Functional analysis of the murine cytomegalovirus genes m142 and m143

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Aknowledgment

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Summary

Human cytomegalovirus (HCMV) infection causes clinical symptoms in immunocompromised individuals such as transplantant recipients and AIDS patients. The virus is also responsible for severe complications in unborn children and young infants. The species specificity of HCMV prevents the direct study of mechanisms controlling the infection in animal models. Instead, the murine cytomegalovirus (MCMV) is used as a model system. Human and murine CMVs have large double-stranded DNA genomes, encoding nearly 170 genes. About 30% of the genes are committed to essential tasks of the virus. The remaining genes are involved in virus pathogenesis or host interaction and are dispensable for virus replication. The CMV genes are classified in gene families, based on sequence homology.

In the present work, the function of two genes of the US22 gene family was analyzed. The MCMV genes m142 and m143 are the only members of this family that are essential for virus replication. These genes also differ from the remaining ten US22 gene family members in that they lack 1 of 4 conserved sequence motifs that are characteristic of this family. The same conserved motif is missing in the HCMV US22 family members TRS1 and IRS1, suggesting a possible functional homology.

To demonstrate an essential role of m142 and m143, the genes were deleted from the MCMV genome, and the mutants were reconstituted on complementing cells. Infection of non-complementing cells with the deletion mutants did not result in virus replication. Virus growth was rescued by reinsertion of the corresponding genes. Cells infected with the viral deletion mutants synthesized reduced amounts of viral DNA, and viral late genes were not expressed. However, RNA analyses showed that late transcripts were present, excluding a role of m142 and m143 in regulation of gene transcription. Metabolic labelling experiments showed that total protein synthesis at late times postinfection was impaired in cells infected with deletion mutants. Moreover, the dsRNA-dependent protein kinase R (PKR) and its target protein, the translation initiation factor 2α (eIF2α) were phosphorylated in these cells. This suggested that the m142 and m143 are required for blocking the PKR-mediated shut-down of protein synthesis. Expression of the HCMV gene TRS1, a known inhibitor of PKR activation, rescued the replication of the deletion mutants, supporting the observation that m142 and m143 are required to inhibit this innate immune response of the host cell.

Zusammenfassung

Die Infektion mit dem humanen Cytomegalovirus (HCMV) kann bei immunsupprimierten Personen wie Transplantatempfängern oder AIDS Patienten, aber auch bei Neugeborenen klinische Symptome hervorrufen. Die Spezies-Spezifität des humanen CMV lässt keine Untersuchung viraler Mechanismen im Tiermodell zu, jedoch steht mit dem murinen CMV (MCMV) ein geeignetes und verbreitetes Modell zur Verfügung. Beide CMVs besitzen große doppelsträngige DNA Genome, die ca. 170 Gene beinhalten. Hiervon sind ca. 30% essentiell für die virale Replikation. Die anderen Gene sind für die Pathogenesse und Interaktion mit den Wirtszellen von Bedeutung. Die Gene des CMV werden auf Grund von Sequenzhomologien in Familien gruppiert. In der vorliegenden Arbeit wird die Funktion der Gene m142 und m143 des MCMV analysiert. Beide Gene sind die einzigen für die Virusreplikation essentiellen Mitglieder der US22 Genfamilie. Darüber hinaus unterscheiden sie sich von den anderen 10 US22 Mitgliedern darin, daß ihnen eine von vier konservierten Sequenzmotiven fehlt. Dieses fehlende Motiv kommt auch bei den HCMV US22 Mitgliedern TRS1 und IRS1 nicht vor, was einen möglichen Hinweis auf eine funktionelle Homologie gibt.

Um die essentielle Rolle der m142 und m143 Gene zu belegen, wurden letztere aus dem MCMV Genom entfernt und die Virusmutanten auf komplimentierenden Zellen rekonstituiert. Die Infektion nicht komplimentierender Zellen mit den Virusmutanten erzeugte keine Infektion, konnte jedoch mit der Reinsertion der Gene wieder hergestellt werden. Infizierte Zellen, die mit den Virusmutanten infiziert wurden, produzierten geringere Mengen viraler DNA. Obwohl die Expression später viraler Gene nicht stattfand, konnten späte virale Transkripte nachgewiesen und somit eine Rolle von m142 und m143 bei der Regulation der viralen Transkription ausgeschlossen werden. In Experimenten, in denen Zellen metabolisch markiert wurden, wurde gezeigt, daß die Gesamtproteinsynthese zu späten Zeitpunkten nach Infektion mit den Virusmutanten gehemmt war. Des weiteren wurde eine Phosphorylierung der dsRNA-abhängigen Proteinkinase R (PKR) sowie des Zielproteins, des Translations Initiationsfaktors 2α (eIF2α), nachgewiesen. Dies läßt vermuten, daß m142 und m143 die PKR-vermittelte Stillegung der Proteinsynthese verhindern. Durch Expression des HCMV TRS1 Gens, einem bekannten Inhibitor der PKR-Aktivierung, konnte die Replikation der

