. Acute HCMV infection is probable when HCMV-specific IgM antibodies and a fourfold increase in HCMV-specific IgG antibody titers are present. Serologic results require a comparison to baseline titers. Since these are often not available, serologic testing is limited in its utility. Previously, cultures had to be held for one to six weeks until cytopathic effects were observed (Chou, 1990). To surpass slow viral growth, detection of HCMV can be achieved within 24 to 72 hours by detecting immediate early proteins

using immunofluorescence staining. This overnight tissue-culture method (shell vial assay) involves centrifugation enhancement and an immunochemical detection technique employing monoclonal antibodies to an immediate early CMV antigen (pp65 protein). HCMV can be detected from multiple sites, including blood, urine, throat washings, cerebrospinal fluid, bronchial washings, and biopsy specimens. Employing PCR one can detect viral DNA of immediate early and glycoprotein genes. Prenatal diagnostic is performed with transabdominal ultrasound and if necessary is followed by analysis of amniotic fluid in the early-to-mid trimester for viral DNA.

2.4 Therapy of cytomegalovirus infection

There are several agents that are available for the systemic therapy of CMV infection. These include ganciclovir, and its prodrug valganciclovir, foscarnet and cidofovir. Ganciclovir is phosphorylated intracellularly by a viral kinase to a triphosphate, which competively inhibitis the incorporation of dGTP by the viral DNA polymerase. This disrupts viral DNA synthesis. Ganciclovir is used in the therapy of HIV positive patients with CMV retinitis, CMV gastrointestinal tract disease, CMV pneumonitis, and CMV neurologic disease. In transplant recipients of solid organs and bone marrow, ganciclovir is also used as a preemptive treatment. As an alternative, foscarnet or cidovir can be used in cases with kinase mutations that lead to ganciclovir resistance. Foscarnet, another agent, is a pyrophosphate that stops DNA chain elongation and has additional antiretroviral activity. Cidovir is a nucleotide analogue and its incorporation into viral DNA disrupts further chain elongation. It is mainly used in the treatment of CMV retinitis in patients with AIDS.

Currently, there is no effective intervention for a primary CMV infection during pregnancy. A nonrandomized study by Nigro et al. (2005) showed that hyperimmune globulin therapy was associated with a significantly lower risk of congenital CMV infection (adjusted odds ratio 0.32; 95% confidence interval, 0.10 to 0.94; $p = 0.04$). Treatment of pregnant women with CMV-specific hyperimmune might be effective in prevention of congenital CMV infection. A controlled trial of this agent will follow.

In the future vaccines against CMV could help to control CMV disease in high-risk patient populations. Evidence from several animal models of CMV infection indicates that a variety of vaccine strategies are capable of inducing immune responses sufficient

to protect against CMV-associated illness following viral challenge (Cicin-Sain et al., 2003; Gonzalez-Armas, et al., 1996; MacDonald et al., 1998). Currently, CMV vaccines are in various stages of preclinical and clinical testing and include: protein subunit vaccines, DNA vaccines, vectored vaccines using viral vectors such as attenuated pox- and alphaviruses, peptide vaccines, and live attenuated vaccines (Schleiss and Heineman, 2005).

3 Generation of recombinant cytomegalovirus

Functional characterization of viral genes, by generating virus mutants and investigating the resulting changes in phenotype, is important for understanding the molecular aspects of herpesvirus replication and pathogenesis, and to provide a basis for the development of new vaccines and chemotherapeutics.

3.1 Methods of mutagenesis used before bacterial artificial chromosome technology

3.1.1 Chemical mutagenesis in infected cells

Genetic alterations, which result in phenotypic alterations, are of great importance to gain insight into the functional role of viral genes. The classical genetic approach using mutagenic chemicals to introduce random mutations was applied 30 years ago and allowed the production of a large number of mutants that can be screened for interesting phenotypes (Fig. A 5). Characterization of conditional alleles, such as temperature-sensitive (ts) mutants (Schaffer, 1975), was favored because operational viability of the virus of interest is a prerequisite for analysis. Generating such mutants is straightforward, but the most demanding and difficult step of this forward genetic approach is mapping the mutations and excluding second-site mutations.

3.1.2 Site-directed mutagenesis by homologous recombination in CMV-infected cells

In the early 1980s, a method for reverse genetics by the targeted mutation of individual genes was developed for HSV (Mocarski et al., 1980, Post and Roizman, 1981) and lat-

er adopted for other members of the herpesvirus family (Manning and Mocarski; 1988; Spaete and Mocarski, 1987). In this method, the mutation together with a marker gene is introduced into the target sequence by the cellular recombination machinery (Fig. A 5).

As the wild-type virus is over-represented in the resulting virus pool, selection procedures are required. This step is laborious and, if the mutant has a growth deficit, isolating the recombinant virus can be difficult. Null mutants of genes that are indispensable for viral growth (essential genes) can only be produced on complementing cell lines, that provide the essential gene product *in trans* (DeLuca et al., 1985).

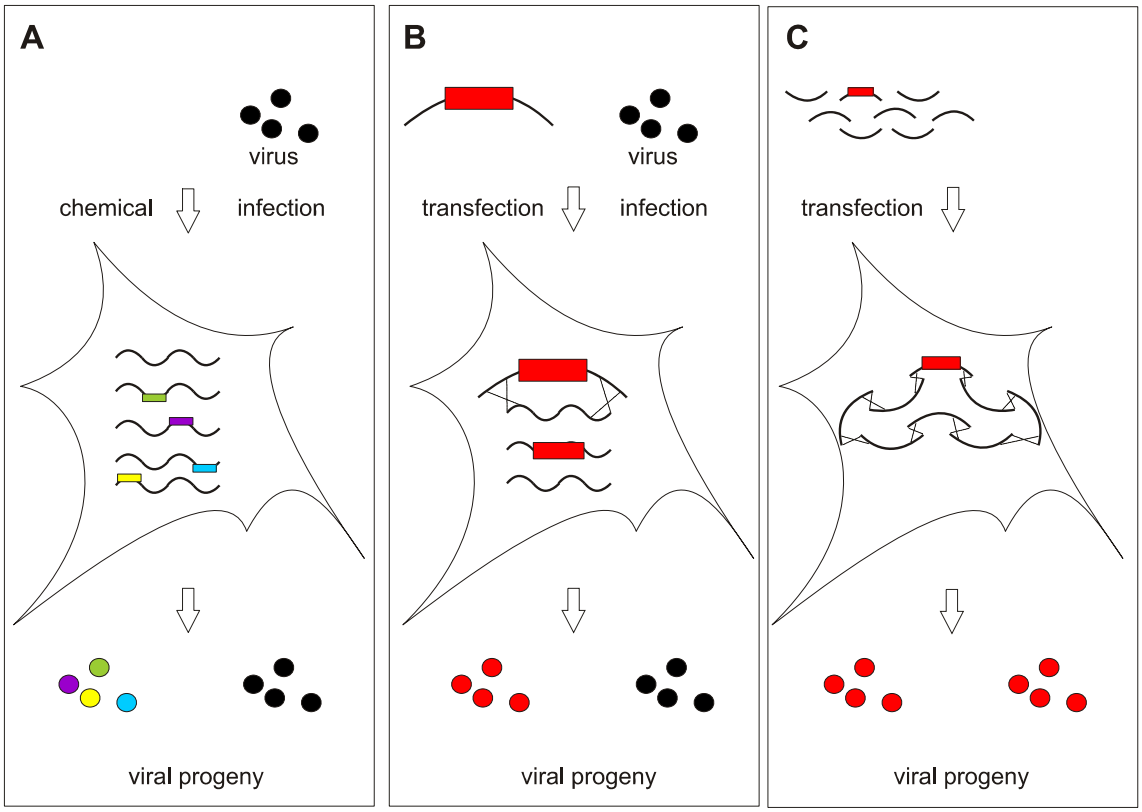

Fig. A 5 Methods of herpesvirus mutagenesis before BAC technology. (A) Chemical mutagenesis. Mutagenic chemicals introduce large number of random mutations. **(B)** Site-directed mutagenesis in eukaryotic cells. A linear DNA fragment that contains the mutation, a marker gene (red box) and flanking homologies to the viral target sequence is transfected into infected cells. The marker gene is inserted into the viral genome by homologous recombination (dashed lines). Recombinant and wt virus need further separation. **(C)** Production of infectious virus from overlapping cosmids. Overlapping fragments spanning the entire viral genome are linearized and transfected into permissive cells, in which the viral genome is reassembled by homologous recombination and viral progeny is produced.

3.1.3 Regenerating virus from overlapping cosmid clones

In the late 1980s, a novel approach was introduced for mutating herpesvirus genomes (van Zijl et al., 1988). The large viral genomes were subcloned in *E. coli* as a set of overlapping clones. Mutations were introduced into one of the subcloned fragments. After cotransfection of cells with the cosmid set, infectious virus can be reconstituted by multiple homologous recombinations via the overlapping sequences (Fig. A 5). Because only the mutant genome is reconstituted using this method, the introduction of markers and selection against wild-type virus is not required. Limitations of this system include the difficulty of finding suitable restriction sites in large cosmids and of mutating regions of cosmid overlap. In addition, because many recombination events are needed to reassemble the full-length genome in the target cell and because recombination procedures in cells cannot be controlled, mutagenesis is often associated with unwanted mutations. Extensive post-mutagenesis analysis of the isolated mutants is therefore required. Finally, it is not easy to construct revertants, which are needed to prove that the altered property is a function of the targeted mutation and not of an unwanted mutation elsewhere in the genome, with the cosmid technology.

3.2 Cytomegalovirus mutagenesis with bacterial artificial chromosomes in *E. coli*

Until recently, mutagenesis of the large CMV genomes was difficult because most mutagenesis procedures were performed in eukaryotic cells and recombinant virus was only obtained after these poorly controlled recombination events occurred. Production of null mutants was not possible. The genetic analysis of the mutant virus for illegitimate mutations could only start after the virus had been reconstituted and isolated, which could take several months.

3.2.1 Cloning herpesvirus genomes as bacterial artificial chromosomes

Cloning a complete herpesvirus genome as a single plasmid was difficult as the size of the herpesvirus genome exceeds the cloning capacity of conventional plasmids and cosmids. Yeast artificial chromosomes (YACs) were the first cloning vectors with a coding capacity of up to 2 Mbp. However, YACs were not suitable for cloning the herpesvirus genome. They are not easy to handle and they cause deletions and frequent spontaneous rearrangements between the repetitive sequences contained in herpesvirus

genomes (Ramsay, 1994). To simplify and accelerate mutagenesis of herpesvirus genomes, Messerle and colleagues (Messerle et al., 1997) chose a completely new approach. They used F-factor-derived bacterial artificial chromosomes (BACs), which can maintain foreign DNA of up to ~ 300 kb in *E. coli*, to clone the MCMV genome. In contrast to YACs or cosmids, BACs showed surprising stability (Shizuya et al., 1992). The strict control of the F-factor replicon maintains a single copy of the BAC per bacterial cell, reducing the risk of recombination events via repetitive DNA elements present in herpesvirus genomes. To clone the herpesvirus genome as a BAC, MCMV infected cells were transfected with a linearized BAC, flanked by sequences homologous to the desired insertion site into the viral genome. The sequences for the BAC vector are thus introduced into the herpesvirus genome by conventional homologous recombination into infected cells. The linear double-stranded DNA genome circularizes during replication. By isolating the circular replication intermediate of the BAC mutant and transfecting it into *E. coli*, the herpesvirus BAC can then be propagated and mutated in *E. coli*. Because inserting the BAC cassette resulted in an overlength of the MCMV genomes (8 kbp) that are not well packaged, spontaneous deletions occurred within the MCMV-BAC genomes. To obtain a stable BAC-clone, 8 kbp of non essential sequences had to be deleted. Once in *E. coli*, the deleted viral sequences were reintroduced into the viral BAC-genome in a way that allows excision of the BAC cassette upon virus reconstitution (Wagner et al., 1999). Methods of prokaryotic genome engineering can now be used and adapted to mutate the virus genome. Infectious virus is simply reconstituted by transfecting the mutated viral BAC into permissive eukaryotic cells. In the last years, the strategy of cloning an entire viral genome as a BAC has been adopted for the Epstein-Barr virus, HSV, pseudorabies virus, human CMV, and others (Adler et al., 2000; Borst et al., 1999; Delecluse et al., 1998; Horsburgh et al., 1999; McGregor et al., 2001; Rudolph et al., 2002; Schuhmacher et al., 2000; Smith and Enquist 1999). BAC-technology has been employed in forward and reverse genetics.

3.2.2 Random mutagenesis of cytomegalovirus BACs with transposons

BAC-technology has been applied to transposon mutagenesis. Transposons are mobile genetic elements that insert randomly into the genome (Berg et al., 1998). Using transposons that insert preferably into plasmid DNA (BAC DNA), a forward genetics proce-

dure was developed (Brune et al., 1999) for constructing transposon libraries (Fig. A 6). After reconstitution of the mutant virus, genes of interest can be identified based on the resulting phenotype. This is a fast and straightforward method to identify essential and nonessential genes.

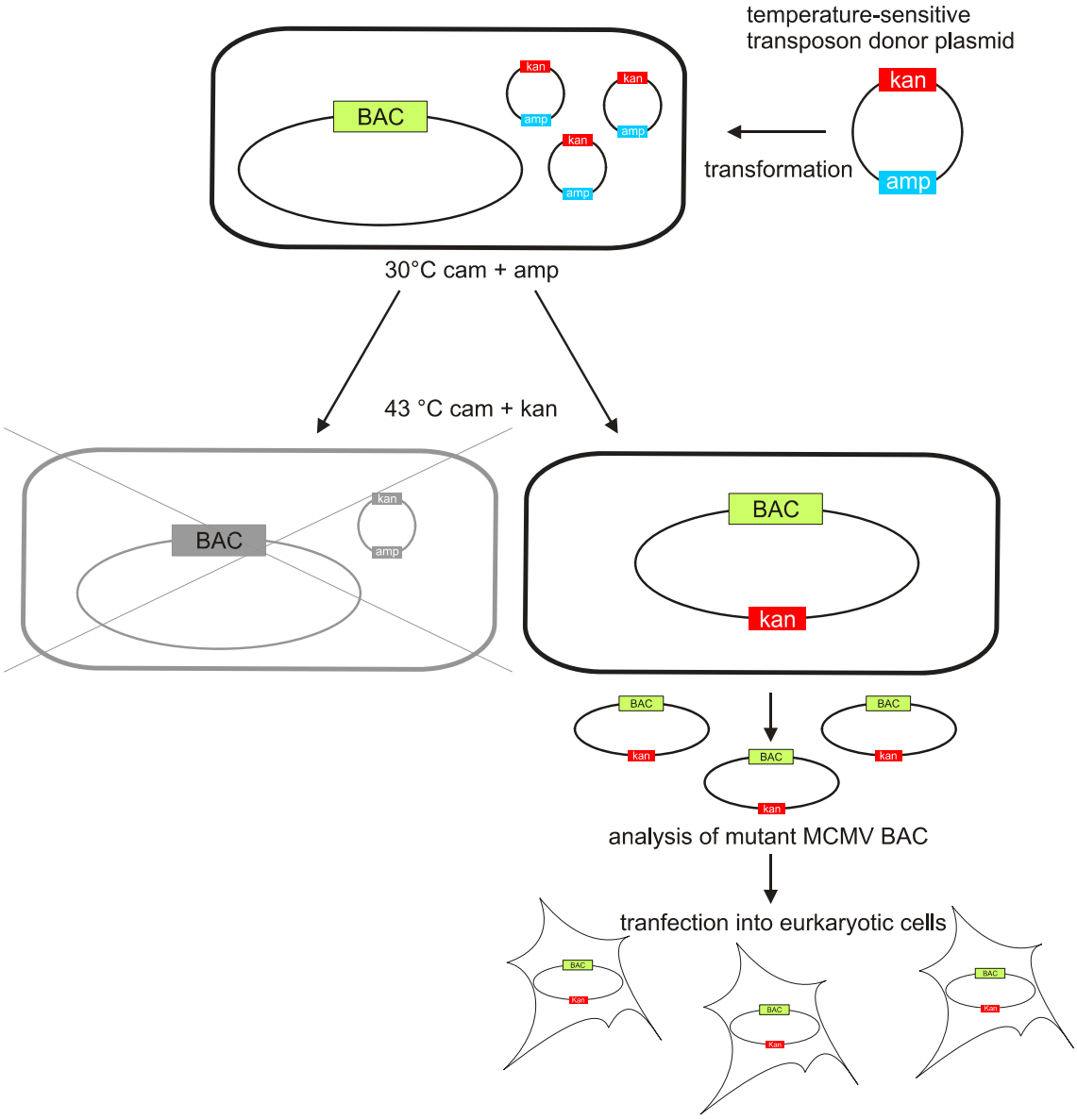

Fig. A 6 Random transposon mutagenesis of MCMV BAC plasmids. The MCMV BAC and the transposon donor plasmid are maintained in *E. coli* at 30°C. The BAC is selected for by chloramphenicol (cam), and the transposon is selected for by ampicillin (amp). To select for transposition events, bacteria are plated on agar plates and incubated at 42°C selecting with cam and kanamycin (kan, red box). The Tn donor plasmid does not replicate at 42 °C, only those bacteria in which a transposition event occurred were able to form colonies. BAC DNA

extracted from bacteria can be analyzed by restriction enzyme digestion and direct sequencing. Finally, mutant virus is reconstituted by transfection of mutated genomes into fibroblasts.

3.2.3 Allelic exchange using linear DNA fragments

In 1998, Stewart and colleagues described a one-step mutagenesis method named ET cloning (Zhang et al., 1998). This method uses the recombination functions of recombination proteins *recET* from prophage Rac or *redαβ* from bacteriophage λ to introduce mutations into a circular DNA molecule by DNA fragments (Muyrers et al., 2000; Zhang et al., 1998). Recently, our group adapted this method to introduce mutations into viral BACs (Wagner et al., 2003; Wagner et al., 2002a). A linear fragment, containing a selectable marker and homologous sequences to the target sequence, is transferred into recombination-proficient *E. coli* with *redαβ* under the control of an inducible promoter (Fig. A 7). To circumvent degradation of the linear DNA, exonuclease-negative bacteria must be used or the exonuclease inhibitor redγ (*gam*) from bacteriophage λ must be expressed. The selectable marker is introduced along with the mutation by a double cross-over event. This one-step recombination method has several advantages compared to the *RecA*-mediated two-step recombination with shuttle plasmids (see Wagner et al., 1999). The recombination functions used allow homologous recombination between homologies as short as 25-50 nucleotides. These sequences can be provided by the synthetic oligonucleotides used to amplify the linear DNA fragment and therefore no cloning is required. The mutagenesis is independent of the presence of restriction enzyme recognition sites and the homology sequences can be chosen freely. This method allows the deletion of sequences starting from very few bps (such as an ATG) to up to several hundreds of kbps within a few days. In addition, sequences with short tags or large genes can be introduced. The drawbacks of the method are the potential instability of the viral BAC and the need to insert a selectable marker along with the mutation. But the marker gene can be removed after mutagenesis with flanking FLP recognition target (FRT) (Macleod et al., 1986) or *loxP* (locus of crossing over P1 phage) sites. These sites allow excision of the marker by expression of the site-specific recombinases FLP (Cherepanov et al., 1995) or Cre, leaving only 48 or 34 nucleotides of bacterial sequence at the mutation site.

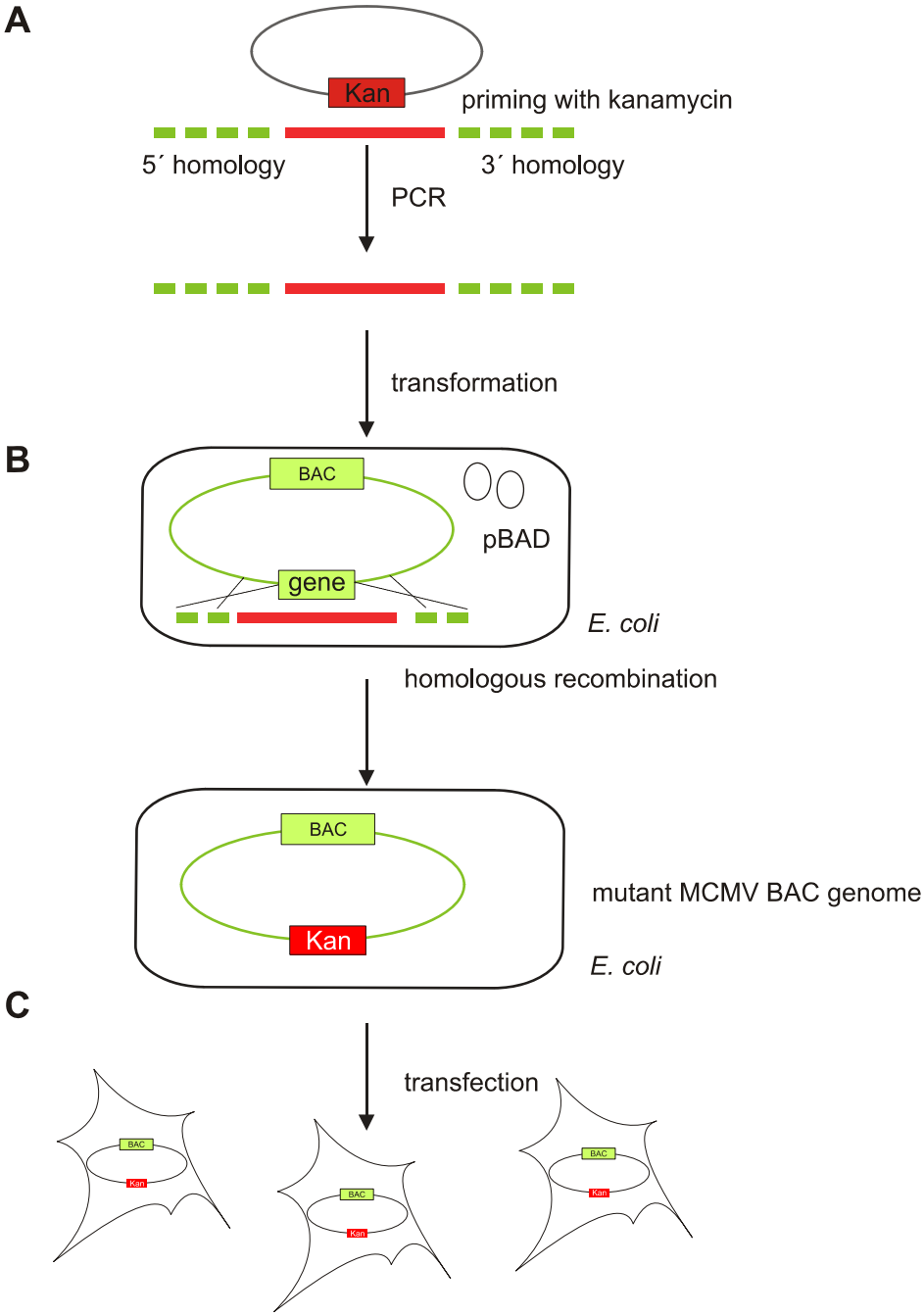

Fig. A 7 MCMV BAC mutagenesis. (A) A contiguous primer pair is used for PCR amplification of a selectable marker gene (red box). The primers contain homologies to the viral sequence at their 5´- ends (dashed lines) and priming regions to the selectable marker. **(B)** The linear PCR fragment is transformed into *E. coli* with the MCMV BAC genome and the plasmid pBADαβγ that expresses recombinases redα, redβ and exonuclease inhibitor redγ. **(C)** Transfection of the mutated MCMV BAC plasmid into permissive cells leads to reconstitution of recombinant virus only. The BAC sequence is efficiently removed by homologous recombination. Thus, the MCMV vector genome is devoid of any residual bacterial sequence.