Virusmutanten wieder hergestellt werden. Dies unterstützt die Ansicht, daß m142 und m143 für die Inhibition der Angeborenen Immunanwort der infizierten Wirtszelle erforderlich sind.

1 INTRODUCTION

1.1 Cytomegalovirus infection

Human cytomegalovirus (HCMV, family *Herpesviridae*, subfamily *Betaherpesvirinae*, genus *Cytomegalovirus*, species *Human cytomegalovirus*) is a common pathogen world wide. CMV spreads at an early age and infects a large majority of the population, nearly 60-80% are affected (Trincado et al., 2001). The prevalence of infection and age at initial acquisition of the virus vary according to the living circumstances. CMV is not highly contagious and requires direct contact with infectious material. In many cases, transmission from mother to foetus or newborn occurs during birth. The vertical mode of transmission plays an important role in maintaining CMV infection in the population. Clinical features of the infection vary according to the mode of transmission. The virus can cause severe damage to the central nervous system (CNS) in congenital infection. Hepatomegaly and pneumonitis are associated with CMV infection in young infants.

HCMV is one of the most important opportunistic pathogens that complicate the care of immunocompromised patients. Infection may occur because of reactivation of latent virus (organ transplantant recipients), re-infection, or primary infection (HIV patients). The infection correlates with the degree of immunosupression. The most severe cases are found in patients with allogeneic transplantation or the acquired immunodeficiency syndrome (AIDS). CMV infection is generally asymptomatic in immunocompetent individuals but it can cause infectious mononucleosis and it has been associated with vascular diseases (Nerheim et al., 2004; Melnick et al., 1993). Immunocompromised patients can develop a multisystem disease that might be life-threatening.

Studies on HCMV pathogenesis are important to develop an effective therapy and prevention. Because of the strict species specificity, HCMV has never been

successfully introduced into another animal. Therefore closely related CMVs with rodent and primate hosts are used as model systems: murine, guinea pig, rat, and rhesus macaque cytomegalovirus. Murine CMV is the most widely studied infection model. Viral functions influencing appoptosis, cytokine activation, leukocyte recruitment, lymphocyte surveillance, and antibody recognition have been characterised in both human and murine CMV, and many have been observed to influence the outcome of infection in mice. Murine cytomegalovirus (MCMV) and HCMV share similar structural features and pathogenic properties. Both viruses cause acute or persistent latent infections, depending on the immune status of the host. The infection of mice with MCMV provides a useful model for studying CMV pathogenesis and the host immune response against cytomegalovirus agents (Hudson, et al., 1979; Ho, 1995). However, MCMV and HCMV have some biological differences, as transplacental transmission of MCMV has not been demonstrated, and mouse models of foetal infection involve direct inoculation of MCMV into the CNS or uterus (Tsutsui et al., 1993).

1.2 CMV virion

1.2.1 Virion components

The cytomegalovirus virion consists of an icosahedral capsid and a 235 kb dsDNA genome, surrounded by a tegument, and enveloped with a lipid bilayer carrying various virus-encoded glycoproteins (Fig 1).

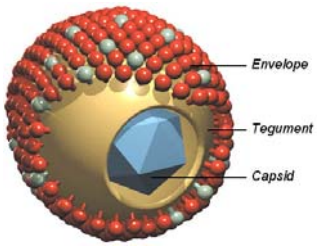


Fig. 1. CMV virion.

HCMV and MCMV have a linear double-stranded DNA genome. Unlike other DNA viruses, CMV contains two types of RNA. One is tightly packed with the DNA at the origin of replication (Prichard et al., 1998) and the other is located at the tegument, expressed after entry into the cell. The HCMV genome consists of two covalently linked segments: unique long and unique short (UL and US) region, flanked by repeated terminals *b* and *c* and inverted sequences *b'* and *c'* (Fig 2). The directly repeated sequence *a* is also present at the termini and in inverted orientation at the junction between UL and US. The genomes of other β–herpesviruses are linear and

lack internal repeats. The MCMV genome consists of a single unique sequence with direct repeats at either end (Ebeling et al., 1983).