4 Murine cytomegalovirus as a model for human cytomegalovirus infection

4.1 Murine cytomegalovirus infection

HCMV infects only humans and thus cannot be studied in animals. Murine, guinea pig, rat, and rhesus macaque CMVs have been employed as surrogate models of human CMV infection, latency and disease, providing insights into CMV pathogenesis. Among these, MCMV is the best analyzed and mostly used model system since MCMV and HCMV share great homology and a similar course of infection (Hudson, 1979). As in HCMV infection, infection of immunocompetent mice with MCMV is controlled by the immune system and leads to latent infection. Infection of immunosuppressed mice leads to life-threatening disease with interstitial pneumonia, hepatitis, and inflammation of the adrenal glands (Brody et al., 1974; Mayo et al., 1977; Sutherland et al., 2001). Furthermore, it has been shown that MCMV can be reactivated from latency through special reactivation protocols (Hamilton and Seaworth, 1985; Jordan et al., 1978; Reddehase et al., 1994). By generating MCMV mutants with functional deletions of individual genes and investigating them in cell culture and the mouse, it is possible to identify new gene functions which are important for viral pathogenesis of MCMV and possibly HMCV infection.

4.2 Function of open reading frames in human and murine cytomegalovirus

Despite the fact that members of the β-herpesviruses have significantly diverged in different animal species, as well as within the same animal species, significant amino acid sequence homology in approximately 80 ORFs in the central region of the β-herpesvirus genomes can be found. Similarly, amino acid sequence homology in as many as 46 herpesvirus-common ORFs controlling DNA replication, genome packaging, and virion morphogenesis, shows that β-herpesviruses are related to all mammalian herpesviruses (Chee et al., 1990). The right and left termini of the MCMV genome contain multiple genes with mainly nonessential and unknown functions.

CMVs have a high coding capacity compared to other viruses. Their genome comprises approximately 230 kb and represents the largest mammalian virus genome. Several herpesviruses genomes have been sequenced (Chee et al., 1990; Gompels et al., 1995; Nicholas, 1996; Rawlinson et al., 1996; Vink et al., 2000), and after sequencing

of the HCMV laboratory strain AD169, 208 potential ORFs were predicted to encode polypeptides containing more than 100 amino acids (Chee et al., 1990). In the original and less passaged HCMV strains Toledo and Towne, 22 additional ORFs were identified, which were lost during the frequent passages of the laboratory strain AD169 (Cha et al., 1996). Sequencing of the MCMV Smith strain resulted in 170 potential ORFs (Rawlinson et al., 1996).

The function of the majority of the CMV genes is still unknown. Altogether, about 49% of the MCMV ORFs share homology to other known proteins, and between MCMV and HCMV 80 genes share significant homology to each other (Rawlinson et al., 1996). Among these 80 genes, 46 genes are conserved in all herpesviruses (Chee et al., 1990). Some of the CMV genes probably arise from gene duplication (Chee et al., 1990; Mocarski et al., 2001; Rawlinson et al., 1996). A specific function was experimentally proven for about 26% of the ORFs in HCMV and 25% in MCMV. For many genes it is not known whether they are essential or nonessential for virus replication in cell culture or *in vivo*. Essentiality has been proven for approximately 35% of the genes in HCMV and 36% in MCMV.

Because of the complex interplay between the CMVs with their host, it is likely that only a minority of the many genes are required for virus morphogenesis, whereas the others are committed to evade the host immune response, optimize viral growth, and promote viral dissemination and latency. Constructing single gene knock-out mutants and analyzing them *in vitro* and *in vivo* compared to wild-type-MCMV are important goals for identifying new gene functions and understanding the biology of CMVs (Wagner et al., 2002b).

4.3 Gene families in human and murine cytomegalovirus

HCMV and MCMV contain families of ORFs that result from gene duplication events during evolution that are present in all β-herpesviruses (Chee et al., 1990; Mocarski et al., 2001; Rawlinson et al., 1996). HCMV encodes nine gene families, with the six largest clustered at each end of the genome. MCMV has four gene family homologs in HCMV. Two families are unique to MCMV. MCMV sequence has revealed homologs to the HCMV gene families US22, UL25, UL 82 and GCRs. Not much is known about the function of these gene families. Given the conservation of gene families in β-

herpesviruses it seems reasonable to assume that their functions are important in the infection of the natural host and that gene duplication may account for the larger size of β-herpesviruses (Weston and Barrell, 1986).

Tab. A 1 US 22 gene family homologs in MCMV and HMCV.

HCMV ORF	MCMV homolog	Gene products in HCMV and MCMV
UL 23	M23 m25.1	HCMV: tegument
UL 24	M24	HCMV: tegument
UL 28	no homolog	unknown function
UL 29	no homolog	unknown function
UL 36 (Exon 1) UL 36 (Exon 2)	M36 (Exon 1) M36 (Exon 2)	HCMV: tegument, inhibitor of apoptosis
UL 43	M43	HCMV: tegument, cell tropism
US 22	m25.2 m128 m139	unknown function HCMV: tegument, immediate early gene MCMV: macrophage cell tropism
US 23	m140 m143	MCMV: macrophage cell tropism MCMV: essential for viral replication
US 24	m141	MCMV: macrophage cell tropism
US 26	m142	MCMV: essential for viral replication
TRS 1	no homolog	HCMV: tegument, transcriptional transactivator

The US22 gene family comprises twelve members in both HCMV and MCMV (see (Tab. A 1) and eleven in rat CMV. The product of the HCMV US22 gene is a nuclear protein which is expressed at early and late times (Mocarski et al., 1988). Members of the US22 gene family are characterized by stretches of hydrophobic and charged residues, as well as up to four conserved sequence motifs which are specific for β-herpesviruses (Hanson et al., 1999b; Kouzarides et al., 1988). The functions of most of the US22 genes are unknown. For some of these, TRS1/IRS1 and UL36 of HCMV, and m128 of MCMV, a transcriptional transactivation function was described (Cardin et al., 1995; Colberg, 1996; Colberg et al., 1992; Iskenderian et al., 1996; Romanowski and

Shenk, 1997). The ie2 gene product of m128 has been shown to be dispensable for growth of MCMV *in vitro* and *in vivo* (Cardin et al., 1995). The report on the combined failure of macrophage growth *in vitro* and altered tissue type distribution *in vivo* of mutants RV7 (encompassing genes m137 through m141) and RV10 (encompassing US22 family genes m139 to m141) (Cavanaugh et al., 1996; Hanson et al., 1999) prompted us to study the US22 gene family members for cell tropism and to analyze their growth in macrophages.

5 Goal of this thesis

To test the role of all US22 members in cell tropism, Menard et al. (2003) analyzed the growth properties of MCMV mutants carrying transposon insertions in all 12 US22 gene family homologs in different cell types. Growth in macrophages of mutants with insertions in genes m139, m140, and m141 was severely affected while mutants of genes m142 and m143 did not show viral growth. These results indicated that mutant genes m139, m140 and/or m141 can confer macrophage tropism and that gene m142 and/or m143 is essential for viral growth. Genes m139 to m143 represent a complex transcriptional unit (Hanson et al., 1999b). Since genes m139 to m141 and genes m142 to m143 are transcribed collinearly, a transposable unit introduced into a gene upstream of this unit could influence the transcription or transcript stability of the downstream gene or genes. The goal of this thesis was to construct and to analyze additional targeted mutants of the genes m139, m140 and m142 in order to exclude polar effects on the downstream genes and to define the gene functions (cell tropism, essentiality) attributable to genes m139, m140 and m142.

5.1 Generation of US 22 gene homolog mutants m139, m140, and m142 in MCMV

Mutagenesis of viral genes is the basic strategy to investigate the function of the herpesvirus ORFs. Due to the large genome size and the slow replication kinetics in vitro of CMV, mutagenesis of viral genes in the context of the viral genome by conventional recombination methods in cell culture has been difficult. That is why the recently developed BAC-technology was employed. This technique allows the maintenance of viral genomes as a plasmid in *E. coli* and the reconstitution of viral progeny by transfection of the BAC plasmid into eukaryotic cells. Using prokaryotic recombination pro-

teins in *E. coli* it is possible to introduce any genetic modification of the viral genome. This allows the generation of mutant viruses and facilitates the analysis of herpesvirus genomes cloned as infectious BACs.

Targeted mutants were constructed by site-directed BAC mutagenesis of the MCMV BAC to analyze the gene functions of ORF m139, m140 and m142. In order to minimize possible effects on transcript or protein stability the introduced mutation was restricted to the start codon or at most to the second ATG, resulting in the deletion of a few base pairs.

5.2 Characterization of MCMV mutants m139, m140 and m142

Once recovered, mutants of the US22 gene family were analyzed for replication in fibroblasts and macrophages. Macrophages play a major role in the pathogenesis of CMV infection. They presumably initiate early antiviral immune response (Heise et al., 1998; Orange and Biron, 1996), but have also been shown to serve as target cells in infected organs, as disseminators of the virus throughout the host and to harbor latent MCMV DNA (Michelson, 1997; Pollock et al., 1997). Because they play a key role in CMV dissemination as well as in tissue tropism and latency, viral gene products, which regulate growth in this cell type, are of great interest and are predicted to significantly affect MCMV pathogenesis *in vivo*. Analysis of the proteins expressed from ORF m139, which contributes to the common macrophage phenotype of mutants of m139, m140, and m141, was performed.

Transposon mutants of genes m142 and m143, also homologs to the HCMV US 22 gene family, were shown to be essential for viral replication. By definition essential genes are imperative for replication of CMV. The insertion of a transposon into an open reading frame usually results in truncation or functional inactivation of the corresponding gene product. Due to the complex transcriptional unit of genes m142 and m143 and the possible polar effects of the 3-kb transposable unit on the neighboring genes, it was not possible to attribute essentiality to m143 and m142. That is why a targeted mutant of gene m142 needed to be constructed and analyzed for potential viral growth in cell culture. In case of absent viral growth, revertants were constructed.

B. MATERIALS AND METHODS

1 Materials

1.1 Reagents

acrylamide/bisacrylamide (ProtoGel)	National Diagnostics, USA
agar	Life Technologies, Germany
agarose	Biozym, Germany
ammonium persulphate (APS)	Bio-Rad, USA
ampicillin	Roche, Germany
arabinose	Roth, Germany
bacto yeast and tryptone	Life Technologies, Germany
BCA protein assay kit	Pierce, USA
bromphenol blue	Serva, Germany
carboxymethylcellulose	Sigma, Germany
chloramphenicol	Life Technologies, Germany
dimethylsulfoxide (DMSO)	Serva, Germany
DNA purification kit	Qiagen, Germany
DNA-ladder (marker III)	Roche, Germany
dNTPs	Roche, Germany
Dulbecco´s modified Eagle´s medium (DMEM)	Gibco, USA
ECL Western blotting detection reagents kit	Roche, Germany
ethanol	Riedel-de Haen, Germany
ethylenediamintetraacetate sodium acid (EDTA)	Roth, Germany
Expand high fidelity PCR system polymerase	Roche, Germany
fetal calf serum (FCS)	PAA, Germany
films (BioMax MR)	Kodak, USA
GFX microplasmid prep kit	Amersham, England
GFX PCR DNA and gel band purification	Amersham, England
glycerol	Roth, Germany
glycine	Serva, Germany
HEPES	Roth, Germany

high-copy plasmid kit	Amersham, England
isopropanol	Riedel-de Haen, Germany
Klenow polymerase	New England Biolabs, USA
methylcellulose	Fluka, Schweiz
10 x modified eagle medium	Life Technologies, Germany
newborn calf serum (NCS)	PAA, Germany
N,N-methylenbisacrylamide	Roth, Germany
Nucleobond BAC-DNA purification kit)	Machery & Nagel, Germany
penicillin	Life Technologies, Germany
phenol/chloroform	Roth, Germany
phosphate buffered saline (PBS)	Gibco, Germany
potassium acetate	Riedel-de Haen, Germany
potassium chloride	Riedel-de Haen, Germany
protein kd ladder	Bio-Rad, USA
proteinase K	Roche, Germany
QiaEx gel purification kit	Qiagen, Germany
Qiaprep spin plasmid miniprep kit	Qiagen, Germany
Qiaprep spin plasmid maxiprep kit	Qiagen, Germany
QiaQiuck PCR purification kit	Qiagen, Germany
rapid ligation kit	Roche, Germany
restriction enzymes	Roche, Germany and NEB, USA
RNAse A	Roche, Germany
RPMI 1640	Life Technologies, Germany
sodium dodecyl sulphate (SDS)	National Diagnostics, USA
shrimp alkaline phosphatise (SAP)	Roche, Germany
sodium acetate	Riedel-de Haen, Germany
sodium hydroxide	J.T. Baker B.V., Holland
Superfect reagent	Qiagen, Germany
T4-DNA ligase	Roche, Germany
Taq-DNA polymerase	Roche, Germany
trypsin	Life Technologies, Germany
tryptone	Difco, Germany
Western blue	Promega, USA

zeocin	Invitrogen, USA

All other chemicals were purchased from Merck (Darmstadt) und Sigma (Deisenhofen).

1.2 Bacteria

DH10B (recA-)	Life Technologies, Germany
DH5-α	Life Technologies, Germany

1.3 Plasmids

Commercially available plasmids

pUC19	New England Biolabs, USA
pCR3	Invitrogen, Germany
pEGFP-C1	Clontech, USA

Plasmids from cooperating institutions

pBADαβγ	Zhang et al., 1998
pCP20	Cherepanov et al., 1995
pSLFRTkan	Atalay et al., 2002
pSM3fr	Messerle et al., 1997

Plasmids from our institution

pSM3fr/GFP	Mathys, S., LMU Munich, unpublished
pUC*Hin*dIII-I	Menard, C., LMU Munich
pORI6kZeo	Bubeck, A., LMU Munich

Generation of plasmids

a) High-copy plasmids

plasmid pORI6kZeo-m142

Fragment *Hin*dIII-I was used as template DNA in order to amplify ORF m142 by PCR with primers 200748 up-m142-Eco and 199441down-m142-Xba. The amplified PCR fragment was inserted into the EcoRI and XbaI restriction sites of the multicloning site of plasmid pCR3. Digestion with restriction enzyme DrdI resulted

in a 2.6 kb fragment with the wild-type m142 ORF under the control of the human CMV immediate-early promoter-enhancer and a polyadenylation site. The fragment was treated with the blunt cutter *Eco*RV and inserted into plasmid pORI6kZeo with the zeocin resistance gene and a 48-bp FRT site. Thus, plasmid pORI6kZeo-m142 contains the wild-type m142 ORF under the control of the human CMV immediate-early promoter-enhancer, a conditional origin of replication, a zeocin resistance gene and a 48-bp FRT site.

b) *MCMV BAC plasmids*

MCMV plasmids were generated by site-directed mutagenesis in *E. coli*. The technique is based on homologous recombination of a linear PCR fragment flanked by 45 to 50-nucleotide sequences homologous to the up- and downstream regions of the target gene sequence of the MCMV BAC plasmid pSM3fr or pSMfr/GFP.

BAC plasmid pΔATG-m139 (parental MCMV BAC: pSM3fr)

For the deletion of the first two ATGs of ORF m139, the PCR fragment m139-PCR that contains the kanamycin cassette flanked by 34-bp minimal FLP recombinase recognition target (FRT) sites was generated using plasmid pSLFRTkan (Atalay et al., 2002) as the DNA template. For PCR the following primers were used: primer 5´-ΔATG-m139 (5´ - GGG GGA AGG CTC CTC TCG TCC ACG CCG CCG TAT TCT CCG AAC TTC TGG TCC GTC GTG GAA TGC CTT CGA ATT - 3´) and primer 3´-ΔATG-m139 (5´ - CGT GAG TTG ACG GCG CCG GCG CCA GAC GGA GCA GAC AGA GAG AGA GAA GGA CAA GGA CGA CGA CGA CAA GTA A - 3´). *E.coli* with the parental MCMV BAC plasmid pSM3fr were used for the generation of plasmid pΔATG-m139. Subsequently, the kanamycin resistance cassette, flanked by the minimal FRT sites, was excised by the FLP recombinase. The first two ATGs of ORF m139 (84 bp apart) were replaced with an 86-bp extraneous sequence.

BAC plasmid pΔATG-m142 (parental MCMV BAC: pSM3fr)

For the deletion of the first ATG of ORF m142, the PCR fragment m142-PCR that contains the kanamycin cassette flanked by 34-bp minimal FRT sites was generated using plasmid pSLFRTkan (Atalay et al., 2002) as the DNA template. For PCR the

following primers were used: primer 5´-ΔATG-m142 (5´ - CTG GTC TCT GAA GTG ATC CGA TCG GAT CGC CGC GCA CAG GGC GTC CGT CGT GGA ATG CCT TCG AAT TC - 3´) and primer 3´-ΔATG-m142 (5´ - CCA CCC TTC TCC ACC CGT GTT CCC GCT GCC GCC CGT CGC CCT CGC CAC AAG GAC GAC GAC GAC AAG TAA - 3´). *E.coli* with the parental MCMV BAC plasmid pSM3fr were used for the generation of plasmid pΔATG-m142. The kanamycin resistance cassette, flanked by the minimal FRT sites, was excised by FLP recombinase. The native ATG of ORF m142 was deleted and replaced with an 86-bp extraneous sequence.

BAC plasmid pΔATG-m142/FRT (parental MCMV BAC: pSM3fr/GFP)

The pSM3fr BAC, that contains a FRT site and the GFP gene in position of the nonessential gene m152 (pSM3fr/GFP), was first subjected to site-directed mutagenesis to delete the native ATG of ORF m142 with the same PCR fragment used for the generation of the ΔATG-m142 mutant genome. The kanamycin resistance cassette was excised by FLP recombinase. This generated plasmid ΔATG-m142/FRT

BAC plasmid pΔATG-m142/m142E (parental MCMV BAC: pSM3fr/GFP)

Plasmid pORI6kZeo-m142 with a conditional origin of replication, a zeocin resistance gene, a 48-bp FRT site and the wild-type m142 ORF under control of the human CMV immediate-early promoter-enhancer from plasmid pEGFP-C1 (Clontech, USA) was inserted into the FRT site of ΔATG-m142/FRT. This generated plasmid ΔATG-m142/m142E.

BAC plasmid pSM3fr/GFP

The m152 gene of pSM3fr was deleted and replaced by the GFP gene under the control of the HCMV promotor from the pEGFP-C1 plasmid and a FRT sequence. This plasmid was constructed by Sybille Mathys.

1.4 Antibodies

Primary antibodies

MCMV m139 rabbit polyclonal antiserum	Hanson et al., 2001
rabbit polyclonal m140* antibodies	Eurogentec, Belgium
rabbit polyclonal m141* antibodies	Eurogentec, Belgium

*15-amino-acid peptides from the C terminus (SVLTTRPDRNRDTRT, amino acid position 431 to 446) and the N terminus (ATGGDQNARRRAIER, amino acid position 25 to 40) were used to generate rabbit polyclonal antibodies for the m140 and m141 proteins.

Secondary antibodies

Pox conjugated goat anti-rabbit antiserum	Eurogentec, Belgium

1.5 Cells and viruses

Cells

IC-21*	simian virus 40-transformed murine macrophages from C57BL/6-mice (ATCC TIB-186)
MEF	murine embryo fibroblasts from BALB/c-mice, prepared according to Wagner et al.(2002), see chapter B. 2.11
M210B4*	b1 marrow stromal line (ATCC CRL-1972)
NIH3T3*	murine fibroblasts from BALB/c-mice (ATCC CRL-1658)

*purchased from the American Type Culture Collection (ATCC).

Viruses

M97.01	derived from plasmid pSM3fr (Messerle et al., 1997)
M152/GFP-MCMV	derived from plasmid pSM3fr/GFP (Sybille Mathys)
Tn-m152	Menard et al., 2003
Δ-m139	Menard et al., 2003
ΔATG-m139-MCMV	MCMV with deletion of the first 2 ATGs of m139
ΔATG-m142-MCMV	MCMV with deletion of the first ATG of m142
ΔATG-m142/FRT-MCMV	MCMV with deletion of the first ATG of m142
ΔATG-m142/m142E-MCMV	MCMV revertant for ORF m142

1.6 Oligonucleotides

Oligonucleotides purchased from Metabion (Germany) were used for cloning, mutagenesis and sequencing. Oligonucleotides longer than 50 base pairs were purified by HPLC. The sequences of the oligonucleotides are listed in Tab. B 1.

Tab. B 1 Sequences of oligonucleotides.

oligonucleotide	sequence	nt
5´-m140-nt-197427	5´ - GCT CCC CGC GGT TCG ACT GGT GGC GGT AGA AGT CGC GGA ACG CGA GCG TCG TGG AAT GCC TTC GAA TTC - 3´	69
3´-m140-nt-197517	5´ - CCC GTT TTT CTC TAG ACC ATT ATC ACC ATC ATC TCA TCC GGC GAG ACA AGG ACG ACG ACG ACA AGT AA - 3´	68
3´-m140-nt-197477	5´ - GAT CCG GCA CCC TAT CGG CAC GCC ACC CGC TCA TCG TTT CTC CGC GTG ACA AGG ACG ACG ACG ACA AGT AA - 3´	71
5´-ΔATG-m139	5´ - GGG GGA AGG CTC CTC TCG TCC ACG CCG CCG TAT TCT CCG AAC TTC TGG TCC GTC GTG GAA TGC CTT CGA ATT - 3´	72
3´-ΔATG-m139	5´ - CGT GAG TTG ACG GCG CCG GCG CCA GAC GGA GCA GAC AGA GAG AGA GAA GGA CAA GGA CGA CGA CGA CAA GTA A - 3´	73
5´-ΔATG-m142	5´ - CTG GTC TCT GAA GTG ATC CGA TCG GAT CGC CGC GCA CAG GGC GTC CGT CGT GGA ATG CCT TCG AAT TC - 3´	68
3´-ΔATG-m142	5´ - CCA CCC TTC TCC ACC CGT GTT CCC GCT GCC GCC CGT CGC CCT CGC CAC AAG GAC GAC GAC GAC AAG TAA - 3´	69
5´-m139-nt 195739	5´ - CGC GAT CTA TGA ACA GGT CAG AC - 3´	23
3´-m139-nt-196200	5´ - CAA CCG AGA CAC CCG AAC CCT - 3´	21
200748up-m142-Eco	5´- GTG AAT TCA TGG ACG CCC TGT GCG CGG-3´	28
199441down-m142-Xba	5´- CTT CTA GAT CAG TCG TCA TCG TCG GCG TC-3´	29
m142-199541 up	5´-ACG TCG AGG TGG CGG ACG CGT-3´	21

2 Methods

The following methods were carried out according to the procedures in Sambrook et al. (1989) if not stated otherwise. The standard protocols were used with the purchased kits. The components of the required solutions are shown below in boxes.