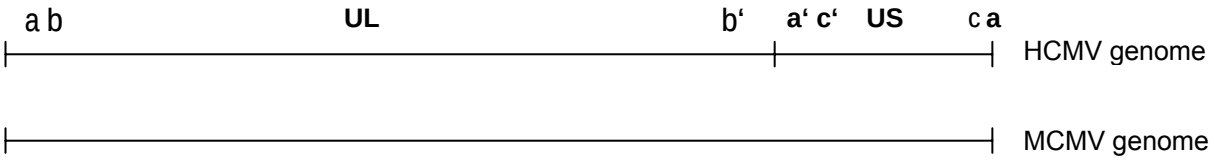


Fig. 2. Genome organisation of HCMV and MCMV.

Sequence analysis of HCMV revealed a high divergence rate among different strains. The consensus sequence representing wild type HCMV is based on data from several low passage strains as well as clinical isolates (Dolan et al., 2004). The complete MCMV sequence was done for the Smith strain, which consists of 230 kb and about 170 predicted genes. The genome is essentially colinear with HCMV over the central 180 000 bp. During evolution, duplications of viral genes occurred and formed families of ORFs, common for all β-herpesviruses. A typical feature of this subfamily is the high amino acid homology. The gene families are characterised by the presence of certain conserved motifs (Fig 3).

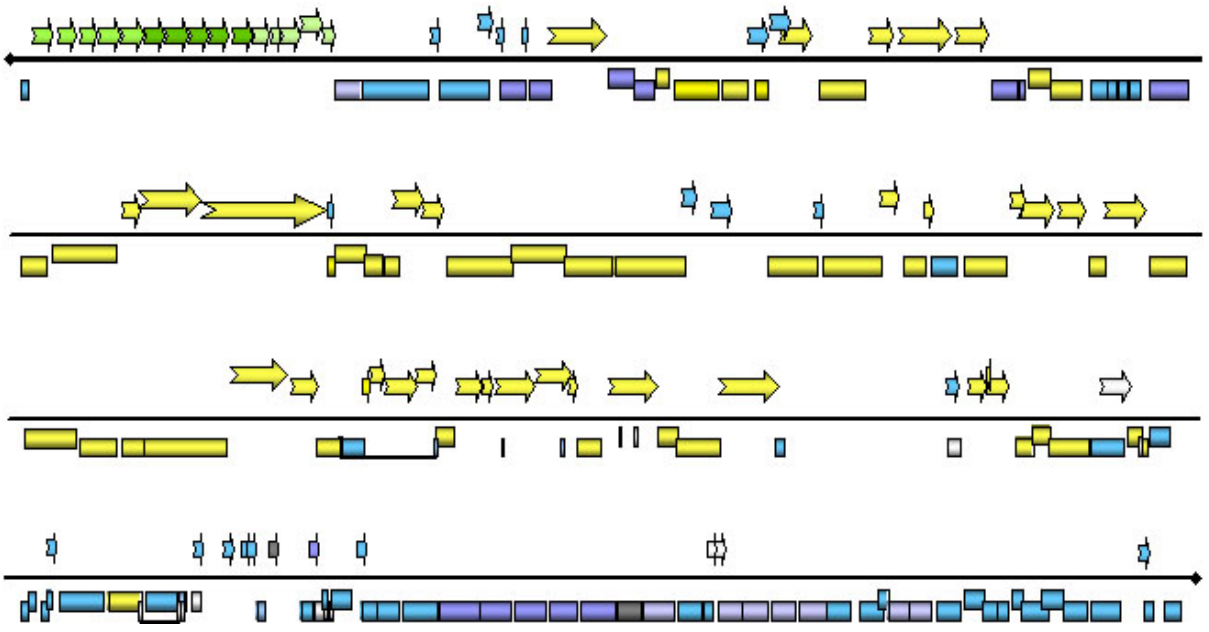


Fig. 3. Genetic map of MCMV. The colours represent dofferent gene families.

Genome analysis revealed that about 30% of the encoded genes are committed to essential tasks of replication (Mocarski., 2004). These are herpesvirus-common genes, found in all characterised mammalian and avian herpesviruses

(Davison et al., 2002). They form seven conserved blocks. The arrangement of the conserved sequences is unique to the β-herpesviruses (Fig 4).

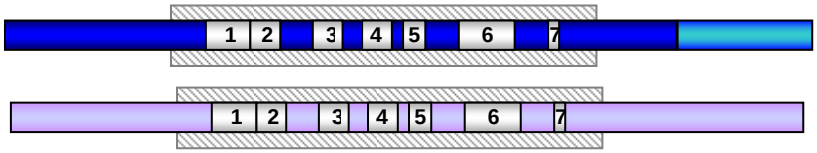


Fig. 4. Conserved sequence blocks in HCMV and MCMV.

Although the analysis of individual genes in HCMV and MCMV is still incomplete, it is known that nearly 70 ORFs are dispensable for virus replication in cell culture. This fact indicates that a large number of viral genes are devoted to optimising virus growth, cell tropism and pathogenesis in the host. About 46 genes are predicted to be nonessential as a result of spontaneous deletion during passaging in cell culture.

1.2.2 Virus replication

During natural infection HCMV replicates in various cell types: epithelial, macrophages, monocytes, endothelial, differentiated fibroblasts, smooth muscle cells, neuron and hepatocyte cells. However, in cell culture it has a restricted host range, and only few cell lines support productive replication (Sinzger et al., 1995). HCMV was isolated and propagated on human foreskin fibroblasts, which were shown to be most productive. Undifferentiated, transformed or aneuploid cell are non-permissive.

The replication cycle begins with viral attachment and penetration. Initially, the virus interacts with heparan sulphate proteoglycan complexes on the cell surface via virus-encoded glycoprotein complex gM/gN and binds to the epithelial growth factor receptor (EGFR) through another virus-encoded glycoprotein gB (Wang et al., 2003). However, not all permissive cells express EGFR, suggesting an involvement of additional receptors, most likely other growth factor receptors. Ultimately, the virus fuses with the plasma membrane, depositing the virion particles in the cell cytoplasm. The heterotrimeric complexes gH/gL/gO and gB are required for membrane fusion. Additionally, cellular integrins are known to serve as co-receptors. They interact with

EGFR and synergise with signal pathways. On the other hand, the interaction of gB with certain β1-integrins plays a crucial role for the entry (Compton, 2003, 2004).

Viral entry results in activation of cellular metabolism. Several signalling pathways are affected, including Ca^{2+} homeostasis, activation of arachidonic acid and its metabolites (Fortunato et al., 2000). The same effect is observed when UV-inactivated virus enters the cell, suggesting that structural components of the virus are responsible for activation during virus-cell contact and/or virus entry. Entry activates also cellular transcription factors – cfos/jun, myc, NF-kB, SP-1, mitogen activated protein kinases ERK1, ERK2 and p38 (Kowalik et al., 1993; Yurochko et al., 1995; Boyle et al., 1999; Boldogh et al., 1991). The virus-induced changes alter the host cell gene transcription. Mostly these changes do not require virus gene expression (Browne 2001; Simmen et al., 2001). Activation of host cell functions upon HCMV infection is associated with progression of the cell cycle and optimisation of the environment for virus replication.

After entering the cell, virus replication starts supported by the cellular machinery. Transcription of viral genes is directed by host cell RNA polymerase II and the associated machinery. This process is regulated by virus-encoded transactivators that are able to modulate virus gene as well as host gene transcription. Viral gene transcription is initiated also by EGFR, which has a strong mitogenic activity. HCMV has two major loci that are subjected for transcriptional control. One is the ie1/ie2 region which encodes a family of regulatory proteins (major immediate early complex – MIE) from differentially spliced transcripts and uses one of the strongest enhancers among the mammalians. The other is a sequence upstream of the US3 gene. Both have repetitive sequences and binding sites for cellular transcriptional factors. They are subject to negative regulation via the virus-encoded protein IE2 and host factors. Suppression occurs with progression of the infection and is an independent process from viral gene activation. Similarly, the MCMV IE3 regulates gene expression by activation of early promoters and is able to repress transcription from the MCMV MIE (Angulo et al., 2000). Several tegument proteins are involved in transcription initiation together with host factors. One of the abundant tegument proteins, UL82 is a general activator of MIE (Liu, et al., 1991, Baldick et al., 1997). Others cooperate with viral regulatory factors and act as co-transcription activators, such as pp69 and TRS1/IRS1, to ensure expression of the viral genes.