2.1 Isolation und purification of nucleic acids

2.1.1 Analytical isolation of plasmid DNA from bacteria (mini preparation of plasmid DNA)

We used the GFX Micro Plasmid Prep Kit from Amersham to extract plasmid DNA. 1.5 ml of Luria Bertani (LB) medium with the appropriate antibiotics were inoculated with the bacterial colony and incubated overnight at 37°C or at 30°C for temperature-sensitive plasmids. 1.5 ml of the overnight culture was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 14,000 rpm for 30 s to pellet the cells. The pellet was completely resuspended with vigorous vortexing in 150 µl of solution I (25 mM Tris/HCl and 10 mM EDTA, pH 8.0), an isotonic solution that contains RNase, after removal of the supernatant. For alkaline lysis and denaturation of chromosomal DNA and proteins 150 µl of solution II (0.2 M NaOH, 1% SDS) was added for a few minutes and mixed by inverting the tubes 10-15 times. 300 µl of solution III (3 M potassium acetate, pH 4.8) was added and mixed by inverting the tube until a flocculent precipitate appeared and was evenly dispersed. After centrifugation at 14,000 rpm for 5 min to pellet the cell debris the supernatant was loaded onto a glass matrix column, incubated for 1 min and then centrifuged at full speed for 30 s. The flow-through was discarded and 400 µl of washing buffer (Tris-EDTA buffer) was added to the column. The column was centrifuged at 14,000 rpm to remove the buffer and dry the matrix and incubated for 1 min at room temperature. The plasmid DNA was eluted with 100 µl of distilled water by centrifugation at 14,000 rpm for 1 minute. Yields are typically 3-6 µg of plasmid DNA/ml of culture.

LB medium	10 g bacto tryptone
	5 g yeast extract
	5 g sodium chloride
	dissolve in 1 l water

2.1.2 Quantitative isolation of plasmid DNA from bacteria (maxi preparation of plasmid DNA)

The QIAGEN Plasmid Maxiprep Kit from Qiagen was used for the preparation of up to 500 µg of high-copy plasmids. 100 ml of LB medium was inoculated with 100 µl of a bacterial starter culture and was then incubated overnight at 37°C or 30°C. The bacterial cells were harvested by centrifugation at 4,000 rpm with a Sorvall 1500 centrifuge for 15 min at 4°C. The bacterial pellet was resuspended in 10 ml of resuspension buffer P1 (50 mM Tris Cl pH 8.0, 10 mM EDT, RNase A). The bacteria were lysed with vigorous inverting in 10 ml of the lysis buffer P2 (200 mM NaOH, 1 %SDS w/v) and a 5 min incubation at room temperature. Precipitation of SDS, chromosomal DNA and protein is enhanced with chilled neutralization buffer P3 (potassium acetate pH 5.5) and incubating on ice for 15 min, followed by centrifugation at 13,000 rpm with a Sorvall centrifuge (SS-34 Rotor) for 30 min at 4°. Ion-exchange columns previously equilibrated with 10 ml of equilibration buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% isopropanol (v/v), 0.15% TritonX-100 (v/v)) were loaded with the supernatant. The columns were washed twice by gravity-flow with 30 ml of washing buffer QC (1.0 M NaCl, 50 mM MOPS pH 7.0, 15% isopropanol (v/v)). After elution of the DNA with 15 ml of elution buffer QF (1.25 M NaCl, 50 mM Tris·Cl pH 8.5, 15% isopropanol (v/v)) the DNA was precipitated with room-temperature isopropanol at 11,000 rpm for 30 min at 4°C. The pellet was washed with 5ml of 70 % ethanol, centrifuged at 11,000 rpm for 10 min, dried on air for 5-10 min and redissolved in 200 µl of Tris-EDTA (TE) buffer.

TE buffer	10 mM Tris/HCl (pH 8.0)
	1 mM EDTA

2.1.3 Analytical isolation of BAC DNA from bacteria (mini preparation of BAC DNA)

We used the solutions P1, P2 and P3 from the Qiagen Plasmid Maxiprep Kit for the isolation of BAC DNA. To reduce shear to the large MCMV BAC DNA (size of over 200 kbp), we generally omitted vigorous shaking. 10 ml of LB medium was inoculated with a bacterial colony and incubated overnight at 37°C. The overnight bacterial suspension was transferred to a 15 ml tube and centrifuged at 3,500 rpm with a Heraeus

centrifuge for 5 min. The pellet was resuspended in 300 µl of solution P1 and transferred to a 2 ml microcentrifuge tube. 300 µl of solution P2 was added and efficient alkaline lysis was achieved by gentle inversion and incubation for max. 5 min. Then SDS, chromosomal DNA, and protein were precipitated on ice with 300 µl of solution P3. After centrifugation at 14,000 rpm with a 5415 Eppendorf centrifuge for 10 min at 4°C, the supernatant (ca. 1 ml) was extracted with an equal volume of 1 ml of phenol/chloroform (1:1). The phases were mixed by gentle rocking for 10 min and to separate the phases centrifuged at 14,000 rpm for 5 min at 4°C. The aqueous layer was recovered avoiding the interface and the DNA was precipitated with isopropanol and centrifuged at 14,000 rpm for 20 min. The pellet was washed with 70% ethanol, dried on air and dissolved in 100 µl TE buffer.

2.1.4 Quantitative isolation of BAC DNA from bacteria (maxi and midi preparation of BAC DNA)

We used the Nucleobond Kit from Macherey & Nagel to extract BAC DNA. First 400 ml (maxi prep) or 100 ml (midi prep) LB medium was inoculated with approximately 100 µl of a bacterial culture and incubated overnight at 37°C. The bacteria were centrifuged at 6,000 rpm in a Kontron-Hermle centrifuge (Centrikon H-401) for 10 min at 4 °C. The appropriate solution volumes were used as stated in the manufacturer's protocol for BAC maxi and midi preparation. The pellet was resuspended in solution S1 (50 mM Tris/HCl, 10 mM EDTA, RNase A 100 µg /ml, ph 8.0). Solution S2 (200 mM NaOH, 1% SDS) was added for cell lysis and incubated for 2-3 min, and SDS, chromosomal DNA and protein was precipitated with pre-cooled solution S3 (KAc 2.8 M, pH 5.1) and incubated on ice for 5 min. The bacterial lysate was clarified by filtration with a folded filter. The ion-exchange column was then equilibrated with solution N2 (100 mM Tris, 15% ethanol, 900 mM adjusted with H_3PO_4 to pH 6.3, 0.15% Triton X-100). The cleared lysate was loaded twice onto the column, washed with solution N3 (100 mM Tris, 15% ethanol, 1150 mM potassium chloride, adjusted with H_3PO_4 to pH 6.3) and eluted with preheated solution N5 (100 mM Tris, 15% ethanol, 1000 mM potassium chloride adjusted with H_3PO_4 to pH 8.5). After DNA precipitation with isopropanol and the DNA was washed with 70% ethanol, the DNA pellet was resuspended in 200 µl or 100 µl TE buffer.

2.1.5 Concentration of DNA by ethanol precipitation

Sodium acetate at a final concentration of 300 mM and two volumes of ethanol were added to the DNA solution in order to precipitate the DNA. The DNA was recovered by centrifugation at 14,000 rpm for 20 min at 4°C and the resulting pellet was washed with 70% ethanol to remove residual salts. The pellet was dried on air to evaporate residual ethanol and finally redissolved in the desired buffer.

2.1.6 Determination of DNA concentration

The DNA concentration was determined by UV spectrophotometry at a wavelength of 260 to 280 nm with a spectral photometer from Amersham. An absorption quotient of 1.8 to 1.95 that was measured at 280 and 260 nm indicated pure DNA solutions.

2.2 Cloning of DNA

2.2.1 Digestion of DNA with restriction enzymes

The digestion of plasmid DNA was performed in a total volume of 40 - 60 µl. 1 µg of DNA was incubated for 1-2 h at the recommended temperature with 10 units of restriction enzyme and 1/10 of the volume of a compatible 10x restriction enzyme buffer. 1 µg of DNA was incubated for 3-4 h with 20-40 units of restriction enzyme for the digestion of BAC DNA with restriction enzymes. If necessary the restriction enzyme was heat inactivated according to the catalog information. In most cases heat inactivation was performed at 65°C for 15 min.

2.2.2 Dephosphorylation of DNA

Alkaline phosphatases are said to prevent recircularization and religation of linearized cloning vehicle DNA by removing phosphate groups from both 5′ termini. Shrimp Alkaline Phosphatase (SAP) catalyzes the dephosphorylation of 5′ phosphates from DNA. SAP is active on 5′ overhangs, 5′ recessed and blunt ends. Per pmol of linear DNA at least 1 unit of SAP (Boehringer Mannheim) was added to the restriction enzyme digest. The DNA was incubated for 1 h at 37°C and SAP was inactivated at 65°C for 15 min prior to ligation.

2.2.3 Treatment of DNA with Klenow polymerase

DNA with non-complementary sticky ends can be treated with Klenow polymerase to create blunt ends. Klenow polymerase represents the subunit of the DNA polymerase and fills in the 5´-protruding ends of DNA fragments with dNTPs to generate blunt end DNA. For the reaction 1 µg of DNA in an aqueous solution was incubated with 1/10 of the volume of the 10x Klenow buffer (10 mM Tris/HCl, pH 7.5; 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT), 1/10 of the volume of a 40 mM dNTP solution and 1 unit of Klenow polymerase (Gibco) for 30 min at 37°C. If necessary, the enzyme was inactivated at 65°C for 15 min.

2.2.4 Purification of DNA fragments from agarose gels

Digested DNA was separated on a 0.6-1.5% agarose gel stained with ethidium bromide (1 µg/ml was added to the agarose gel) and visualized by UV light. The desired DNA band was then cut out and purified with the PCR DNA and Gel Band Purification kit from Amersham according to the standard protocol.

2.2.5 Ligation of DNA

DNA fragments were ligated with T4 DNA ligase at a molar vector:insert ratio of 1:4. The DNA was incubated with 1 unit of T4 DNA ligase and 1/10 of the supplied 10x buffer for 3 h at room temperature. Blunt end DNA was incubated overnight at 16°C.

2.2.6 Preparation of electro-competent bacteria

200 ml of LB medium with the appropriate antibiotics were inoculated with 2 ml of a fresh overnight culture and incubated at 37°C for the preparation of electrocompetent bacteria. After 2 h the optical density (OD_{600}) of the bacterial culture was first measured. Once an OD_{600} of 0.5 to 0.6 was reached, the bacteria were chilled on ice for 15 min and then centrifuged at 7,000 rpm for 10 min at 0°C. All further steps were performed on ice. The supernatant was decanted and the pellet was resuspended in 10 ml of cold 10% glycerol to remove salts and centrifuged at 7,000 rpm for 10 min at –4°C. This step was repeated twice. The resulting bacterial suspension was dispensed in 60 µl aliquots, flash frozen with liquid nitrogen and stored at –80°C.

2.2.7 Preparation of chemical-competent bacteria

50 ml of LB medium was inoculated with 1 ml of an overnight culture and incubated at 37°C for the preparation of chemically competent bacteria. The diluted culture was grown to an optical density (OD_{600}) of 0.25 to 0.5 and the bacteria were spun down at 2,300 rpm for 10 min at 4°C. All further steps were performed on ice. The supernatant was decanted and the pellet was resuspended in 15 ml of ice cold transformation buffer (TfB) I and incubated on ice for 40 min. The cells were centrifuged at 2,300 rpm for 10 min at 4°C and the pellet was cautiously resuspended in 2 ml of prechilled TfB II buffer and incubated on ice for 15 min. The bacteria were dispensed to 60 µl aliquots, flash frozen with liquid nitrogen and stored at –80°C.

TfB I buffer	100 mM $RbCl_2$
	10 mM $CaCl_2$
	30 mM KAc
	50 mM $MnCl_2$
	15% glycerol
	adjust pH to 5.8, filter sterilize

TfB II buffer	10 mM $RbCl_2$
	75 mM $CaCl_2$
	10 mM MOPS (pH 7.0)
	15% glycerol
	filter sterilize

2.2.8 Electroporation of bacteria

The DNA solution was first dialysed on a 200 µm filter membrane on water for 30 min to reduce the salt concentration. The electro-competent cell aliquots were then thawed on ice. Less than 2 µl of DNA was added to the 60 µl aliquots of electrocompetent bacteria on ice, gently mixed and transferred to a chilled cuvette (diameter 0.2 µm). The bacteria were pulsed at 2.5 kV, 400 Ω und 25 µFD with the Gene Pulser from Perkin Elmer. 200 Ω were used for electroporation of MCMV BAC DNA into DH10B. The cuvette was removed from the chamber and immediately 1 ml of LB medium was added. The cells were transferred to a microcentrifuge tube and incubated with shaking

at 37°C or 30°C for 1 h. The cells were plated onto selective agar plates and incubated overnight at 37°C or 30°C.

2.2.9 Chemical transformation of bacteria

The 60 µl aliquots with chemical-competent bacteria were thawed on ice for chemical transformation. DNA was added onto the competent cells and incubated on ice for 30 min. The cells were heat shocked for 1 minute at 42°C and incubated on ice for 2 min in 1 ml of LB medium and incubated at 37°C or 30°C for 1 h. Then the cells were centrifuged at 6,000 rpm for 1 minute, the supernatant was decanted and were plated onto selective LB plates.

2.2.10 Preservation of bacteria

An overnight culture was mixed with 50% glycerol to a final concentration of 20% (v/v) and stored at –80°C for the long-term storage of bacteria.

2.3 Analysis of DNA

2.3.1 Agarose gel electrophoresis

DNA was separated and analyzed on non-denaturating agarose gels with gel concentrations 0.6-1.5%. BAC DNA was separated on 0.8% gels. Ethidium bromide was added to the gels to a final concentration 1 µg/ml in order to visualize the DNA with an ultraviolet transilluminator after electrophoresis. Either Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE) buffer was used to prepare and run the agarose gel electrophoresis and 1/10 a 10x running buffer (200 mM EDTA, 40% ficoll 400, 0.02% (w/v) bromphenol blue) was added to the DNA samples before loading the samples onto the solidified agarose gels. The gels were run with voltages of 70-120 mV for 1-2 h and MCMV BAC DNA was run with 70 mV for 14-16 h.

TAE buffer	40 mM Tris/HCl
	20 mM glacial acetic acid
	1 mM EDTA
	adjust pH to 8.0

TBE buffer	90 mM Tris
	90 mM boric acid
	1 mM EDTA
	pH 8.3

2.3.2 Sequencing of DNA

The sequencing services of sequiserve (Germany) were used to sequence cloned DNA and PCR products. The sequencing reaction required 1 µg of plasmid DNA and 5 µg of MCMV BAC DNA. BAC DNA in 200 µl of TE buffer was additionally treated with ultrasound (120 W) for 3 s. The DNA was then ethanol-precipitated and resuspended in 10 µl of distilled water.

2.4 Polymerase-chain reaction (PCR)

We used the *Expand High Fidelity PCR System* polymerase from Roche for all PCR reactions. PCR was performed in the thermocycler T 2400 from Perkin Elmer. Each PCR sample was prepared with 10 ng to 100 ng of template DNA, 300 nM of each primer, 200 µM of dATP, dCTP, dGTP, dTTP, 1/10 of 10x Expand HF buffer and 3.5 units of *Expand High Fidelity PCR System* polymerase. See table B 2 for PCR conditions. Chapter B 2.5.1 describes the PCR conditions for the amplification of PCR products for site-directed BAC mutagenesis.

Tab. B 2 Conditions for PCR.

cycles	conditions for PCR
1	at 95°C for 5 min
28	at 94°C for 30 s at T_H***** for 1minute at 68°C or 72°C****** for 1 – 3 min (elongation time*****)**
1	at 68°C or 72°C****** for 6 min

***** The temperature for hybridization (TH) of a primer was calculated using this simplified equation:

T_H (°C) = 60 + [(G + C) x 41 / Nt Number] – (600/NtNumber)

(G + C equals the number of cytosine und guanidine in a primer sequence, Nt_{Number} corresponds to the length of the primer). The temperature for hybridization was 2°C lower than the smaller T_H of both primers.

** For DNA fragments smaller/larger than 1 kbp 72°C/68°C was chosen.

*** For each 500 bp of template DNA an elongation time of 30 s was set, with a minimum of 1 min.

2.5 Mutagenesis of MCMV BAC plasmids

The mutagenesis of the parental and mutated MCMV BAC plasmid was performed by homologous recombination in *E.coli* bacteria with the one-step mutagenesis according to Zhang et al. (1998).

2.5.1 Generation of PCR fragments with viral homologies

68 to 73 base pair large synthetic oligonucleotides were used as primers for PCR. These contained 22 to 23 base pair recognition sites at their 3´-end that are complementary to sequences of the template DNA pSLFRTkan. The 5´-end contained a 45-50-bp homology to the desired MCMV sequence. The following conditions were used to amplify the PCR fragment with the kanamycin resistance gene and the chosen viral homologies: We used the *Expand High Fidelity PCR system* polymerase from Roche at a temperature of 68°C and PCR was performed with a maximum of 10 ng of template DNA. PCR was performed in principal as described in chapter B 2.4 for conventional PCR, but touch-down conditions were applied to avoid unspecific binding. During the first 18 cycles the annealing temperature was decreased every round from 62°C to 45°C by 1°C. This was followed by 17 cycles at 45°C and an elongation time of 2 min.

2.5.2 Purification of PCR fragments

First, 4 µl of the 100 µl PCR reaction were analyzed by agarose gel electrophoresis to confirm successful amplification. The remaining 96 µl were purified with the Qiaquick PCR Purification kit from Qiagen according to the standard protocol and eluted in 50 µl. The eluted DNA was digested with 20 units (1 µl) of restriction enzyme DpnI for 1.5 h at 37°C. DpnI digests methylated DNA of bacteria and thus removed the template DNA. The DNA was then precipitated with 6 µl of 3 M sodium acetate and 180 µl of ethanol (3 vol.) at –80°C and centrifuged at 14,000 rpm for 15 min at 4°C. The pellet washed

with 70% ethanol, dried on air and resuspended in 10 µl of water. The DNA was stored at –20°C.

2.5.3 Generation of electro-competent and arabinose-induced DH10B bacteria

5 ml of LB medium with 25 µg/ml of chloramphenicol and 100 µg/ml of ampicillin were inoculated with 5 µl of a glycerol culture of DH10B bacteria that contained the MCMV BAC and the pBADαβγ plasmid. After overnight incubation at 37°C 2 ml of the starter culture were used to inoculate 200 ml LB medium with 25 µg/ml of chloramphenicol and 100 µg/ml of ampicillin. 2 ml of fresh arabinose solution (10% w/v) was added to a final concentration of 0.1 % (w/v) at an optical density (OD_{600}) of 0.15-0.18. The cells were further incubated at 37°C until the required OD_{600} was between 0.25-0.35. The appropriate OD_{600} was reached after 30 min in most cases. All materials and solutions were cooled to temperatures slightly below 0°C in the following steps. The bacterial culture was incubated on ice for 15 min and centrifuged at 7,000 rpm in a prechilled (-5°C) GSA Sorvall rotor for 10 min. The supernatant was decanted and the bacterial pellets were resuspended in 10% glycerol. The bacteria were centrifuged and resuspended in glycerol again. Finally, the supernatant was discarded and the residual supernatant was cleared with a paper towel. The pellet was resuspended in the residual supernatant in the tube and was dispensed into 60 µl aliquots that were frozen at –80°C.

2.5.4 Transformation of the PCR fragment into the arabinose-induced bacteria

The electrocompetent DH10B bacteria were thawed on ice and transferred to a chilled cuvette. Then, 5 µl of the PCR fragment were added to the bacteria and pulsed with a gene pulser from Perkin Elmer at 2.5 kV, 200 Ω and 25 µFD. 1 ml of LB medium was added immediately and the bacteria were incubated with shaking at 37°C for 1.5 h. The transformed bacteria were plated onto selective agar plates with chloramphenicol and kanamycin (25 µg/ml) and incubated overnight at 37°C.

2.5.5 Deletion of the FRT-flanked selection marker

Plasmid pCP20 was transformed into electro-competent bacteria with previously mutated MCMV BAC. The pCP20 plasmid expresses the FLP recombinase that can

excise sequences between two FRT sites. The transformed bacteria were incubated at 30°C for 1 h and then 1/10 vol. of bacteria was plated onto selective agar plates with chloramphenicol (25 µg/ml) und ampicillin (100 µg/ml) and incubated overnight at 30°C. The FLP recombinase encoded on plasmid pCP20 excised the kanamycin cassette between the flanking FRT sites at 30 °C. The next day, 4 colonies were plated onto agar plates with chloramphenicol and incubated overnight at 43°C to loose plasmid pCP20 (Fig. B 1). Then 50 colonies were tested for sensitivity for the previously excised kanamycin cassette.