CMV gene expression is temporally regulated and classified in three kinetic classes, depending on the time and sensitivity to different inhibitors (DNA, RNA and protein synthesis). The first genes expressed are immediate early (α) genes. They are independent of any newly synthesized viral proteins and some of them encode regulatory *trans*-acting factors. The next genes expressed are early (β) genes. They require the presence of functional IE products. Some β gene products encode proteins which have a role in DNA replication, DNA repair or immune evasion. The third kinetic class are the late (γ) genes. They are expressed after the onset of DNA replication. The products of late genes are involved in modulating immune responses (envelope glycoproteins) or have a structural role (capsid proteins).

α genes. Immediate-early proteins are produced first and regulate the subsequent early protein synthesis, DNA replication, and late protein synthesis. Four regions of α gene expression have been mapped on the human CMV genome: ie1/ie2 (major immediate early complex, MIEP), UL36 and UL37, TRS1/IRS1 and US3. Five exons are encoded downstream of the MIEP. The first three exons are spliced to either exon 4, generating the *ie1* transcript, or to exon 5, generating the *ie2* transcript. In HCMV, the *ie1* transcript is translated into the acidic 72-kDa IE1 phosphoprotein. The HCMV *ie2* transcript gives rise to the 86-kDa IE2 phosphoprotein. The corresponding IE transcripts of MCMV encode the 89-kDa acidic IE1 phosphoprotein pp89 (Keil et al., 1987) and the 88-kDa IE3 protein (Messerle et al., 1992). Both IE1 and IE2 are involved in regulation of viral genes expression. IE1 augments its own expression by positive autoregulation of the MIEP. It also has a costimulatory function in activation of early gene promoters and mediates the disruption of nuclear structures (promyelocytic leukaemia protein-PML-associated nuclear bodies or nuclear domains ND10) to ensure an optimal environment for viral replication. The IE2 protein is a transactivator of early genes and also of heterologous viral and cellular genes. It governs late gene expression as well (Mocarski et al., 1996; Stenberg, 1996). IE2 down-regulates transcription from its own promoter by binding to the *cis*-repression signal (*crs*) target site near the transcription start site of *ie1/ie2*, thereby mediating autoregulation of its own expression (Lang and Stamminger, 1993; Macias and Stinski, 1993; Wu et al, 1993). Moreover, it was reported that IE2 has a role in immune evasion by blocking IFNβ expression (Taylor and Bresnahan, 2005, 2006).

All studied animal CMVs have an analogous MIE locus, but they vary depending on the presence of additional α genes. Some ancillary α products contribute to the regulation of gene expression or have different roles. Two IE products, TRS1 and/or IRS1 have been shown in transient assays to cooperate with IE1 and IE2 proteins and activate the transcription of early gene promoters. Later studies have shown that these genes are involved in immune evasion and virus assembly. The immediate early gene US3 encodes differentially spliced products, which are endoplasmatic reticulum-resident and block egress of peptide-loaded MHC class I proteins, representing a very early mechanism of immune evasion (Ahn et al, 1996, Jones and Muzithras, 1992). Another two IE gene products, UL36 and UL37 are involved in blocking apoptosis. UL37 encodes a viral analogue of the cellular antiapoptotic protein Bcl2 which can inhibit the downstream caspase 9 pathway (Goldmacher, 1999, 2001). By conrast, the UL36 gene product inhibits apoptosis by blocking caspase 8 activation. The synergetic work of all α genes ensures effective productive replication as it triggers the expression of further viral genes.

β genes. Early proteins are required for viral DNA synthesis, cleavage and packaging of viral genomes, and assembly of viral particles. Additionally, they have a role in maintaining an optimal environment for viral gene expression and DNA replication (McElroy et al., 2000). The products of genes UL112-UL113 regulate the expression of core replication genes. The replicative complex components, DNA polymerase, and the DNA processivity factor are encoded by the β genes UL54 and UL44, respectively. Early genes are involved also in immunomodulation. US11 encodes a protein which down regulates the expression of antigen-presenting molecule MHC class I. This function contributes to the IE US3 gene product and ensures the virus to escape from the host cell immune response. Another early gene, UL4 encodes a glycoprotein which has role in transcriptional and posttranscriptional control. The most abundant transcripts β1.2 and β2.7 are thought to have regulatory function but their translated products have not been studied yet.

γ genes. Late genes encode proteins involved in modulating the immune response or have a structural role. The UL99 product is a highly immunogenic glycoprotein that localizes near the capsid surface of the virion (Landini et al., 1987). The HCMV glycoprotein gB initiates the antiviral response by activation of the interferon regulatory factor 3 (IRF3) (Boehme et al., 2004). Another late gene

product, glycoprotein H is a target for the complement-independent neutralizing antibodies (Rasmussen et al., 1984).