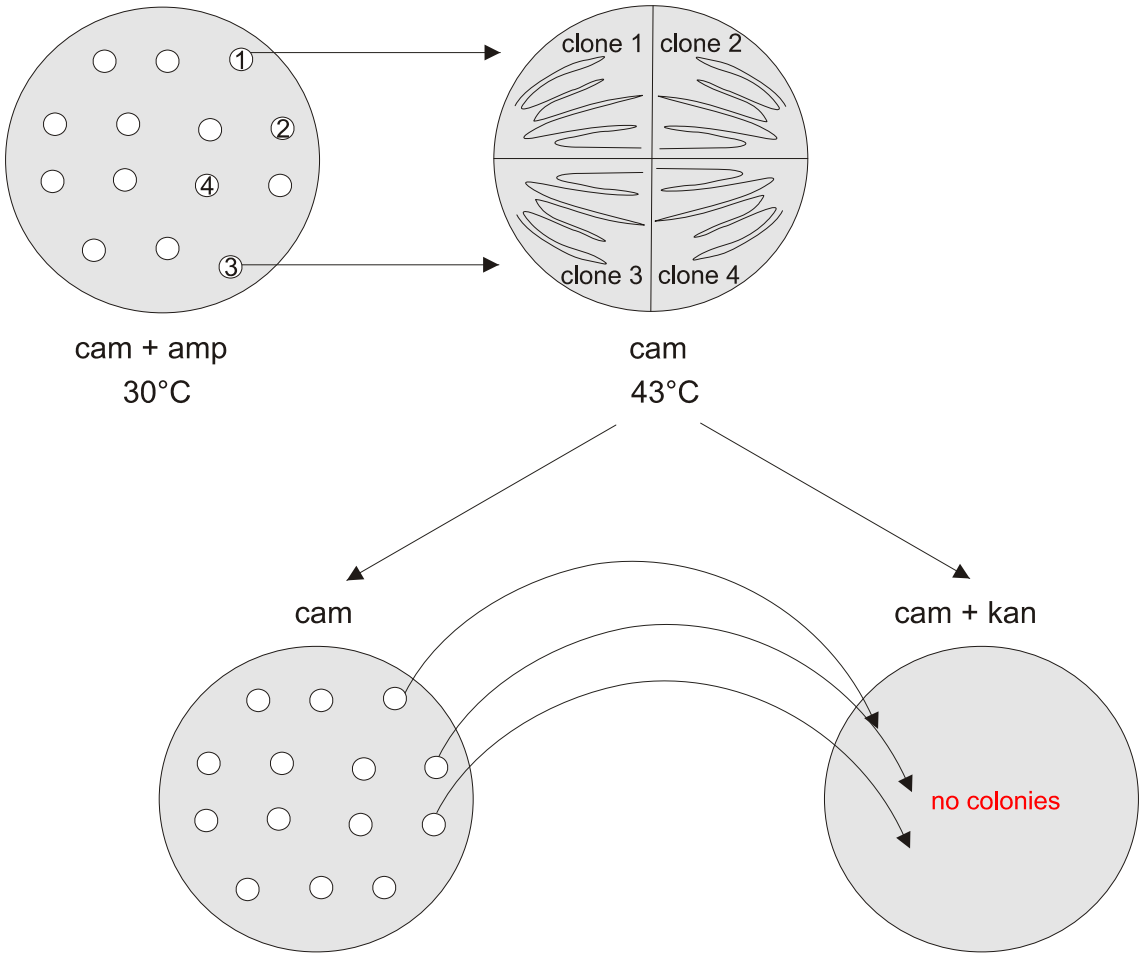

Fig. B 1 Efficacy of the excision of the kanamycin cassette. The plasmid pCP20 was transformed into electro-competent bacteria with the mutated MCMV BAC and incubated on LB agar with chloramphenicol und ampicillin overnight at 30°C. Four colonies were plated on agar plates with chloramphenicol the next day and incubated at 43°C overnight leading to the loss of the pCP20 plasmid. Then 50 colonies were tested for sensitivity for the previously excised kanamycin cassette.

2.5.6 Viral reconstitution of MCMV BAC plasmids

Wild-type and mutant viruses were reconstituted by transfection of BAC DNA into MEFs with calcium-phosphate precipitation (see chapter B2.7.3) or into NIH 3T3 cells with the Superfect transfection reagent from Qiagen (see chapter B2.7.4). 1-2 µg of plasmid DNA was transfected into 80% confluent MEFs for the reconstitution of recombinant MCMVs from the MCMV BAC plasmid with calcium precipitation. 3-15 viral plaques were formed after 4 (wt virus) to 10 days (attenuated virus). After a couple more days all cells showed a cytopathic effect. Alternatively, transfection with Superfect transfection reagent from Qiagen was performed according to the manufacturer's instructions (see chapter B 2.7.4). One ml of the viral supernatant was used to infect 30% confluent NIH3T3 fibroblasts to subsequently make larger viral stocks (see chapter B 2.8). The residual supernatant was stored at –80°C as a primary viral stock.

2.6 Proteins in infected cells

2.6.1 SDS-PAGE gel electrophoresis

Proteins were separated on 12.5% polyacrylamide gels under denaturing conditions (SDS-PAGE). A ready-to-use 30% acrylamide stock solution with 0.8% bisacrylamide was used to pour gels. The stacking gels were poured with 4x stacking gel buffer while the resolving gel was poured in 4x resolving buffer. The samples were heated at 95°C for 5 min and loaded into the sample wells in a maximum volume of 40 µl of a 1X sample buffer. The gels were run in running buffer at a maximum of 180 V.

4x stacking gel buffer	0.5 M Tris 0.4% SDS pH 6.8

4x resolving buffer	1.5 M Tris 0.4% SDS pH 8.8

1x sample buffer	2% SDS 20% glycerol 25 mM Tris pH 6.8 1% β-mercaptoethanol 0.02% bromphenol blue

running buffer	25 mM Tris 10% SDS 250 mM glycin

2.6.2 Western blotting

The cell lysate of $2x10^5$ infected cells was used for Western blot analysis. After electrophoresis of the protein samples on an acrylamide gel, the gel was blotted onto a Hybond P PVDF membrane that was pre-wetted in blotting buffer and swiped in methanol. Three layers of Whatman paper equilibrated with blotting buffer were placed above and below the gel and membrane. The transfer was conducted with a semidry-blotting apparatus from Bio-Rad (USA) for 30 min at 18 V. If necessary, the transfer of proteins was confirmed with ponceau dye.

10x blotting buffer	390 mM glycine 480 mM Tris/HCl pH 8.3 0.37% SDS

ponceau dye	0.2% Ponceau-S 3% acetic acid

To block unabsorbed protein binding sites the membrane was placed in 5% BSA or milk in Tris Buffered Saline with Tween (TBST) buffer for 30 min at room temperature or at 4°C overnight. The membrane was then incubated with the primary antibody (diluted 1:1000 to 1:3000) for 1 h and washed at least five times with TBTS at room temperature. Then the secondary antibody (diluted 1:2000 to 1:7000 in TBST) was added for 1 h at room temperature. After washing the membrane another five times, the peroxidase that is conjugated to the secondary antibody was visualized photochemically

with the ECL Western blotting detection reagents kit from Roche according to the manufacturer's instructions. To detect the protein bands, the membrane was placed on an X-ray film for 1-5 min.

TBST buffer	150 mM NaCl
	10 mM Tris/HCl (pH 8.0)
	0.02% Tween 20

2.7 Cell culture

All cell lines were cultured with 5% CO_2, 95% humidity and at 37°C in a Heraeus incubator. NIH3T3 fibroblasts were cultured in DMEM supplemented with newborn calf serum (NCS). IC-21 and MEFs were cultured in DMEM supplemented with fetal calf serum (FCS). Confluent cells were subcultured 1:3 to 1:5 every 3 to 4 days. MEFs were subcultured 1:2 every 6 days. First, the cells were washed in phosphate-buffered saline (PBS), incubated with trypsin/EDTA for 3 min at room temperature, harvested, and added to 10 ml of DMEM. Then, the cells were centrifuged with a Heraeus centrifuge at 12,000 rpm for 3-5 min at room temperature, resuspended in supplemented DMEM and transferred to an appropriate cell culture dish.

trypsin/EDTA	0.5 g trypsin in 1:250 in PBS
	0.2 g EDTA/l

supplemented	90% DMEM
DMEM	10% FCS or NCS
	0.3 mg/ml L-glutamine
	1.3% (w/v) streptomycin
	0.6% (w/v) penicillin

2.7.1 Thawing cells

Frozen cells were thawed rapidly at 37°C and were then added to supplemented DMEM. After centrifugation for 5 min with a Heraeus centrifuge at 1,200 rpm and room

temperature the cells were resuspended in supplemented DMEM and transferred to cell culture dishes.

2.7.2 Freezing cells

Cells that were approximately 90% confluent were trypsonized, pelleted, and added to 1 ml of freezing medium. The cells were first frozen at –80°C overnight and then in liquid nitrogen.

Freezing medium	50% FCS 40% DMEM 10% dimethyl sulfoxide (DMSO)

2.7.3 Transfection of eukaryotic cells with calcium-phosphate precipitation and glycerol shock

Mouse embryonic fibroblasts (MEF) were freshly thawed from –80°C, seeded on 10 cm tissue culture dishes and subcultured two days later to produce 40-80% confluent cells on the day of transfection. Then, 1 to 2 µg of MCMV BAC DNA were added to 31 µl of 2 M $CaCl_2$ and TE buffer was added to a final volume of 250 µl. After careful mixing 250 µl 2x HEPES buffered saline (HBS) solution was added and the solution was mixed again. The suspension of calcium-phosphate precipitated DNA was incubated at room temperature for 15 min and pipetted drop by drop onto 40-80% confluent cells in a 6 cm cell culture dish.

The cells were glycerol shocked to increase the amount of transfected DNA. The cells were washed with PBS after 5 h and the supernatant was removed. 1 ml of a 15% glycerol solution in 1x HBS solution was added to the cells for 2.5 min. The glycerol solution was then aspirated and the cells were rinsed twice with PBS and new supplemented DMEM was added.

2x HBS solution	50 mM HEPES 1.5 mM Na_2HPO_4 x 2 H_2O 0.28 M NaCl pH 7.13

2.7.4 Transfection of eukaryotic cells with Superfect transfection reagent

Wild-type and mutant viruses were reconstituted by transfection of BAC DNA into NIH3T3 cells with the Superfect transfection agent from Qiagen according to the manufacturer's instructions. 2 to 3 µg of BAC DNA was incubated with 100 µl of medium without serum and 10 µl of Superfect transfection reagent. The mixture was added to approximately confluent 3 x 10^5 NIH3T3 fibroblasts. Cells were washed with phosphate-buffered saline 4 h later, cultured with fresh medium, and passaged when necessary. Plaques usually appeared 4 to 7 days after transfection.

2.8 Generation of MCMV viral stock

Ca. 30% confluent M2-10B4 cells in 14.5 cm tissue culture dishes were infected with an MOI of 0.1 to generate 8-10 MCMV viral stocks. 1 ml of viral supernatant from a 10 cm cell culture dish of cells with a complete cytopathic effect was used to generate first-time viral stocks. The cells and the supernatant were harvested after 3-5 days and transferred to a 250 ml centrifugation tube. The following steps were performed at 4°C or on ice. The supernatant with the virions was stored on ice and the cell pellet was resuspended in 4 ml of DMEM after centrifugation at 6,000 rpm with a Sorvall centrifuge for 15 min. The cells were homogenized 20 times with a glass dounce homogenisator to isolate cell-associated virions from the cells. The suspension was centrifuged again at 12,000 rpm for 10 min with a Sorvall centrifuge to remove cell debris and the supernatant was added to the previously stored supernatant. The combined supernatants were centrifuged at 13,000 rpm with a Sorvall centrifuge for 3 h to pellet the virions. The resulting viral pellet was covered with 1 ml of DMEM and was stored at 4°C overnight. After resuspending the viral pellet in 4 ml DMEM, the virions were dounced 20 times with the homogenisator and then transferred to 7 ml of 15% sucrose in VSB buffer in a SW28 centrifuge tube. The pure viral pellet was resuspended in VSB buffer and dounced again 20 times with the homogenisator after 1 h of

centrifugation at 20,000 rpm with a Beckmann SW 28 rotor. The viral preparation was stored in 50 µl aliquots at –80°C.

VSB buffer	0.05 TRIS/HCl
	0.012 M KCl
	0.005 M EDTA
	pH 7.8

2.9 Viral titration of MCMV with standard plaque assay

MEFs were prepared in 48 well plates and after reaching 100% confluence were infected with dilutions of the viral suspension ($1/10^4$ to $1/10^9$ in DMEM) to determine viral titers. After 1 h of adsorption, the cells were washed with phosphate-buffered saline and covered with carboxymethylcellulose medium to prevent formation of secondary plaques. Growth of each virus was quantified at least twice or in triplicate. At 6 days post infection (p.i.) the plaques were counted under the light optical microscope and viral titers were calculated with the following equation:

$$\text{Viral titer (PFU/ml)} = \frac{\text{counted plaques} \times \text{dilution of counted well}}{\text{volume of virus dilution}}$$

carboxy-	15% glycerol solution (in 1x HBS solution)
methylcellulose	3.75 g carboxymethylcellulose
medium (for 0.5 l)	25 ml FCS
	50 ml 10x MEM
	5 ml L-glutamine
	2.5 ml solution of non-essential a.a.
	5 ml penicillin/streptomycin
	425 ml water
	24.7 ml of a 7.5% $NaHCO_3$ solution

2.10 Viral titration of MCMV with the $TCID_{50}$ (median tissue culture infectious dose) method

Viral stock titers were determined by plaque assay on monolayers of NIH3T3 fibroblasts. Confluent NIH 3T3 fibroblasts from a 10 cm tissue culture dish were subcultured 1:15 and seeded on 96 well microplates. The next day dilutions of the wild-type or mutant virus (10^4 to 10^9) were added to each lane. 1 to 2 h after viral adsorption, the viral supernatant was aspirated and replaced by new DMEM. The $TCID_{50}$ is defined as the dilution of virus required to infect 50% of the cell culture inoculated. Once CPE was obtained and the results were recorded, the virus infectivity titers were calculated to determine the 50% endpoint.

2.11 Isolation of mouse embryonic fibroblasts from the mouse

Mouse embryonic fibroblasts had been isolated from BALB/c mice as described previously (Wagner et al., 2002a, Wagner et al., 2000) and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. These cells were used for virus reconstitution from recombinant MCMV BAC plasmids.

C. RESULTS

1 Mutants ΔATG-m139 and ΔATG-m142 were generated by site-directed mutagenesis

In 2003 Menard et al. had generated, a transposon library of mutant BACs in order to analyze the members of the US 22 gene homolog family in MCMV. Transposon mutants for the US22 gene homolog members m139, m140, m141, and m143 were generated rapidly. ORFs m139 to m141 and m142 to m143 represent a complex translational region and are transcribed collinearly. The insertion of a transposon donor plasmid into an upstream ORF of this region can destabilize the ORFs downstream. This could lead to difficulties in attributing a phenotype to a specific ORF.

Additional site-directed mutants with a minimal genetic change were generated in this thesis. Linear PCR fragments with the kanamycin resistance marker flanked by viral homologies were transformed into recombination-deficient bacterial strains that contain the MCMV BAC DNA. The desired mutation was introduced by homologous recombination between the viral sequences of the linear PCR fragment and the MCMV genome. The mutation was successfully introduced if the kanamycin cassette replaced the desired sequences of ORF m139, m140 and m142 and was later excised by recombinases from Saccharomyces cerevisiae.

1.1 PCR fragments with flanking viral homologies to ORF m139, m140 and m142 were generated

The generation of linear PCR products was the first step in the PCR-based mutagenesis of MCMV. 68 to 73 base pair large synthetic oligonucleotides were used as primers to generate the linear PCR fragments for site-directed BAC mutagenesis of ORF m139, ORF m140 and ORF m142. The primers contained 22-23 bp recognition sites at their 3´-end that were complementary to sequences of the template plasmid pSLFRTkan. Plasmid pSLFRTkan contains the kanamycin resistance cassette and minimal FRT sites with 34 bp. These minimal FRT sites are sufficient for homologous recombination (McLeod et al., 1986). The 5´-end of the primers contained 45-50 bp homologies to the MCMV sequence of ORF m139, ORF m140 and ORF m142. Thus, the linear fragments generated by *touch-down* PCR with plasmid pSLFRTkan (Atalay et al., 2002) as tem-

plate DNA contained the kanamycin resistance gene flanked by Flp recognition target sites (FRT), non-coding priming regions, and viral homologies (see Fig. C 1). The viral homologies were chosen so that the kanamycin resistance cassette replaced the ATG of ORF m139, m140 and 142 in MCMV. The resistance marker was introduced into the PCR fragment in order to select for the rare event of homologues recombination in *E. coli*. This selection marker was later excised by the flanking FRT.

This one-step mutagenesis method based on double crossing-over does not require any prior cloning steps. Only synthetic oligonucleotides have to be generated in order to construct the desired mutant MCMV genome.

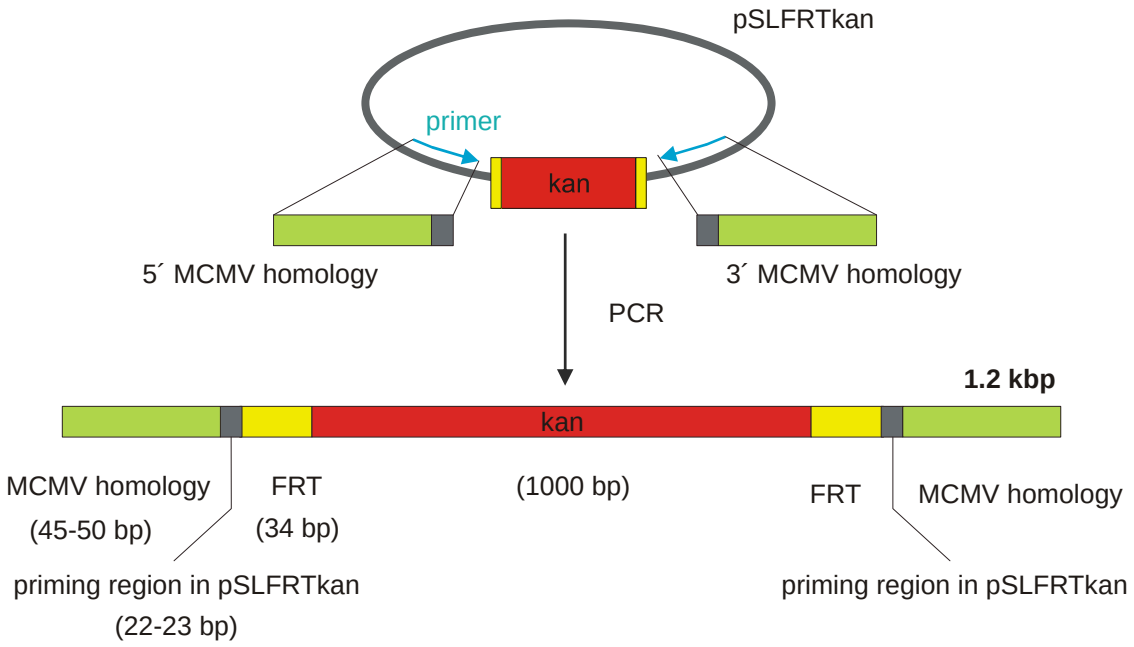

Fig. C 1 Generation of linear PCR fragments. The plasmid pSLFRTkan was used as a template for PCR. All primers (blue arrows) had a priming region (grey box) to plasmid pSLFRTkan and viral homologies (green box) to the desired MCMV sequence. The amplified PCR fragments contained the kanamycin cassette (red box) flanked by Flp recognition target sites (FRT, yellow box), the priming regions in pSLFRTkan, and viral homologies.

Successful amplification of the PCR fragments was confirmed by electrophoresis on ethidium bromide-stained agarose gels (see Fig. C 2). Approximately 1.2 kbp large linear PCR fragments were generated. The purified PCR fragments were digested with *Dpn*I to remove methylated template DNA from the *dam* positive bacteria.

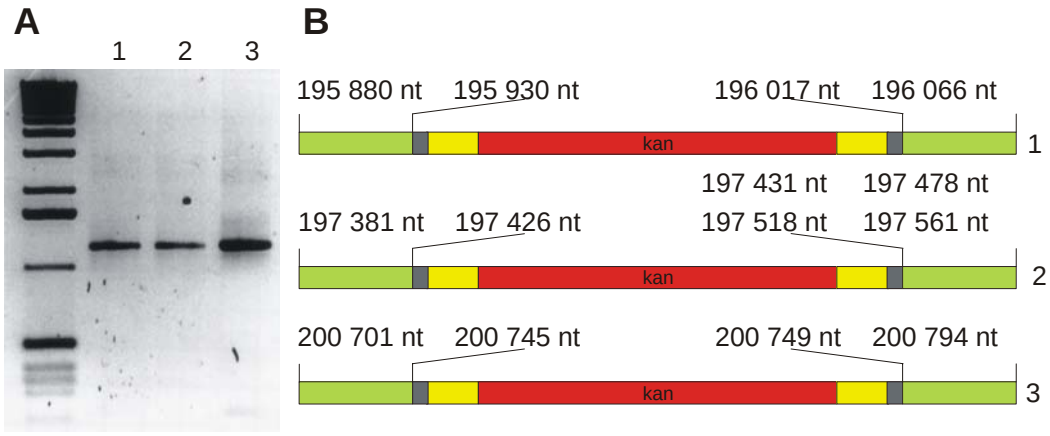

Fig. C 2. Linear PCR fragments. (A) The ethidium bromide-stained agarose gel shows linear DNA fragments amplified for the generation of site-directed mutants ΔATG-m139 (lane 1), ΔATG-m140 (lane 2) and ΔATG-m142 (lane 3). **(B)** PCR fragments PCR-m139 (1), PCR-m140 (2), and PCR-m142 (3) are shown with the respective viral homologies (green box). The priming regions are shown as grey and the FRT sites as yellow boxes.

1.2 Site-directed mutants of ORF m139 and m142 were generated

Plasmid pSM3fr in *E. coli* was used for homologous recombination between the linear PCR fragments PCR-m139, PCR-m140, PCR-m142 and the MCMV BAC (Wagner et

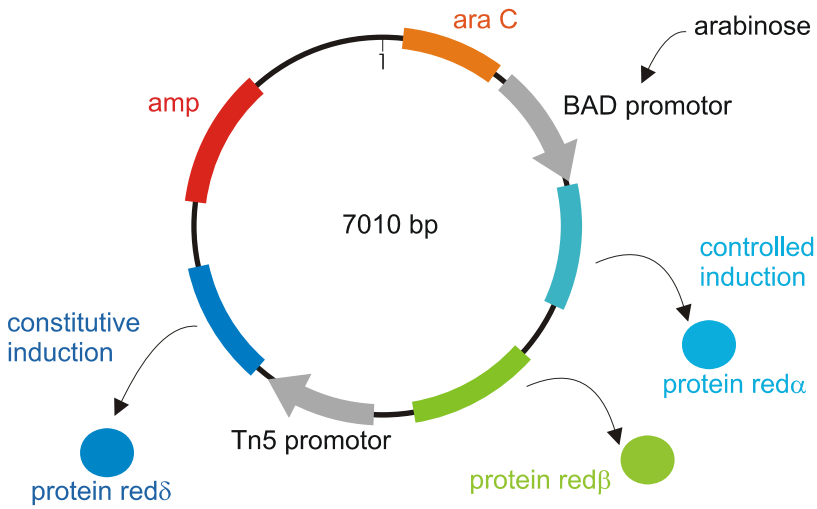

Fig. C 3 Plasmid pBADαβγ. Plasmid pBADαβγ contains the inducible recombination enzymes of phage λ. The redα–gene is under the control of the BAD promoter that can be inhibited by the regulatory protein araC. Arabinose activates the release of araC from the BAD promoter. The redβ–gene is under the control of promoter EM7 and the redγ–gene is under constitutive control of promoter Tn5.

al. 2002a; see also Materials and Methods). The MCMV BAC plasmid pSM3fr contains the entire MCMV genome and the BAC backbone. The construct loses the bacterial sequences and virus MV97.01 is reconstituted with wild-type properties after transfection into fibroblasts or infection of mice.

For site-directed mutagenesis 1 μg of a PCR fragment was transformed into electrocompetent DH10 B that contain the plasmids pSM3fr and pBADαβγ (Fig. C 3). Plasmid pBADαβγ expresses the inducible recombination enzymes of phage λ. The PCR fragment was inserted by the flanking 45-50 bp homologies to the viral target sequences. Viral homologies 25 to 60 bp long are sufficient for homologous recombination (Murphy et al., 1998). The double crossover between the viral homologies of the PCR fragment and the MCMV BAC plasmid was conferred by the transiently arabinose-induced recombinases redα and redβ, expressed by plasmid pBADαβγ.

The transformation reaction was streaked out onto agar plates with chloramphenicol and kanamycin for selection of the mutant MCMV BAC plasmid. Bacterial colonies were counted and mutant BAC DNA was isolated from *E. coli* cultures with an alkaline lysis procedure (Sambrook et al., 2001) and purified with Nucleobond AX100 columns (Machery & Nagel) after overnight incubation. Table C 1 summarizes the primers used for the generation of mutants ΔATG-m139, ΔATG-m140, ΔATG-m140.1 and ΔATG-m142.

Tab. C 1 Overview of the generated mutant MCMV BAC plasmids.

mutant	ΔATG-m139	ΔATG-m140	ΔATG-m140.1	ΔATG-m142
primers	5´-ΔATG-m139 3´-ΔATG-m139	5´-m140-nt-197427 3´-m140-nt-197517	5´-ΔATG-m139 3´-m140-nt-197477	5´-ΔATG-m142 3´-ΔATG-m142
Deleted viral sequence (nt position)	195 931 - 196 016	(197 427 - 197 517)* * Estimated deletion	(197 430 - 195 931)* * Estimated deletion	200 476 - 200 478

The used primers and the deleted viral sequences are listed for mutants ΔATG-m139, ΔATG-m140, ΔATG-m140.1, and ΔATG-m142.

Mutagenesis of ORF m139 and m142 was successful. The viral sequences from nucleotide 195 931 to 196 016 were deleted from the m139 ORF and the viral sequences from nucleotide 200 476 to 20 478 were deleted from the m142 ORF. Correct mutagenesis was confirmed by restriction pattern analysis (Fig. C 4, Fig. C 5) and sequencing. The first two start codons of ORF m139 were deleted and replaced by the kanamycin cassette introducing an additional *Eco*RI cleavage site into the 4.7 kbp *Eco*RI fragment. This rendered a single 4.7-kbp band, a triple band at 2.4 kbp, and an additional 3.4 kbp-band on the ethidium bromide-stained gels (see ΔATG-m139+kan in Fig. C 4). In ORF m142 only the first start codon was deleted and replaced by the kanamycin cassette. Due to the insertion of the kanamycin cassette the 9 kbp band disappeared and was replaced by a 7.7 fragment and a triple band at 2.4 kbp (see ΔATG-m142+kan Fig. C 5).

Site-directed mutagenesis of the first two in-frame start codons of the m140 ORF was not possible. Only isolated bacterial colonies could be recovered after transformation of the PCR-m140 fragment into electro-competent DH10 B. The purified low-scale BAC DNA was contaminated with plasmid DNA that could not be eliminated by re-transformation. In the process, the MCMV BAC plasmid was lost repeatedly. BAC mutagenesis with the PCR fragment PCR-m140.2, constructed to delete an alternative not in-frame start codon at position 197 479, did not produce bacterial colonies.

1.3 The kanamycin cassette was excised from mutant ΔATG-m139+kan and mutant ΔATG-m142+kan

The kanamycin resistance cassette, flanked by the minimal FRT sites, was excised with the FLP recombinase expressed from plasmid pCP20 (Fig. C 4). Fifty colonies plated simultaneously on agar plates with both chloramphenicol and kanamycin or chloramphenicol only grew on the later (see Material and Methods, chapter B 2.5.5). This confirmed the successful excision of the kanamycin resistance marker.

Digest analysis of mutant ΔATG-m139+kan with *Eco*RI produced a 3,386 and 2,402 bp DNA fragment. After excising the kanamycin cassette, the 3,386 bp fragment disappeared (see Fig. C 4). The MCMV sequence from nucleotide 196 016 to nucleotide 195 931 was deleted rendering the mutant ΔATG-m139. This viral sequence includes the first two in-frame ATGs of ORF m139.

Restriction digest analysis of mutant ΔATG-m142+kan with *Eco*RI produced two additional DNA fragments of 7,706 and 2,452 kbp. After excising the kanamycin cassette the 7,706 band disappeared and was replaced by a 6,706 kbp DNA fragment (Fig. C 5). Nucleotides 200 476, 200 477 and 200 478 representing the first ATG in ORF m142 were deleted. This rendered the mutant ΔATG-m142.

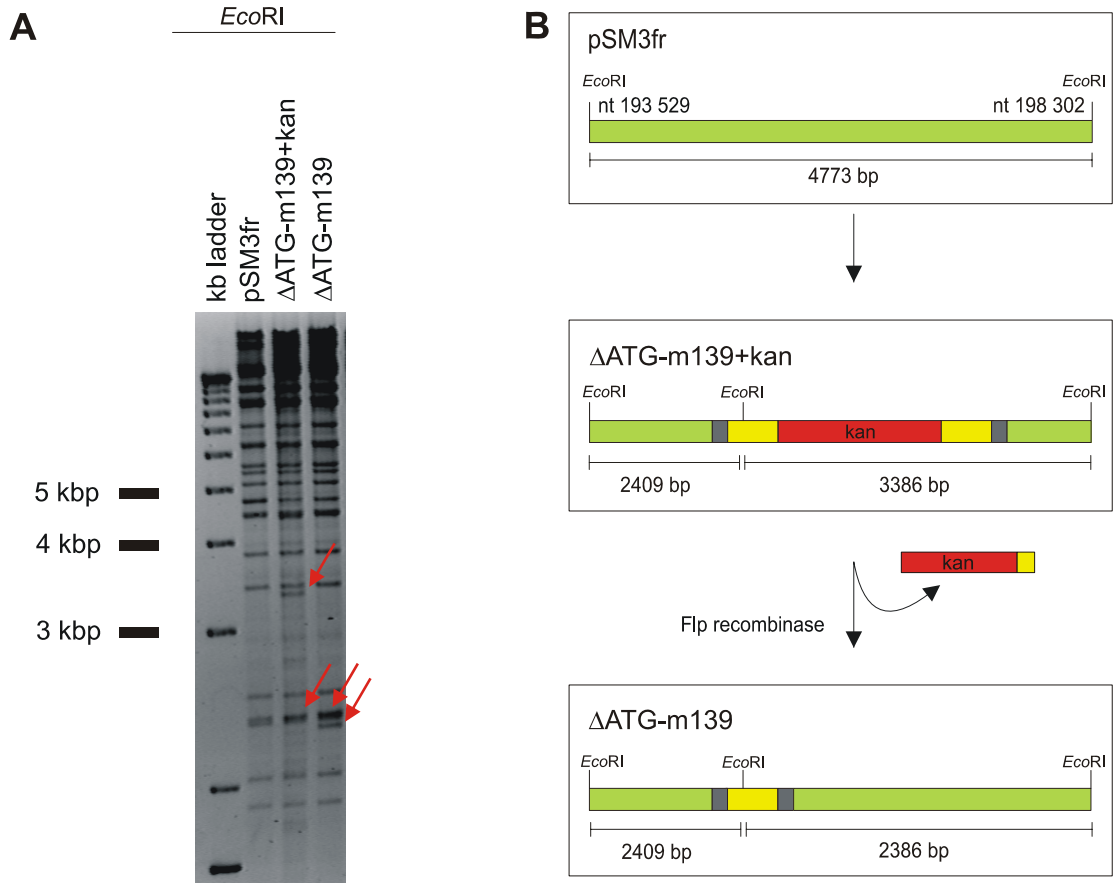

Fig. C 4 Mutant ΔATG-m139 was generated by site-directed mutagenesis of ORF m139. **(A)** Ethidium bromide-stained agarose gel of *Eco*RI-digested BAC plasmids pSM3fr, ΔATG-m139 + Kan and ΔATG-m139 isolated from *E. coli*. Digestion of mutant ΔATG-m139+kan with *Eco*RI produced a 3,386 and 2,402 bp DNA fragment. After excising the kanamycin cassette the 3,386 bp fragment disappeared. **(B)** *Eco*RI (black line) restriction enzyme pattern after excising the kanamycin cassette (red box) from mutant ΔATG-m139+kan. Mutant ΔATG-m139 contains a FRT site (yellow box) and the priming region (grey box) at the position of the inserted mutation. MCMV BAC DNA is shown as a green box.

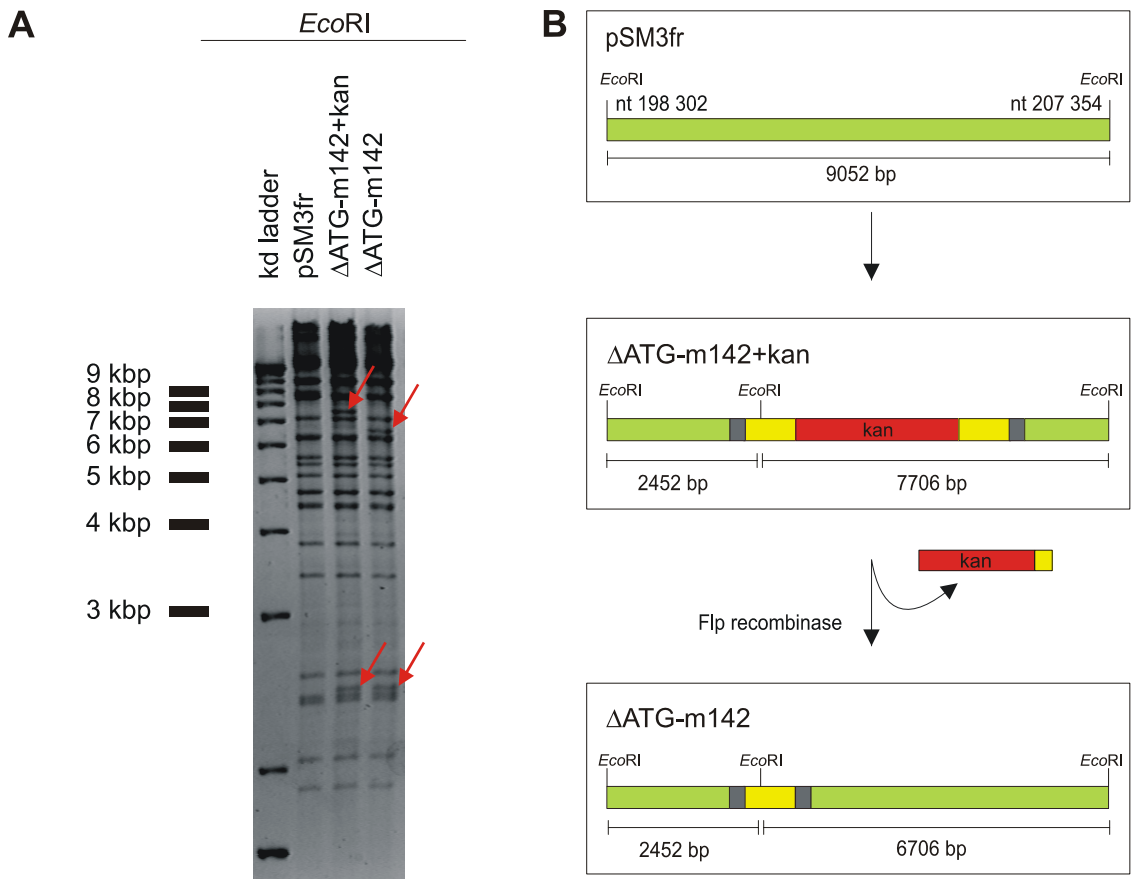

Fig. C 5 Mutant ΔATG-m142 was generated by site-directed mutagenesis of ORF m142. **(A)** Ethidium bromide-stained agarose gel of *Eco*RI digested BAC plasmids pSM3fr, ΔATG-m142 + Kan and ΔATG-m142 isolated from E. coli. Restriction analysis of mutant ΔATG-m142 + Kan with *Eco*RI produced additional DNA fragments at 7,706 and 2,452 kbp. After excising the kanamycin cassette, the 7,706 band disappeared and was replaced by a 6,706 kbp DNA fragment **(B)** *Eco*RI (black line) restriction pattern after excising the kanamycin cassette (red box) from ΔATG-m142 + Kan. Mutant ΔATG-m139 contains a FRT site (yellow box) and the priming region (grey box) at the position of the inserted mutation. MCMV BAC DNA is shown as a green box.

1.4 Site-directed mutagenesis of ORF m139 and m142 introduces a minimal genetic alteration

In mutant ΔATG-m139 the first two putative start codons of ORF m139 that are 84 bp apart were replaced with an 86-bp extraneous sequence (Fig. C 6). The MCMV sequence from nucleotide 195 931 to nucleotide 196 016 was replaced by the 34-bp minimal FRT site and the priming sequence in plasmid pSLFRTkan. Site-directed mutagenesis of ORF m139 was confirmed by sequencing (Fig. C 6). In mutant ΔATG-m142 the native ATG of ORF m142 was deleted and replaced by an 86-bp sequence that contains the 34-bp FRT flanked by a 23-bp sequence of priming in plasmid pSLFRTkan.

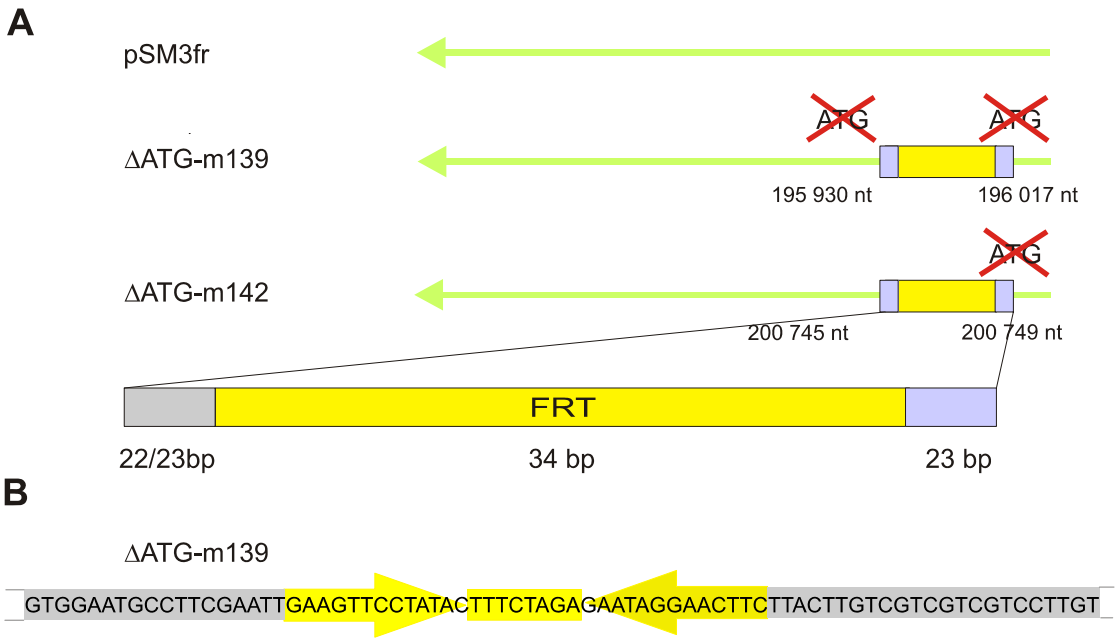

Fig. C 6 Traces of mutagenesis. (A) pSM3fr corresponds to the MCMV wt sequence. In mutant ΔATG-m139 the first two start codons were deleted resulting in a deletion of 87 base pairs, while in mutant ΔATG-m142 only 3 base pairs were deleted. After excision of the kanamycin cassette, an 80-bp (grey and yellow box) sequence remained. MCMV sequence (green arrow), FRT (yellox box), priming region (grey box). **(B)** Sequence analysis of the *Hin*dIII fragment that contained the mutated region of ORF m139 revealed appropriate mutagenesis with a remaining minimal FRT site flanked by the priming sequence (grey box) in pSLFRTKan. The minimal FRT site consists of the spacer (yellow box) and two surrounding 13 bp symmetry elements arranged in inverse orientation (yellow arrows).

2 Mutant ΔATG-m139 has wild-type properties in NIH3T3 fibroblasts, but shows attenuated growth in IC-21 macrophages

2.1 Growth kinetics in NIH3T3 fibroblasts

The mutant ΔATG-m139 was first tested with respect to growth on NIH 3T3 fibroblasts in comparison with wild-type virus. Table C 2 shows a representative experiment. Growth kinetics of mutant ΔATG-m139 was compared to wild-type MCMV virus MW 97.1. In order to see whether growth of mutant ΔATG-m139 was comparable to the corresponding transposon mutant for ORF m139 Tn-m139, the wild-type virus Tn-152 with a transposon insertion 300 bp downstream to the native ATG (nucleotide 211 077) of gene 152, which expresses green fluorescent protein, was added to the experiment as a control. Monolayers of NIH3T3 fibroblasts were infected in triplicate with a multiplicity of infection of 0.1 of the respective viral stock. Virus progeny in tissue culture supernatants was quantified by the $TCID_{50}$ method on NIH 3T3 fibroblasts. After incubation of the NIH3T3 monolayers with viral progeny for one hour the supernatant was harvested and fresh cell medium was added. This supernatant, that had been absorbed one hour post infection, represented day 0. The titers of cell-free viruses were determined on day 0, day 1, day 4 and day 6 postinfection. After absorption of the virus one hour post infection viral plaques and viral spread was detectable after day 1. By day 6 multiple viral plaques had formed.

Tab. C 2 Mean viral titers of wild-type MCMV (MW 97.01, Tn-m152), mutant ΔATG-m139, and transposon mutant Tn-m139 at day 0, 1, 4 and 6 post infection in NIH 3T3 fibroblasts.

MCMV mutant	day 0	day 1	day 4	day 6
wt (MW 97.01)	1.6×10^4	1.3×10^2	1.1×10^4	3.3×10^4
wt (Tn-m152)	1.4×10^4	1.0×10^2	7.5×10^4	9.0×10^4
ΔATG-m139	1.0×10^4	1.5×10^2	6.0×10^3	1.3×10^4
Tn-m139	1.9×10^4	1.8×10^2	3.7×10^4	5.7×10^4

Monolayers of NIH 3T3 fibroblasts were infected with an MOI of 0.1 with wild-type or mutant MCMV. At day 0 to 6 post infection, supernatant was harvested from individual wells and infectious virus was quantified on NIH3T3 fibroblasts by the TCID50 endpoint titration method.

Fig. C 7 shows the corresponding multistep growth curves of the MCMV mutants in these adherent cells. The results demonstrate that the growth rate of each mutant was similar to that of the corresponding wild-type MCMV. A difference in the range of less than one log unit compared to wild-type MCMV was detected. Thus there was no significant difference between wild-type MCMV and the mutant ΔATG-m139 or transposon mutant Tn-m139 with respect to growth on NIH 3T3 fibroblasts, confirming that the mutations introduced into this ORF do not negatively affect MCMV replication in cultured fibroblasts. The transposon and the ATG mutant of ORF m139 acted similarly displaying a growth difference of less than one log unit.

Taken together, neither the introduction of a transposon at ORF m139, as shown before (Menard et al., 2003), nor the deletion of the first two ATGs in ORF m139 impairs growth in fibroblasts. Both m139 mutants show wild-type properties in NIH3T3 fibroblasts.

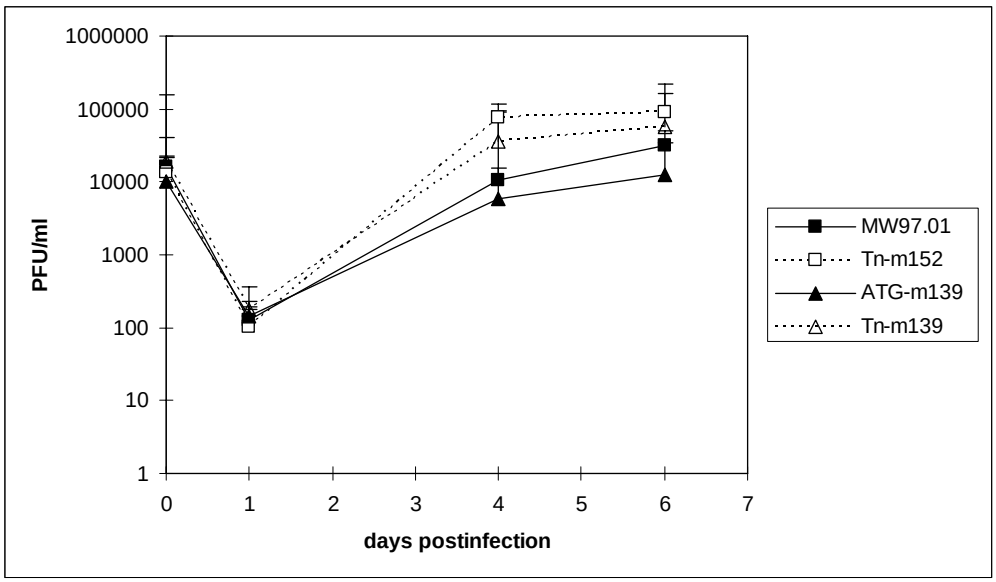

Fig. C 7 In vitro growth of mutant ΔATG-m139 in NIH3T3 fibroblasts. Multistep growth curves of wild-type MCMV (MW 97.01, Tn-m152), mutant ΔATG-m139 and transposon mutant Tn-m139 were determined in NIH3T3 as described in Material and Methods. Each data point represents the average of three separate cultures and error bars indicate the standard deviations between triplicates.

2.2 Growth kinetics in IC-21 macrophages

As a complement to the studies described above, growth of wild-type MCMV and that of mutants ΔATG-m139 and Tn-m139 were compared in the fully permissive peritoneal macrophages cell line IC-21 (Mauel, 1971). As shown previously the transposon mutant of gene m139 and a deletion mutant for m139, that lacks almost the complete m139 ORF, were impaired for growth on IC-21 macrophages compared with wild-type MCMV (Menard et al., 2003). The attenuation of these mutants for growth on IC-21 macrophages was in the range of two to three log units compared to wild-type MCMV.

Tab. C 3 Mean viral titers of wild-type MCMV (MW 97.01, Tn-m152), mutant ΔATG-m139 and transposon mutant Tn-m139 at day 0, 1, and 6 postinfection in IC-21 macrophages.

MCMV mutant	day 0	day 1	day 6
wt (MW 97.01)	3.6×10^3	3.9×10^1	2.6×10^5
wt (Tn-m152)	1.9×10^3	3.7×10^1	1.6×10^5
ΔATG-m139	1.9×10^3	3.7×10^1	2.9×10^2
Tn-m139	4.3×10^3	3.4×10^1	3.2×10^3

Monolayers of IC-21 macrophages were infected with an MOI of 0.1 with wild-type or mutant MCMV. At day 0 to 6 post infection, supernatant was harvested from individual wells and infectious virus was quantified on NIH3T3 fibroblasts by the TCID50 endpoint titration method.

Growth kinetics of mutant ΔATG-m139 was compared to wild-type MCMV virus MW 97.1. Table C 3 shows a representative experiment. In order to see whether growth of mutant ΔATG-m139 was comparable to the corresponding transposon mutant for ORF m139 Tn-m139, the wild-type virus Tn-152 with a transposon insertion 300 bp downstream to the native ATG (nucleotide 211 077) of gene 152, which expresses green fluorescent protein, was added to the experiment as a control. Monolayers of IC-21 macrophages were infected with an MOI of 0.1 of the respective viral stock in triplicate. Virus progeny in tissue culture supernatants was quantified by the $TCID_{50}$ method on NIH 3T3 fibroblasts. After incubation of the IC-21 macrophages with viral progeny for one hour the supernatant was harvested and fresh cell medium was added. This supernatant absorbed one hour post infection represented day 0. The titers of cell-free viruses were determined on day 0, day 1 and day 6 postinfection. After absorption of the virus one hour post infection viral plaques formation for wild-type virus was seen later in IC-21 macrophages than in NIH3T3 fibroblasts. The first viral plaques had formed

starting at days 3 to 4. The ATG and transposon mutant for ORF m139 both grew slowly in general.

Figure C 8 shows the corresponding multistep growth graph of the MCMV mutants ΔATG-m139 and Tn-m139. Both wild-type viruses grew similarly. As described above, the transposon mutant Tn-m139 showed a two log unit growth difference compared to wild-type. A difference in the range of three log units compared to wild-type MCMV was detected for mutant ΔATG-m139. Thus there was a significant difference between wild-type MCMV and the mutant ΔATG-m139 or transposon mutant Tn-m139 with respect to growth in IC-21. The mutations introduced into this ORF affects MCMV replication in cultured macrophages whether the ORF is deleted completely or just the start codon.

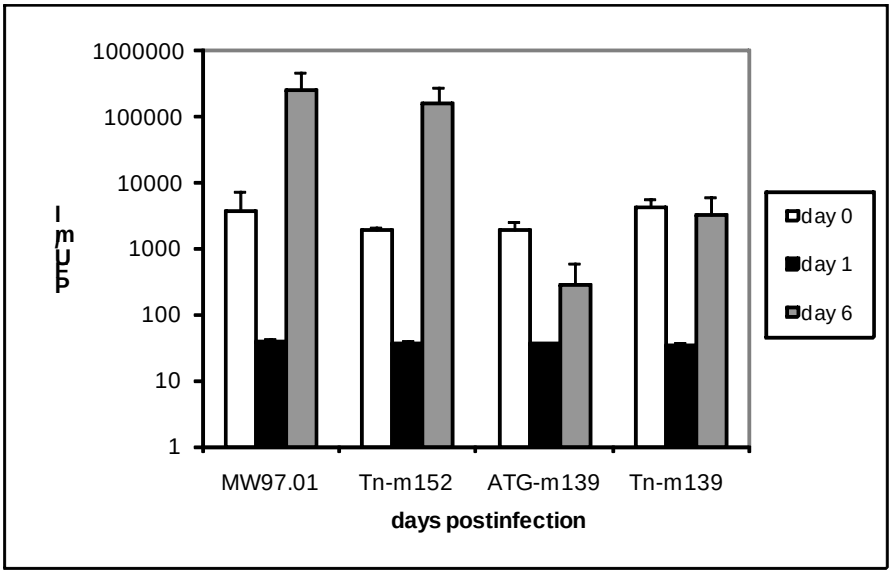

Fig. C 8 In vitro growth of mutant ΔATG-m139 in IC-21 macrophages. Multistep growth graphs of wild-type MCMV (MW 97.01, Tn-m152), mutant ΔATG-m139 and transposon mutant Tn-m139 were determined in IC-21 macrophages as described in Material and Methods. Each bar represents the average of three separate cultures and error bars indicate the standard deviation of the geometric mean.

In summary, both m139 mutants show wild-type properties in NIH3T3 fibroblasts, but are impaired for growth in IC-21 macrophages by two to three log units. These results show that inactivation of the m139 gene of the US 22 gene family affects growth on IC-21 macrophages.

3 The gene products of gene m139 interact with the gene products of genes m140 and m141

3.1 The complex translational region of m139 to m143

The US22 gene members m139 to m143 are localized toward the right end at the *Hin*dIII-I and *Hin*dIII-J region of the MCMV genome (Rawlinson et al., 1996). The m139, m140 and m141 genes and m142 with m143 genes belong to a complex transcriptional unit and have 3´-coterminal transcripts (Fig. C 9). Such sets of 3´-coterminal transcripts, with alternative promoters, are found in multiple regions in HCMV (Stenberg et al., 1989; Welch et al., 1991; Adam et al., 1995; Wing and Huang, 1995) and at least one other region in MCMV (Cranmer et al., 1996), suggesting that this is a common occurrence in cytomegaloviruses.

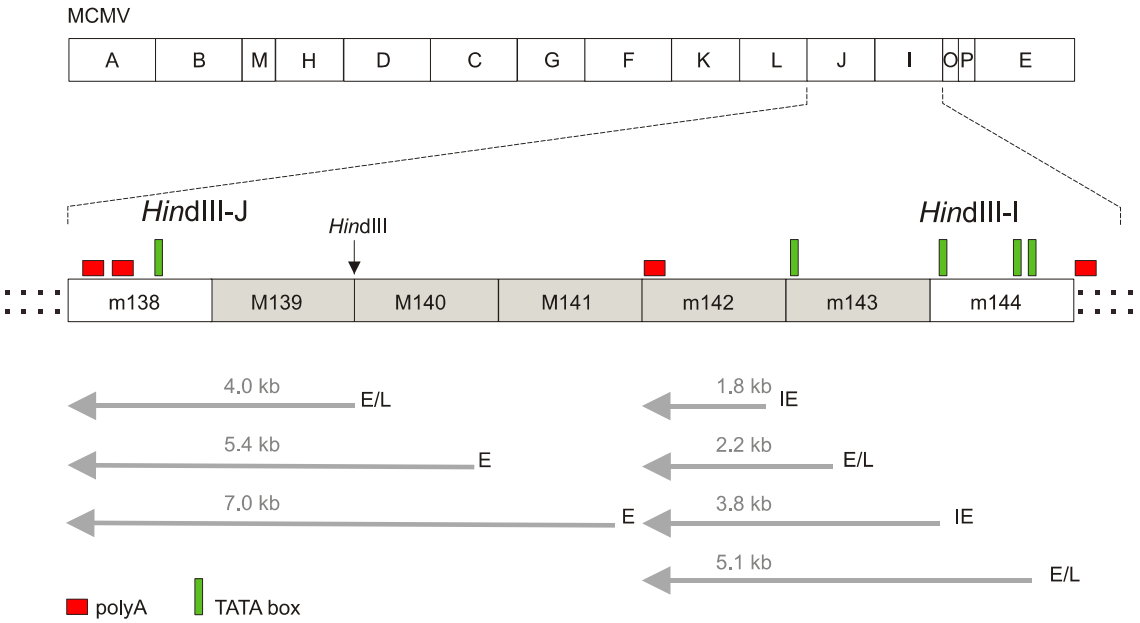

Fig. C 9 Transcript map of genes m139 to m143. Black arrows denote the major transcripts of genes m139 to m141 and genes m142 and m143 according to Hanson et al. (1999b). Potential ORFs within the *Hin*dIII-J and –I regions are indicated by open boxes. The small arrow depicts the indicated restriction site. M or m designation indicates respectively the ORFs with or without sequence homology to HCMV genes (Rawlinson et al., 1996). IE, E and L denote immediate-early, early, and late transcripts. Numbers above the arrows indicate sizes of the transcripts in kb. The red boxes denote potential polyadenylation signals. Green boxes indicate potential TATA boxes.

It is less likely that a transposon insertion in the m141 ORF would affect the transcription of the downstream m139 and m140 genes, because they have independent transcription start sites downstream of ORF m141. However, a transposon insertion in the m139 ORF could affect the transcription rate or the transcript stability of the upstream m140 and m141 genes. To determine whether the transposon insertion in m139 had an effect on expression of the neighboring genes, the targeted mutant of the m139 ORF ΔATG-m139 was constructed by site-directed mutagenesis of the wild-type MCMV BAC plasmid pSM3fr as described above. The first 86 bp of m139 were deleted, thereby deleting both ATGs. After excision of the kanamycin resistance marker by FLP recombination, 86 bp of noncoding sequence was left behind in the MCMV BAC genome, corresponding exactly to the length of the deleted viral sequence. Thus, the first two start ATGs of m139 were deleted without altering the lengths of the m139 to m141 mRNA transcripts. Maintaining the same transcript length should reduce transcript instability.

3.2 Mutant ΔATG-m139 reduces the presence of the m140 and m141 proteins

We first tested whether the proteins encoded by m139, m140 and m141 interact posttranslationally. The transcripts and proteins are expressed at early times within the MCMV replication cycle (Hanson et al., 1999b; Vieirra et al., 1994). Western blot analysis of the mutants ΔATG-m139 and Tn-m139 MCMV was performed with m140-, and m141-specific polyclonal antibodies. For the m140 and m141 proteins, 15-aa peptides at the C-terminus (SVLTTRPDRNRDTRT, starting at position 431) and the N-Terminus (ATGGDQNARRRAIER, position 25), respectively, were used to generate rabbit polyclonal antibodies (Eurogentec, Belgium).

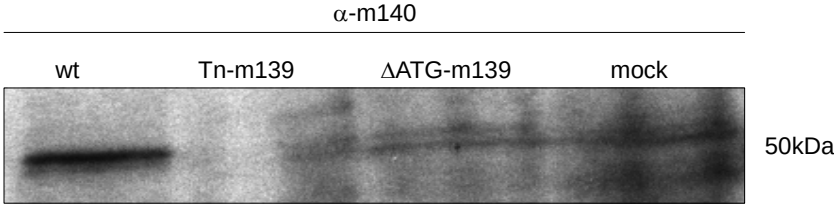

Fig. C 10 Western blot analysis with polyclonal antisera against protein m140. NIH 3T3 cells were infected at an MOI of 1 with wild-type MCMV (wt) or MCMV mutants ΔATG-m139 and Tn-m139. Cell lysates were harvested 24 h postinfection and separated by polyacrylamide gel electrophoresis. Western blot analyss with rabbit polyclonal antisera against protein m140 was performed.

The viral proteins were quantified using the bicinchoninic acid (BCA) protein assay reagent and comparable viral protein was loaded. The protein load was separated under denaturing conditions and then blotted. The peptide antisera to the m140 and m141 products detected single viral proteins in wild-type MCMV-infected fibroblasts with the expected size of 56 kDa and 52 kDa, respectively. This is consistent with previous data (Hanson et al., 1999). In both the transposon mutant and the site-directed mutant of the ORF m139 the proteins expressed by m140 and m141 were marginally detectable. Thus mutation of gene m139 strongly reduced the presence of the m140 and m141 proteins (see Fig. C 10 and 11).

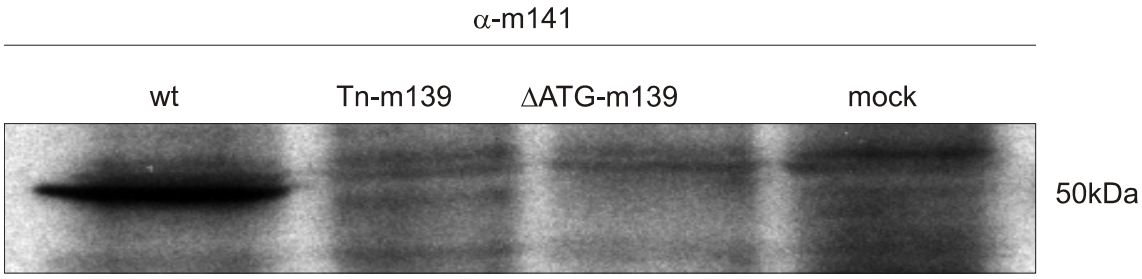

Fig. C 11 Western blot analysis with polyclonal antisera against protein m141. NIH 3T3 cells were infected at an MOI of 1 with wild-type MCMV (wt) or MCMV mutants ΔATG-m139 and Tn-m139. Cell lysates were harvested 24 h postinfection and separated by polyacrylamide gel electrophoresis. Western blot analysis with rabbit polyclonal antisera against protein m141 was performed.

3.3 ΔATG-m139 expresses a truncated protein of m139

Western blot analysis was then performed with antisera against the protein expressed from ORF m139. The MCMV m139 rabbit polyclonal antibody is directed against amino acids 57 to 644 of the m139 recombinant protein and was kindly provided by Ann Campbell, Eastern Virginia Medical School. In wild-type MCMV-infected cells, two proteins of 72 and 61 kDa were detected with the m139-specific antiserum (Fig. C 12), consistent with previous data (Hanson et al., 1999b). In cells infected with the Tn-m139 MCMV, no m139-specific signal was seen, whereas with the ΔATG-m139 MCMV mutant, a smaller protein of 61 kDa was still present, suggesting that this protein is encoded by the m139 transcript, probably originating at the third alternative start

ATG of the m139 ORF at nucleotide position 195767. The 61 kDa protein is thus a truncated protein expressed by the m139 ORF.

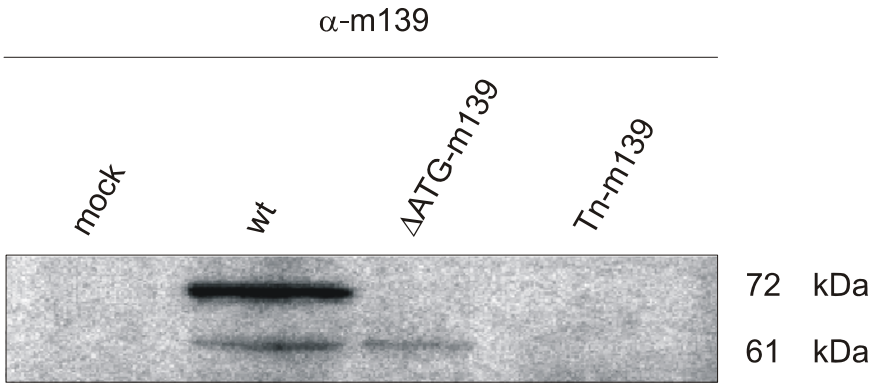

Fig. C 12 Western blot analysis with polyclonal antisera against protein m139. NIH 3T3 cells were infected at an MOI of 1 with wild-type MCMV (wt) or MCMV mutants ATG-m139 and Tn-m139. Cell lysates were harvested 24 h postinfection and separated by polyacrylamide gel electrophoresis. Western blot analysis with rabbit polyclonal antisera against m139 was performed.

3.4 Gene products of m139, m140, and m141 interact on the protein level

These results indicate that the products of genes m139, m140, and m141 interact at the protein level and that the lack of parts of ORF m139 affects the steady-state level of the other proteins of m140 and m141. These data are consistent with previous reports that the m140 and m141 proteins form a stable complex within infected cells (Campbell et al., 2001, Karabekian et al., 2000).

4 Gene m142 is essential for MCMV replication

4.1 Is ORF m142 essential for viral growth?

Genes m139 to m143 belong to a complex transcriptional region. The transcripts of m142 and m143 coterminate downstream of m142 (Fig. C 13). Previous work in our laboratory with transposon mutants showed that three mutant genomes with transposon insertions in m142 and m143 (Tn-m142.A, Tn-m142.B, and Tn-m143) and a targeted deletion mutant of m143 failed to generate infectious progeny (see Menard et al., 2003). Since a transposon insertion into ORF m142 should not have affected the transcription

of the downstream m143 gene, the no-growth-phenotype can be truly attributed to gene m143. But it was not clear, whether the observed lethal phenotype for ORF m142 was due to change in transcription for this gene or due to a destabilized m143 transcript. It is possible that the insertion of the additional 3-kb into gene m142 affected the transcription rate or the transcript stability of the upstream m143 gene. In order to minimize the possible polar effects on m143 mRNA expression, mutagenesis was restricted to the m142 start codon in this thesis. The first ATG was deleted by site-directed mutagenesis of the wild-type MCMV BAC plasmid pSM3fr (See chapters C 1).

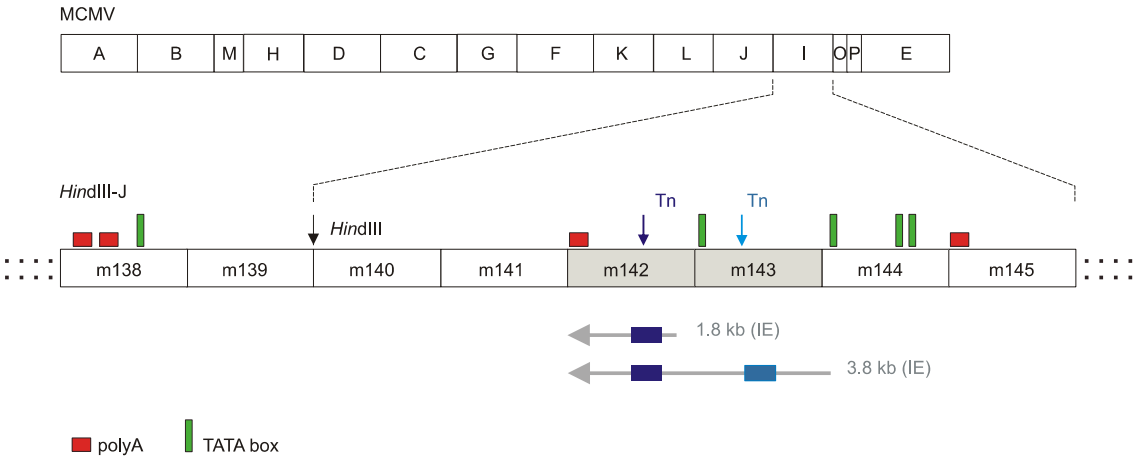

Fig. C 13 Transcript map of genes m142 to m143. The *Hin*dIII map of the MCMV genome is shown at the top. The expanded map of the *Hin*dIII I fragment represents the gene region that encodes for 6 genes including m142 and m143. ORFs are indicated by open and grey boxes. Red and green boxes denote polyadenylation signals and TATA boxes, respectively. Grey arrows denote the major transcripts of genes m142 and m143, according to Hanson et al. (1999b). Only immediate-early (IE) transcripts are shown. Transposon (Tn) insertions into the wild-type MCMV BAC pSM3fr are indicated as blue boxes.

4.2 Construction of site-directed MCMV mutants of ORF m142

Mutant ΔATG-m142 was constructed by site-directed mutagenesis of the parental MCMV BAC genome pSM3fr as described above (see chapter C 1.2, C 1.3, and Fig. C 5). pSM3fr contains the complete MCMV genome cloned into a BAC vector. Its growth

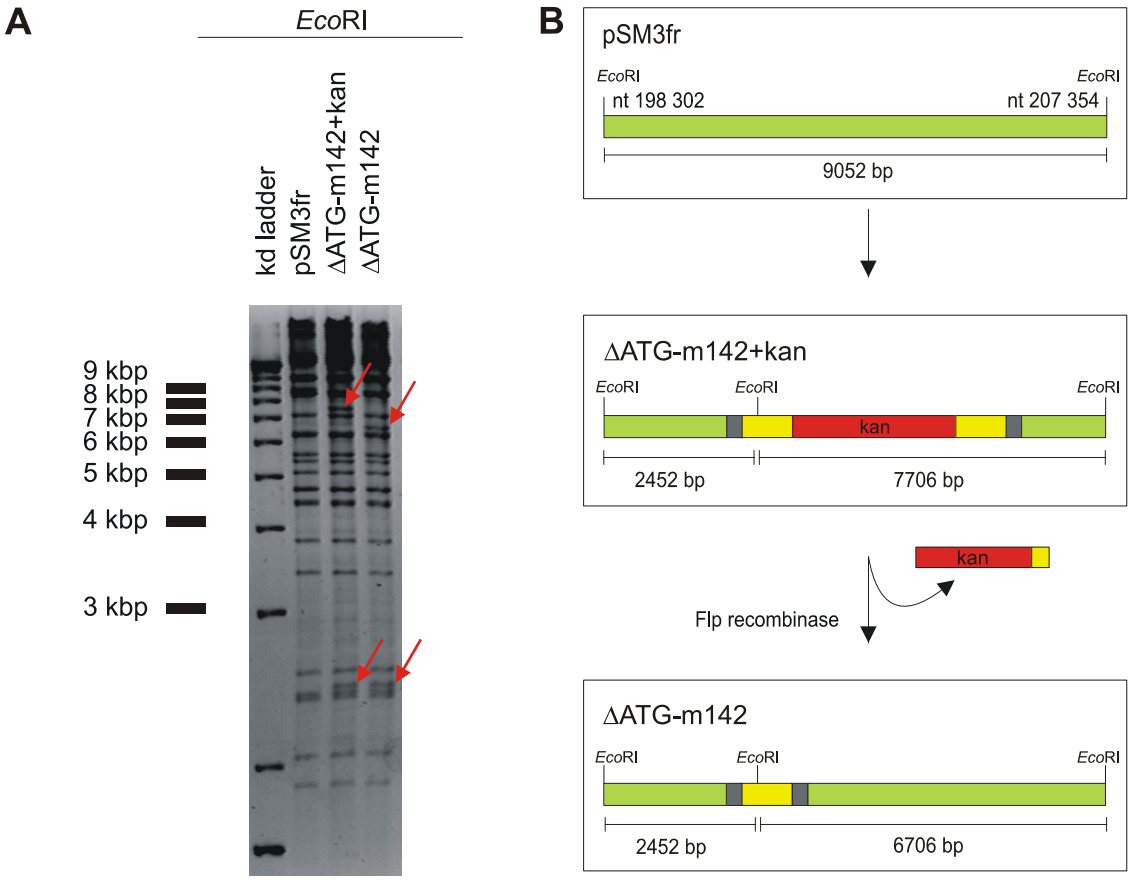

Fig. C 14 Mutant ΔATG-m142/FRT was generated by site-directed mutagenesis of plasmid pSM3fr/GFP. (A) Ethidium bromide-stained agarose gel of *Eco*RI-digested BAC plasmids pSM3fr/GFP, ΔATG-m142 + kan/FRT, and ΔATG-m142/FRT isolated from *E. coli*. Restriction analysis of mutant ΔATG-m142 + kan with *Eco*RI produced additional DNA fragments at 7,706 and 2,452 kbp. After excising the kanamycin cassette, the 7,706 band disappeared and was replaced by a 6,706 kbp DNA fragment. **(B)** *Eco*RI (black line) restriction pattern after excising the kanamycin cassette (red box) from ΔATG-m142 + kan/FRT. The yellow boxes depict FRT sites.

properties are indistinguishable from wild-type MCMV (Wagner et al., 1999) after transfection into permissive cells. In order to be able to insert the entire ORF m142 in future experiments at an alternative position into the MCMV genome the parental virus pSM3fr/GFP was used. pSM3fr/GFP contains the green-fluorescent protein (GFP) and a FRT site at the position of the nonessential m152 gene. The FRT site at this position can be used to introduce a mutation and the expression of the GFP can facilitate the visualization of plaque formation. The pSM3fr/GFP BAC that contains an FRT site and the GFP gene in position of the nonessential gene m152 was first subjected to site-directed mutagenesis to delete the native ATG of ORF m142. The ATG codon was deleted by insertion of a kanamycin marker by PCR based mutagenesis with the same PCR frag-

ment used for the generation of the ΔATG-m142 mutant genome. The kanamycin cassette, flanked by minimal FRT sites, was subsequently excised by the expression of the FLP recombinase. The mutant ΔATG-m142/FRT with the deletion of the first ATG of ORF m142 (nucleotides 200 746, 200 747, 200 748) was constructed. This mutant also contains a GFP marker and a FRT site at the position of the nonessential m152 gene. Thus, the MCMV genome ΔATG-m142 should not be able to encode for functional m142 protein. The structure of the BAC plasmids was analyzed by digestion of plasmid DNA with restriction enzyme *Eco*RI followed by agarose gel electrophoresis (Fig. C 14). The 9.0 kbp *Eco*RI fragment of the parental BAC plasmid pSM3fr/GFP was missing in the BAC plasmid ΔATG-m142+kan/FRT and was replaced by two new fragments of 2.4 and 7.7 kbp. Excision of the kanamycin cassette resulted in a shift of the 7.7 kbp fragment to a new 6.7 kbp fragment and generated ΔATG-m142/FRT. These results show that the intended excision of the kanamycin cassette between the two 34-bp FRT sites was introduced into the MCMV BAC plasmids and that no adventitious deletions or rearrangements could be detected anywhere else in the cloned genome. Specifically, no deletion or inversion was introduced between the 34-bp and 48-bp FRT site.

4.3 ORF m142 has a lethal phenotype

4.3.1 Rescuing essential genes

Genes important for host-virus interaction are not directly involved in replication of the viral genome. These genes are dispensable for replication and are referred to as nonessential genes. Deletion of essential genes does not render viral progeny (Fig. C 15). In order to rescue these viruses, the essential gene has to be introduced in *trans*. Cotransfection of either a small subgenomic fragment overlapping the mutant gene or the introduction of the essential gene ectopically can rescue viral progeny (Fig. C 15 B and C). Subgenomic fragments that contain the disrupted sequence are able to complement the lethal mutation. The wild-type properties are restored by allelic exchange in *E. coli* and viral plaques are readily obtained. In this thesis, both ΔATG-m142 and ΔATG-m142/FRT were tested for viral growth. Mutant ΔATG-m142/FRT was used to demonstrate that ORF m142 is essential for viral growth.

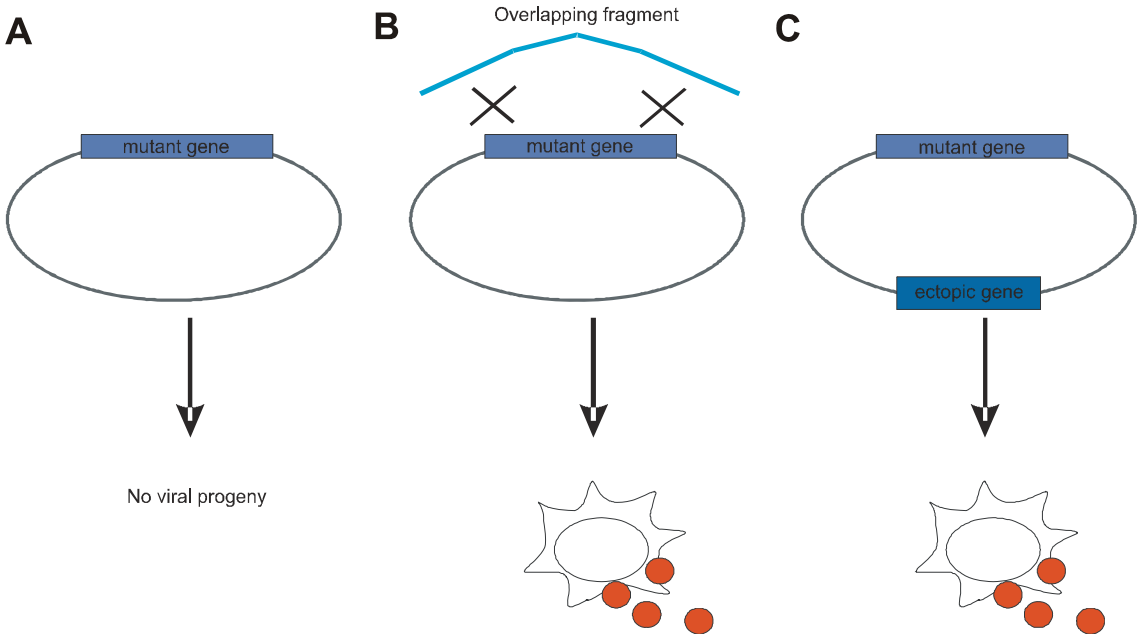

Fig. C 15 Mutants of essential genes. (A) Transfection of mutated MCMV BAC plasmid into permissive cells leads to no viral progeny. **(B)** Cotransfection of small subgenomic fragments overlapping the mutant gene should rescue the defect MCMV BAC plasmid. **(C)** Construction of a revertant MCMV genome by homologous recombination in *E. coli* and transfection into fibroblasts will prove if the affected gene is essential.

4.3.2 Mutant ΔATG-m142 and ΔATG-m142/FRT did not reconstitute viral progeny after transfection

Mutant ΔATG-m142 was transfected into MEFs with calcium-phosphate precipitation and ΔATG-m142/FRT was transfected into NIH3T3 cells with Superfect transfection reagent. Both cell lines are permissive for MCMV infection. Plasmids pSM3fr and pSM3fr/GFP were used as controls for viral infection for ΔATG-m142 and ΔATG-m142/FRT, respectively. The results of the experiments are shown in table C 4. Transfection of the parental BAC plasmid pSM3fr and pSM3fr/GFP reproducibly resulted in the formation of plaques. Plaques usually occurred around day 5 to 10 posttransfection for MEFs and around day 4 to 8 posttransfection on NIH 3T3 fibroblasts, afterwards the infection spread rapidly throughout the monolayers. Cells harbouring the mutant ΔATG-m142 and ΔATG-m142/FRT did not reconstitute viral progeny. These results were observed after multiple attempts and prolonged incubation. Identical results were obtained after transfection of the BAC plasmids into mouse embryonic fibroblasts and

NIH 3T3 fibroblasts. This suggested that ORF m142 is essential for the lytic replication cycle of MCMV.

4.3.3 Fragment *Hin*dIII-I rescued ΔATG-m142/GFP

In a first attempt to prove that the failure of the m142-deficient BAC plasmid to form plaques was due to the disrupted m142 gene, rescue experiments by cotransfection of a subgenomic fragment *Hin*dIII-I were performed. Fragment *Hin*dIII-I, that contains the complete wild-type ORF of m142, was isolated from plasmid pUC19-*Hin*dIII-I by digestion with enzyme *Hin*dIII and gel elution. Recombination between the purified subgenomic fragment and the m142-deficient genome ΔATG-m142/FRT resulted in the reconstitution of replication-proficient genomes. A few plaques appeared after cotransfection of ΔATG-m142/FRT with fragment *Hin*dIII-I. Typically the plaques were first seen at 7 to 8 days post transfection (Tab. C 4). The infection spread slowly throughout the tissue

Tab. C 4. Initial plaque formation after transfection of the MCMV BAC plasmids.

BAC plasmid	ΔATG-m142*	ΔATG-m142/FRT**	ΔATG-m142/m142E**
BAC plasmid only	n***	n***	+++***
Plaque formation	n***	n***	day 6-7
Cotransfection of *Hin*dIII I fragment	+***	++***	-
Plaque formation	day 7-8	day 7-8	-

* Transfection was performed on MEF with calcium-phosphate precipitation.

** Transfection was performed on NIH3T3 with Superfect transfection reagent.

*** Scoring of the transfection was as follows: +++ 10-20 plaques, ++ 5-10 plaques, + 1-5 plaques, n no plaques, - not performed.

culture after occurrence of the first plaques. Transfection with wild-type virus rendered plaques on day 5 which spread quickly. Infected cells in the culture displayed a green fluorescence. Thus, cotransfection ΔATG-m142/FRT with fragment *Hin*dIII-I provided the missing m142 protein. The reduced number of plaques as well as the delayed kinetics in plaque formation was consistent with the expectation that reconstitution of replication–proficient genome by recombination with the complementing subgenomic frag-

ment had to occur prior to plaque formation. Thus, fragment *Hin*dIII-I rescued the mutant virus ΔATG-m142/FRT and led to the production of infectious virus.

4.3.4 The ectopic gene m142 rescued the ΔATG-m142/GFP mutant

In a next step gene m142 was introduced at an ectopic position into the MCMV BAC genome. For this purpose mutant ΔATG-m142/FRT, that contains a GFP marker and a FRT site at the position of the nonessential m152 gene, was constructed by site-directed mutagenesis with the deletion of the first ATG of ORF m142, (see chapter 4.2, Fig. C 14).

For the insertion of ORF m142 at the additional FRT site at ORF m152 of ΔATG-m142/FRT, a plasmid with the entire m142 gene and a promoter and polyadenylation site was constructed (Fig. C16). First, the ORF of m142 was amplified by PCR with primers 200748 up-m142-Eco and 199441down-m142-Xba using fragment *Hin*dIII-I as template DNA. The amplified DNA fragment with ORF m142 was inserted into plasmid pCR3 via the XbaI and *Eco*RI cleavage sites. The *Xba*I cleavage site of pORI6kZeo-m142 was sequenced with primer m142-199541-up and confirmed correct insertion. This plasmid and plasmid pORI6kZeo that contains a 48-bp FRT site, were then submitted to *Drd*I restriction enzyme digestion. After blunt end ligation of these DNA sequences, plasmid pORI6kZeo-m142 with the m142 gene, a promoter, a polyadenylation site and a FRT site was generated. The desired mutant genome was constructed by homologous recombination of the mutant ΔATG-m142/FRT genome and plasmid pORI6kZeo-m142 in *E. coli*. Mutant ΔATG-m142/m142E lacks the m142 at its original position, but has a wild-type copy of the m142 gene instead at the original position of the m152 gene. Correct insertion was analyzed by digestion of plasmid DNA with restriction enzyme *Hin*dIII followed by agarose gel electrophoresis (Fig. C 17). After recombination between ATG-m142/FRT and plasmid pORI6kZeo-m142 two additional bands at 1,9 and 3,6 kbp could be detected.

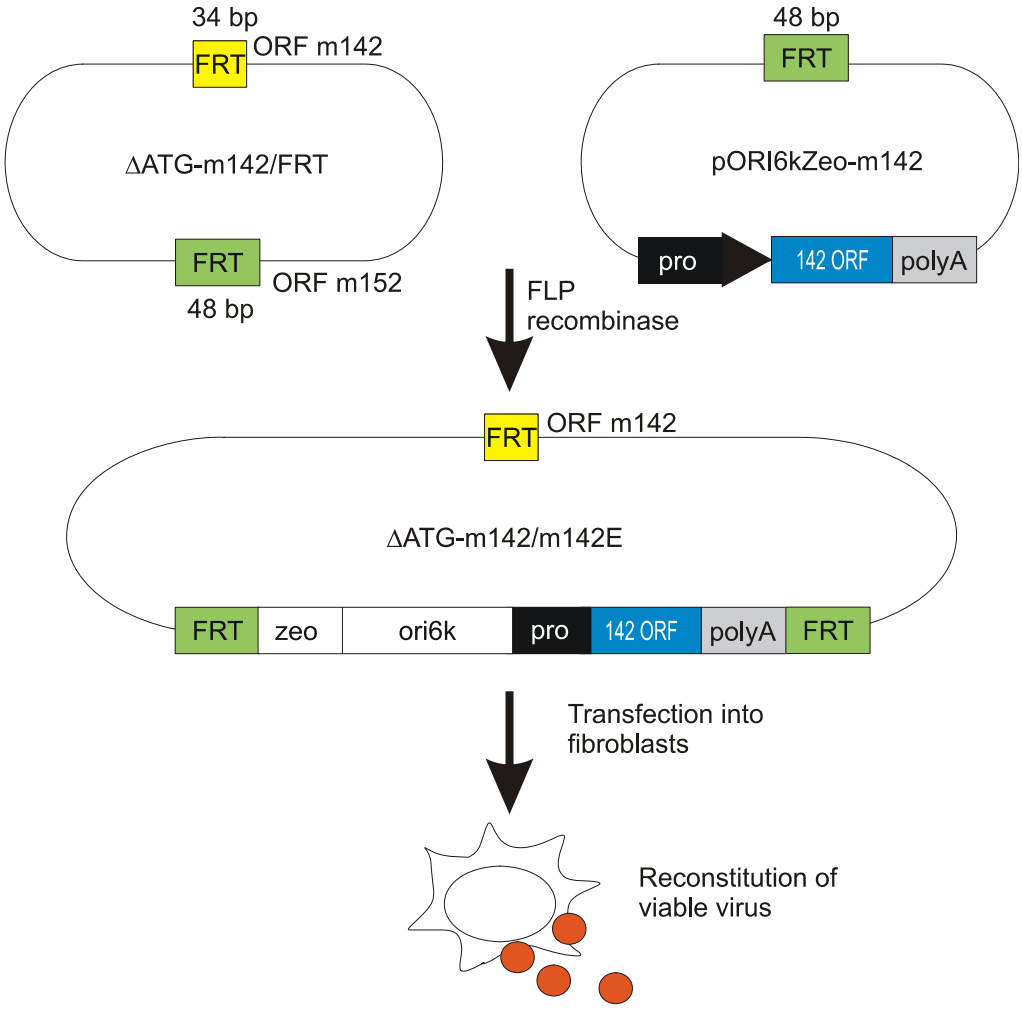

Fig. C 16 Complementation of the ΔATG-m142 genome by insertion of the m142 gene at an ectopic position. The genome of ΔATG-m142/FRT is almost identical to that of ΔATG-m142, but carries a 48-bp FRT site in gene m152. This FRT site was used for insertion of the m142 gene under the control of its native promoter (pro) by FLP recombinase (FLP). Zeocin (Zeo) was used for selection. The resulting genome, ΔATG-m142/m142E, gave rise to viral progeny.

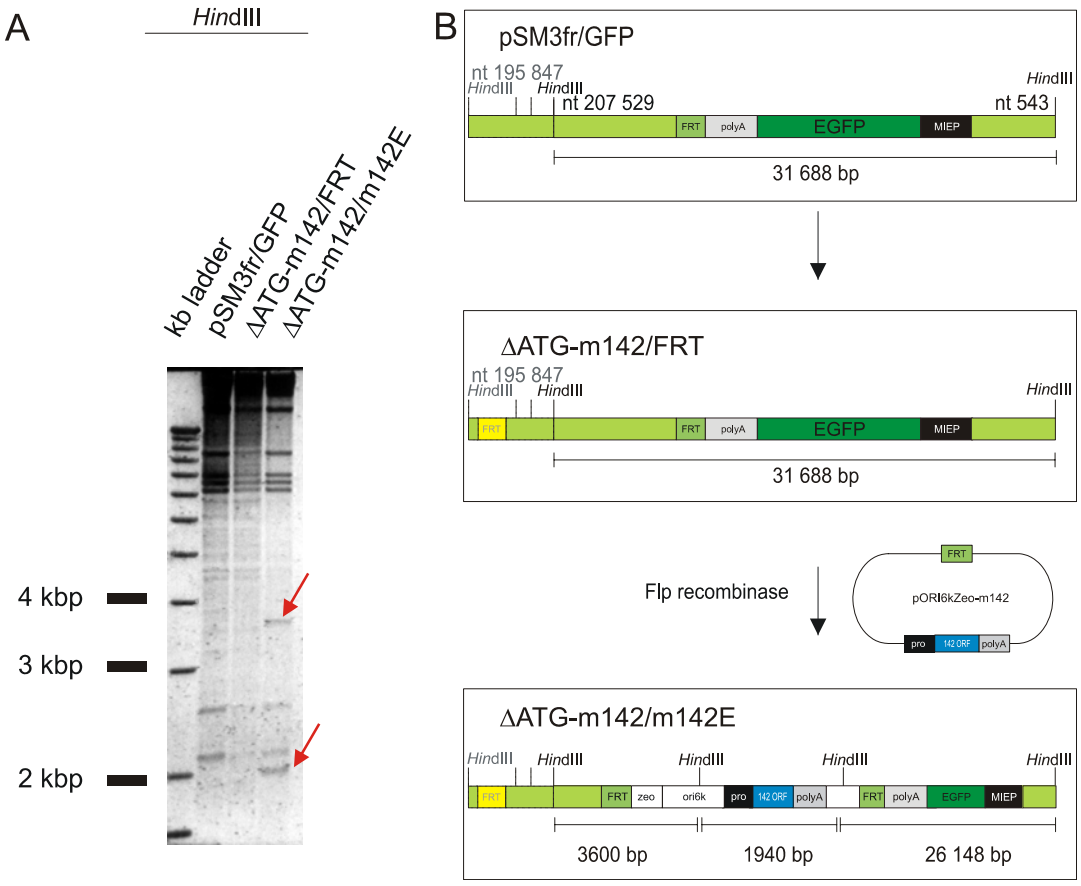

Fig. C 17 Construction of mutant ΔATG-m142/m142E. Ethidium bromide-stained agarose gel of *Hin*dIII digested MCMV BAC plasmids pSM3fr/GFP, ΔATG-m142/FRT, and ΔATG-m142/m142E isolated from *E. coli*. Restriction analysis of mutant ΔATG-m142/m142E with *Hin*dIII detected two additional fragments at 1,940 and 3,600 kbp.

To confirm that m142 is essential for viral growth wild-type pSM3fr/GFP, mutant ΔATG-m142/FRT and ΔATG-m142/m142E were transfected into NIH 3T3 fibroblasts with Superfect transfection reagent. Transfection of NIH3T3 fibroblasts with wild-type pSM3fr/GFP and mutant ΔATG-m142/m142E produced infectious virus at day 5-7 (see Tab. C 4). Once viral plaques had formed infection spread quickly for both the wild-type and the rescue virus. Mutant ΔATG-m142/FRT did not render viral progeny.

Reconstitution of viral progeny after transfection of mutant ΔATG-m142/m142E into fibroblasts proved that the m142 gene that had been inserted at an ectopic position had rescued the missing ORF m142. Thus, ORF m142 is essential for viral growth. The US22 gene homolog family now harbors two genes, m142 and m143, that are essential for viral replication. A new essential gene was identified for MCMV in this thesis.

D. DISCUSSION

1 Targeted and random mutagenesis strategies of BAC cloned herpesvirus genomes

The propagation of herpesvirus genomes as infectious BACs in *E.coli* has made the genes of herpesviruses accessible to the tools of bacterial genetics (Messerle et al., 1997). In the past mutagenesis of large genomes of CMVs was a time consuming task since slow replication in cell culture made mutagenesis by homologous recombination tedious (Mocarski et al., 1996). Approximately 75% of the gene functions of HCMV and MCMV are unknown, leaving 173 ORF of the estimated 230 ORF of HCMV and 128 ORF of the estimated 170 ORF MCMV with an unknown function (Rawlinson et al., 1996). Thus, simple and straightforward methods to generate CMV mutants are vital to characterize the viral functions of MCMV and HCMV.

BACs that contain the MCMV genome can be submitted to techniques of forward or reverse genetics. The aim of forward genetics is to identify mutations that produce a certain phenotype. Once mutants have been isolated, the mutated gene can be molecularly identified. Using transposons that insert into BACs, a forward genetics procedure for MCMV has been developed (Brune et al., 1999). In reverse genetics, as applies to site-directed mutagenesis of BACs, one determines the phenotype that results from mutating a given gene.

Reverse genetic approaches allow candidate genes or genome regions to be tested by the generation of the corresponding deletion mutants. ATG mutants of ORF m139 and m142, both members of the US 22 gene family, were constructed in this thesis to confirm the phenotypes that had been observed for the transposon insertion mutants (see Menard et al., 2003). The applied transposable element had introduced an additional 3-kbp gene sequence that could have possibly increased transcript length and altered protein folding. We hypothesized that this could be omitted by introducing a minimal change, as in the case of the generated ATG mutants. The desired deletion was introduced by homologous recombination between the viral homologies of a linear PCR fragment and the MCMV BAC DNA. Oligonucleotides with a priming region to a template plasmid that contained the kanamycin resistance marker and viral homologies of

were used to amplify the linear PCR fragment. The PCR fragment was then inserted by the flanking 45- to 50-bp viral homologies.

The kanamycin cassette was inserted to select for the rare occurrence of double crossing-over between the corresponding viral sequences. The kanamycin cassette, flanked by short 34-bp FRT sites, was later excised with the Flp recombinase. Flp recombinase recognizes not only 48-bp but also 34-bp long FRT sites (Schlake and Bode, 1994). As described previously homologies of 50 bp were sufficient for successful insertion of the desired mutation (Murphy et al., 1998; Muyers et al., 1999). In contrast, the RecABCD recombination system requires homologies of 2 kbp on both sides for efficient homologous recombination. Since primers larger than 80 nt are costly, difficult to synthesize and PCR with long primers is difficult, primers with a length of 68-73 nt were used in this thesis. This was achieved by incorporating the resistance cassette into the template plasmid, choosing viral homologies not longer than to 45- to 50-bp, and using the shorter 34-bp FRT site.

A recET recombination system which confers homologous recombination between short stretches of 27-60 bp of linear and circular DNA was previously described by Zhang et al. (1998). However, MCMV BACs were not stable in the recET system. Homologous recombination occurred between the 533 bp repeats which were introduced to delete the bacterial sequences in eukaryotic cells. As an alternative, the red recombination system of phage λ, encoded by plasmid pBADαβγ, was used in this thesis (Zhang et al., 1998). This system also confers homologous recombination between short DNA sequences (Szostak et al., 1983). To regulate the expression of the recombinases and thus the recombination activity, the inducible BAD promoter from Invitrogen was introduced. Undesirable recombination between internal repeats was diminished with the induction of the BAD promoter with arabinose. Additionally, the plasmid pBADαβγ expresses the redγ-protein with an exonuclease inhibiting action. That is why it was possible to carry out the mutagenesis in exonuclease positive yet recA negative *E. coli* strain DH10B. To date BACs can be best propagated in DH10B (Shizuya et al., 1992).

But if no candidate gene(s) or gene regions for a specific phenotype are known, unbiased approaches have to be applied. In this case large libraries of mutant genomes must be established. Transposon libraries can provide first insight for analysis of pheno-

types. A library of transposon insertion mutants was constructed for the analysis of the US 22 gene homolog family by Menard et al. (2003). A one-step procedure based on a TnMax16 transposon, bearing the enhanced green fluorescent protein, was used for insertional mutagenesis of MCMV BACs. The distribution of transposon insertion sites was not completely random (Gutermann et al., 2002) and required a collection of 2600-3000 mutants. The mutant genome was easily identified by direct sequencing from primer sites within the transposon (Brune et al., 1999; Hobom et al., 2000; Menard et al., 2003). For rapid recovery of mutant viruses BAC DNA was directly transferred from *E.coli* to mammalian cells (Brune et al., 2001). The transfer of MCMV BAC DNA into eukaryotic cells was conferred by *E.coli* that express the invasion gene of Yersinia pseudotuberculosis and the listeriolysin O gene of Listeria monocytogenes of plasmid pGBΩinv-hly (Grillot-Courvalin et al., 1998). These bacteria invade eukaryotic cells and release the MCMV genome for virus replication. Without knowledge of the transposon insertion site and without previous DNA preparation or transfection, virus mutants can be generated. Viable mutants are easily detected, since they form green fluorescent plaques. This approach allows the random generation of viable MCMV mutants in one step and has already been applied to another herpesvirus, the murine γ-herpesvirus 68 (MHV-68) (Fuchs et al., 2001). The generation of a transposon library does not take more effort than the targeted mutagenesis of about a dozen genes. Transposon libraries can be used to screen viable mutants for the alteration of a specific phenotype and allow the identification of so-far unknown viral gene functions.

With the transposon mutant library one can rapidly determine which MCMV genes are essential for viral growth (Brune et al., 1999) and confirm the observed phenotype with site-directed mutagenesis. Essential genes are of particular importance, as they may serve as targets for antiviral chemotherapy (Benedict et al., 1999). The large size of the herpesvirus genomes is one of the properties that make herpesvirus attractive for gene therapy, and several herpesviruses have been used for gene transfer into mammalian cells (Fink et al., 1997; Messerle et al., 2000; Sclimenti et al., 1998). The nonessential sequences can be replaced by therapeutic genes, and even large genes with their regulatory sequences can be incorporated into herpesvirus vectors (Benedict et al., 1999).

BAC technology has been adapted to many DNA viruses, such as EBV, HSV, HCMV, and MHV-68 (Delecluse et al., 1998; Stavropoulos and Strathdee, 1998; Saeki et al. 1998; Smith and Enquist, 1999; Borst et al., 1999; Adler et al., 2000). Recently, a RNA-virus was cloned as a BAC (Almazan et al., 2000). Thus, BAC technology has introduced a feasible and straightforward method for the mutagenesis of not only herpesviruses and could lead to new developments in clinical virology and basic virology research.

2 Analysis of the MCMV US22 gene family homologs m139 and m142

In the past, constructing, isolating and testing individual mutants of herpesviruses was time consuming. In this thesis site-directed mutagenesis of MCMV BAC DNA, a rapid and straightforward approach, was used to confirm the observations of transposon mutagenesis for mutants of the US 22 gene homolog family. Two members of the US 22 gene homolog family, murine CMV gene m139 and m142, were analyzed.

2.1 Mutant ΔATG-m139 has a macrophage specific growth defect

CMV genes dispensable for growth in NIH3T3 fibroblasts, as seen with the ATG-mutant of ORF m139, are likely to have important functions within the infected host: influencing tissue tropism, dissemination, or immunological responses against the host. These nonessential genes are conserved in herpesviruses, but some may also encode redundant functions in the intact host. The site-directed ATG-mutant of ORF m139 was dispensible for growth in NIH3T3 fibroblasts, but the mutant did not grow to wild-type titers in IC-21 macrophages. Thus, this nonessential gene and member of the US22 gene homolog family seemed to confer a macrophage phenotype.

CMV infection occurs in a variety of cell types, including not only endothelial cells, epithelial cells, stromal cells, neuronal cells, smooth muscle cells, hepatocytes, but also macrophages (Schrier et al., 1985; Sinzger et al., 1995; Soderberg et al., 1993). Macrophages are believed to play a critical role in CMV pathogenesis (Bale and O´Neil, 1989; Brautigam et al., 1979; Collins et al., 1994; Ibanez et al., 1991; Kondo et al., 1994; Saltzman et al., 1988; Schrier et al., 1985; Stoddart et al., 1994; Taylor-Weideman et al., 1991). They have been implicated as sites of CMV latency and persistence (Jarvis et

al., 2002). First, peripheral blood monocytes function to disseminate virus during acute infection (Collins et al., 1994; Stoddart et al., 1994; Taylor-Weideman et al., 1991) and then monocytes and macrophages are the most likely cell types that harbor latent CMV infection (Kondo et al., 1994; Stoddart et al., 1994; Taylor-Weideman et al., 1991). The determinants of MCMV infection in macrophages represent an exciting area of research.

Strict species specificity has hindered the study of HCMV in animals, and infection of mice with MCMV has been used extensively as a model for studying the pathogenesis of CMV infection (Staczek, 1990). Mutations in the viral genome can result in cell tropism for specific cell types. Currently, viral genes associated with growth defect in macrophages have only been identified in the MCMV system. Gene products of M36, M45, m140, and m141 have been shown to be required for normal in vitro replication in macrophages (Brune et al., 2001; Hanson et al., 2001; Hanson et al., 1999a; Hanson et al., 1999b). Among these, genes m140, m141 and M36 are representatives of the US22 gene homolog family. Macrophages are also proposed as 'carrier cells' responsible for the transport of Marek´s disease virus, HHV-6 and MHV-68, all herpesviruses, to lymphoid organs (Barrow et al., 2003; Kondo et al., 2002).

In this thesis, the peritoneal macrophage cell line IC-21 was used to test for macrophage tropism of ORF m139. The IC-21 cell line is a simian virus 40-transformed peritoneal macrophage, which displays properties of a differentiated macrophage, including high phagocytic activity and lysozyme and acid phosphatase production (Mauel et al., 1971). This cell line is highly permissive for growth of wild-type MCMV. Cavanaugh et al. (1996) first reported of a mutant RV7 with genes deleted from the *Hin*dIII-J fragment that grew poorly in this macrophage cell line and not in bone marrow macrophages. The inability of mutant RV7 to replicate to wild-type levels in IC-21 macrophages compared with the bone marrow macrophages was most likely related to differences in the state of differentiation of the two host cells. In mutant RV7 the genes m137, m138, and m139 of *Hin*dIII-J fragment were deleted, in addition to genes m140 and m141 of *Hin*dIII-I. All seven open reading frames have been shown to be nonessential for MCMV replication in NIH3T3 fibroblasts (Vieira et al., 1994). To date only the function of the transcript of m138 is known. It encodes for an Fc receptor for murine immunoglobulin G (Thale et al., 1994).

Preliminary data of Vieira et al. (1994) and Cavanaugh et al. (1996) had first indicated that MCMV *Hin*dIII-J mutants disrupted in ORF m139 grew to high titers in IC-21 macrophages. In subsequent studies Hanson et al. (1999b) could show a macrophage phenotype for a mutant with the deletion of the ORF m139, m140, and m141. In this thesis, mutation of the first two ATGs of m139 affected virus growth on IC-21 macrophages. The difference was in the range of three log units and comparable to the growth differences observed with the transposon and deletion mutant of m139. Thus, not only deletion of the entire ORF m139 or introduction of a transposon mutation into this ORF, but also deletions of minimal sequences of ORF m139 affect its replication in cultured macrophages. Furthermore, the truncated 61-kDa protein could not substitute for the full-length protein and its function.

Apart from the observed macrophage phenotype for ORF m139 little is known about its function. ORF m139 contains all four motifs seen in US22 gene family members (Kouzarides et al., 1988; Nicholas and Martin, 1994). Motifs I and II contain hydrophobic residues. Motifs III and IV are less well defined and have stretches of nonpolar residues (Kouzarides et al., 1988). m139 has an additional acidic domain, common to herpesvirus transcriptional activators, and specifically common to MCMV immediate early proteins 1 and 2 (Cardin et al., 1995; Munch et al., 1992). ORF m139 encodes for a 72 and 68 kDa protein with unknown functions (Hanson et al., 2001) and has been shown to transcribe at early and late times in the nucleus and cytoplasm. Furthermore, the m139 gene product colocalizes to a perinuclear region of the cell juxtaposed to or within the cis-Golgi region, but is excluded from the trans-Golgi region. Gene products of m140 and m141 that are adjacent to m139 are also found at this location. There has been some debate on the interaction of these three genes and their contribution to the macrophage cell tropism.

2.2 Gene products of m139, m140, and m141 interact

To clarify how m139 is responsible for the observed phenotype, the targeted ATG deletion mutant ΔATG-m139 was constructed. Deletion of a short sequence of the ORF m139 should minimize possible polar effects on the mRNA expression of m140 and m141. Since an alternative ATG in frame is located 86 bp downstream of the native ATG, the first 86 bp were deleted by site-directed mutagenesis of the MCMV-BAC.

The deleted sequence was replaced by 86 bp of noncoding sequence after the excision of the kanamycin cassette. Thus, the transcript length was not altered.

All the transcripts characteristic of the 139-141 transcription unit and seen in the wild-type virus, as shown by Hanson et al. (1999b), were present in both fibroblasts and macrophages infected with the ΔATG-m139 mutant (see also Menard et al., 2003). Thus, there does not seem to be a difference in the regulation of IE/E phases of MCMV gene expression in different permissive cells as seen by Kerry et al. (1995) for HCMV in monocytes-derived macrophages. The protein product of ORF m139 must then influence replication in macrophages after transcription. ORF m139 is predicted to transcribe a 72 kDa protein (Rawlinson et al., 1996). In addition to this full-length product, a 61 kDa product has been previously described (Hanson et al., 2001). The ΔATG-m139 mutant produced the alternative smaller 61 kDa m139 product. This protein is probably encoded by the transcript that originates at the third alternative start codon of ORF m139 at nucleotide position 195 767.

In Western blot analysis of the ΔATG-m139 mutant with polyclonal antibodies to m140 and m141 the predicted 56 kDa and 52kDa gene products for m140 and m141 were scant. The mutation of the first two ATGs of ORF must have had an affect on the transcription or translation of the other 3´-collinearily transcribed ORF of m140 and m141. Campbell and colleagues had demonstrated in 2001 an interaction between m140 and m141 at the protein level and showed that mutation of m140 and m141 had an effect on m139, whereas deletion of m139 did not lead to a detectable difference in the levels of the other two protein products. In immunoprecipitation of ΔATG-m139 infected cells, the m141 protein was still synthesized (see also Menard et al., 2003). Nevertheless, the expression of the 61 kDa product did not suffice to stabilize the steady state expression of m140, and m141. Thus, mutagenesis of one of the three genes affects the stability of the other two gene products. Lack of any of these genes affects the steady-state level of the other proteins and causes the common macrophage phenotype. In this work we confirmed that the m140 and 141 gene products act in cooperation and that in contrast to previous observations m139 also influences this protein complex.

It is possible that the Smith substrains kept in the two laboratories have differences in this genomic region. This can be responsible for the divergent results in the two research groups. The described observations may be due to continuous cultivation of cell

lines in vitro without physiological in vivo selection pressure. An alternative explanation for the observed differences is that BAC cloning in *E. coli* requires fewer passages of progeny virus in cell culture than do former methods of cloning. It is possible that the repeated passages required to purify recombinant progeny from wild-type parental virus may select viruses with a compensatory mutation elsewhere in the genome by way of a growth advantage. Resequencing of the MCMV Smith substrains in this region should reveal the differences.

2.3 Gene m142 is essential

Essential genes are of particular importance as they may serve as targets for antiviral chemotherapy. Moreover, inactivation of at least one essential gene is required to create replication-incompetent herpesvirus vectors for gene therapy, while nonessential genes can be removed to gain space for insertion of therapeutic genes (Glorioso et al., 1995) or to generate attenuated vaccines.

The first essential gene in MCMV was described by Messerle et al. in 2000. Messerle and colleagues constructed mutant genomes of gene IE3 by BAC mutagenesis. The ie3-deficient mutant did not replicate in normal mouse fibroblasts, but growth could be restored by a complementing cell line that provided the IE3 protein in *trans*. Since then no other essential MCMV gene has been described.

Cavanaugh et al. (1996) showed that mutant MCMV RV9 with a deletion spanning genes m137 through m143 could not be purified from wild-type virus, while mutants deleted of m137 through m141 were replication competent. This data suggested that either m142 or m143, as individual genes or in combination, might be essential for viral growth.

Menard et al. (2003) could show that viral progeny could not be reconstituted for the transposon mutants of ORF 142 and m143 (mutants Tn-m142 and Tn-m143). In addition to the Tn-m142 and Tn-m143 mutant genomes, a deletion mutant of the complete m143 ORF also failed to produce viral progeny. The transcripts derived from the m142 to m144 region use a common polyadenylation signal downstream of m142 (Hanson et al., 1999 b). It is therefore possible that transposon mutagenesis affects the neighboring ORF. Transcription of m142 is probably not affected by transposon insertion into m143

since m142 and m143 have independent transcripts and transposon insertion in the m143 mutant is located approximately 1.5 kb upstream of the m142 start ATG. It was not clear whether the transposon insertion within m142 could result in destabilization of the m143 transcript. That is why the lethal phenotype was controlled by a targeted mutant of ORF m142.

The first ATG of ORF m142 was deleted and replaced by an 86-bp extraneous sequence. This ATG mutant of the m142 ORF, which should not affect the m143 transcript, failed to produce viral progeny. A revertant virus in which the m142 ORF was restored was able to replicate with wild-type kinetics. This provided evidence that the defective growth phenotype of the m142 mutant was due to disruption in the m142 gene. The m142 revertant, which contained the m142 gene in an ectopic position, rescued virus growth. Thus, not only is m143, but also m142, essential for virus replication. These two genes form a new subfamily of essential US22 family genes expressed at immediate-early times after infection.

Still, not much is known about gene m142 apart from the fact that it is transcribed with immediate-early kinetics. Transcription of IE genes is carried out by the cellular RNA polymerase II and is not dependent on *de novo* synthesis of viral proteins. Viral transactivator proteins that are synthesized during the IE phase activate transcription of early genes and give rise to a more extensive gene expression program during the early phase of the replication cycle. However, a transactivating function has not been shown for m142. This makes it unique among the immediate-early genes of the US 22 gene homolog family. It also lacks motif II, which is present in all other MCMV US22 gene family members. Hanson et al. (2005) could show that the m142 gene product is not expressed until early times post infection and that the protein pm142 is predominantly cytoplasmatic and did not co-localize with markers for either the ER or Golgi. Protein pm142 was present in the nucleus as well and the virion and is predicted to be membrane-associated by sequence analysis.

The closest homolog in HCMV is US26. The m142 and m143 gene homologs in HCMV, US 23 and US 26, have not been classified as essential genes in functional transposon mutagenesis, but as augmenting genes (Dong et al., 2003). It is possible that these genes are actually essential, but the mutants in this study replicated to a limited extent due to the accumulation of a truncated protein, although the insertions were made

in the N-terminal region of ORFs. For the HCMV strain AD169 41 essential , 88 essential and 27 ORFs whose products augment viral growth in fibroblasts have been identified. In general, the essential and augmenting ORFs were located in the central region and the nonessential ORFs cluster at the ends of the viral genome. Over 90 % of the essential genes are conserved among all herpesviruses or beta herpesviruses (Dunn et al., 2003). These core genes represent the minimal genome of all herpesviruses. HCMV may have evolved from the progenitor genome through the acquisition of nonessential genes that are responsible of its infection and pathogeneses in various tissues. The functions of most of the essential gene products still have to be analyzed.

E. SUMMARY

Human cytomegalovirus is a ubiquitous human pathogen, causing disease in the immunocompromised host. Most of its ORFs have not been well studied due to a limited host range and slow growth of HCMV in cultured cells. MCMV, a natural pathogen isolated from mice, constitutes the most amenable animal model for human β-herpesviruses. To date most of its approximately 200 genes have an unknown function. For the analysis of these genes straightforward mutagenesis methods are necessary. With the cloning of herpesviruses as an infectious bacterial artificial chromosome a novel approach of mutagenesis has been established. Herpesviruses are now accessible to the tools of bacterial genetics. Since then site-directed mutagenesis by homologous recombination using linear DNA fragments and random transposon BAC mutagenesis have been introduced to delineate the functions of viral ORFs.

The purpose of this work was to analyze two members of the US 22 gene homolog family, genes m139 and m142, with site-directed mutagenesis. Members of this family are conserved in all herpesviruses and mostly have unknown functions. Transposon mutants showed a macrophage phenotype for m139, whereas m142 was possibly essential for viral replication. Genes m139-m141 and m142-m143 have complex transcriptional regions and have 3´-coterminal transcripts. The insertion of a 3-kb large transposon could destabilize the upstream transcripts. Site-directed mutants of genes m139 and m142, where only the start codon is deleted, should not influence transcript stability and permit confirmation of the results obtained with transposon mutagenesis.

Targeted mutants of MCMV BAC were constructed for ORF m139 (ΔATG-m139) and m142 (ΔATG-m142, ΔATG-m142/FRT) by homologous recombination using linear DNA fragments. Mutant ΔATG-m139 showed attenuated growth in peritoneal macrophages. This mutant, with the first two ATGs deleted, expressed a truncated protein of 61 kDa. Gene m139 seems to act in cooperation with genes m140 and m141 on the protein level. The site-directed MCMV BAC mutant of ORF m142 on the other hand could not reconstitute viral progeny in eukaryotic cells. The ORF of m142 was inserted an ectopic position and viral progeny was reconstituted with this revertant. Thus, it was shown that gene m142 is essential for viral replication.

Further analysis of nonessential and essential genes of cytomegalovirus will be needed to understand CMV viral pathology and to develop vaccines for herpesvirus infection and vectors for gene therapy.

E. ZUSAMMENFASSUNG

Humanes Zytomegalievirus ist ein human pathogenes Virus, das vor allem bei Personen mit eingeschränktem oder unreifem Immunsystem zur Erkrankung führt. Aufgrund seiner strikten Speziesspezifität kann HCMV nur Menschen infizieren und ist damit ungeeignet für die Infektion im Mausmodell. Daher wird murines CMV benützt, um die Funktion der Gene des größten Herpesvirus zu erforschen. Die Mehrzahl der etwa 200 CMV-Gene wurde noch nicht charakterisiert. Mit der Klonierung des gesamten CMV-Genoms in ein bakterielles künstliches Chromosom (BAC) wurde eine neue Mutagenesemethode basierend auf homologer Rekombination in *E. coli* etabliert. Herpesviren sind jetzt den Werkzeugen der Bakteriengenetik zugänglich.

Im Rahmen dieser Arbeit wurden zwei Mitglieder der US22 Genfamilie, m139 und m142, mit zielgerichteter BAC-Mutagenese analysiert. Mitglieder dieser Genfamilie sind in allen Herpesviren konserviert und haben eine noch unbekannte Funktion. Transposonmutanten dieser Gene zeigten einen Makrophagenphänotyp für das Gen m139 und eine mögliche Essentialität für die Virusreplikation für das Gen m142. Gene m139 bis m141 und m142 bis m143 haben eine komplexe Transkriptionsregion. Die Insertion eines 3-kb großen Transposons könnte die Transkription eines aufwärts transkribierten Gens einschränken. Deshalb wurden Mutanten, die lediglich eine Deletion des Startkodons tragen, hergestellt.

Zielgerichtete Mutanten wurden durch homologe Rekombination zwischen einem linearen DNA Fragment und dem MCMV BAC Genom für m139 und m142 hergestellt. Mutante m139 zeigte attenuiertes Wachstum für peritoneale Makrophagen. Diese Mutante, bei der die ersten beiden ATGs deletiert wurden, exprimiert ein verkürztes 61 kDa Protein. Dabei scheinen Gene m139, m140 und m141 in Kooperation zu arbeiten. Zur Bestätigung, dass ORF m142 essentiell für die Replikation von MCMV ist, wurde das Startkodon von ORF m142 deletiert und der Offene Leserahmen von m142 an einer anderen Stelle im BAC CMV Genom inseriert. Mit dieser Mutante konnte das Virus rekonstitutiert werden. Damit ist neben m143 auch m142 essentiell für die virale Replikation von MCMV.

Weitere Untersuchung von essentiellen und nicht-essentiellen Genen sind notwendig, um die Pathogenese von CMV zu erforschen, und um in Zukunft neue Impfstoffe und Vektoren für die Gentherapie entwickeln zu können.

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G. ABBREVIATIONS

AS	amino acid
ATCC	American type culture collection
BAC	bacterial artificial chromosome
bp	base pairs
cam	chloramphenicol
CMV	cytomegalovirus
CTL	cytotoxic T-cells
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco´s modified Eagle´s medium
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
Fig.	figure
FRT	FLP recombinase target
GFP	green fluorescent protein
gp	glycoprotein
HCMV	human CMV
HHV-6, 7, 8	human herpesvirus-6, 7, 8
HSV	herpes simplex virus
IFN	interferon
IL	interleukin
KAc	potassium acetate
Kan	kanamycin
KCl	potassium chloride
kb	kilobase pairs
kDa	kilo dalton
M	molar
Mbp	mega base pairs
ml	mililiter
mM	milimolar
MCMV	murine CMV

MEF	murine embryo fibroblasts
MHC	major histocompatibility complex
MHV-68	murine herpesvirus 68
MOI	multiplicity of infection
NK	natural killer
NCS	new born calf serum
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
PEC	peritoneal exudate cells
PFU	plaques forming units
pi	post infection
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Tab.	table
Tn	transposon
VZV	Varicella zoster virus

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ERKLÄRUNG

Hiermit erkläre ich, Karina Holak, dass die vorliegende Arbeit selbstständig verfasst und keinen anderen als die angegebenen Hilfsmittel verwendet habe. Die aus fremden Quellen stammenden Daten sind als solche kenntlich gemacht. Die vorliegende Arbeit wurde bisher noch keiner anderen Prüfungsbehörde vorgelegt.

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