After entering the cell, expression of viral proteins is triggered to ensure viral replication. DNA replication starts at early time after infection. The genome circularizes and starts a rolling-circle replication, producing concatamers at late times. This process involves viral and host factors. Replication starts at a single origin of replication (*ori* $_{Lyt}$) site, located within the UL region. This position is conserved in all β-herpesviruses. The synthesis of DNA is directed by herpesvirus conserved proteins, and the replicative complex is translocated to subnuclear sites where transcription starts. The DNA to be packaged is cleaved into unit-length pieces and inserted into the preassembled capsids.

The CMV-infected cell produces different types of virus particles. Non-infectious particles are empty capsids that have acquired an envelope. Dense bodies consist of tegument proteins and envelopes. The infectious virions have packaged DNA and an envelope derived from the nuclear or cytoplasmic membrane. Virus egress via exocytic vesicle transport results in release of mature progeny into the extra cellular space.

1.3 Virus – host interaction

1.3.1 CMV influence on the host cell

The CMV infection induces metabolic and structural changes in the host cell that are optimal for viral gene expression, replication and virion morphogenesis as well as survival (Muranyi et al., 2002). Co-infection studies revealed the impact of HCMV on cellular metabolism. HSV-1 and adenovrius replication have been shown to be activated by HCMV infection (McPherson et al., 1985; Spector et al., 1986; Colberg-Poley, et al., 1979). The ability of HCMV to activate cellular genes was associated with chronical proliferative diseases, such as cancers: adenosarcoma, Kaposi Sarcoma (Spector, 1984; Rosenthal, 1993) and possible involvement in vascular disease (Epstein et al., 1996). During HCMV infection the normal expression of the cyclin-dependent kinases is disrupted, and the cell cycle is blocked before replication of the cellular DNA (Bresnahan et al., 1996; Dittmer and Mocarski, 1997; Salvant et al., 1998; Wiesbusch and Hagemeier, 2001). Microarray analyses

provided useful tool to monitor the expression of all virus and host cell mRNAs. They showed that HCMV-infected cells have upregulated mRNA levels of genes coding proteins, which have a role in cell cycle progression (Challacombe et al., 2004). Another study reported a dysregulation of mRNAs encoding proteins that function during mitosis and associate with abnormal mitotic spindles during the late phase of infection (Hertel and Mocarski, 2004). The infection also inhibits the licensing of host cell DNA origins of replication by preventing the assembly of a pre-replication complex (Biswas et al., 2003; Wiebusch and Hagemeier , 2001). All these changes favour optimal viral replication and efficient viral gene expression. On the other hand, HCMV infection induces the expression of cellular RNAs encoding interferon-responsive proteins and pro-inflammatory chemokines (Browne and Shenk, 2003). These changes represent the attempt of the host cell to respond to infection and combat replication and spreading of an invading virus.

1.3.2 Detection of the invader by the host

The earliest response to an invading virus is the innate immune response. A major player of the antiviral defence is interferon type I production. The interferon type I response consists of three phases: induction of IFNβ, signalling, and antiviral gene expression (Fig. 5). The first phase includes activation of the IFNβ promoter upon viral entry. There are two pathways leading to interferon (IFN) production: the classical and Toll-like receptor mediated pathway.

The classical pathway represents type I IFN induction, it is activated by double-stranded RNA (dsRNA). The presence of dsRNA is typical not only for RNA viruses, DNA viruses can generate dsRNA during transcription as a result from intermediate structures consisting of opposing RNA transcripts. Viral dsRNA is detected by two intracellular helicases, RIG1 and MDA5. These proteins are expressed in many tissue types and function in parallel. Binding of dsRNA to the helicase domain induces conformational changes which transmit the signal to the nucleus. Several factors are involved in signal transduction. Helicases interact with the recently discovered protein IPS-1/MAVS (Kawai and Akira, 2006; Seth et al, 2005) that localized at the mitochondrial outer membrane, activates NF-kB promoter and leads to indirect activation of IRF3 (Kawai and Akira, 2006). Other kinases, IKK and TANK-binding (TBK) kinase phosphorylate the transcription factor IRF3

(Fitzgerald et al., 2003). The IFN regulatory factor 3 (IRF3) is a constantly expressed cellular protein, which resides in the cytoplasm. The phosphorylated form homodimerizes and moves to the nucleus, where it activates the expression of IFNβ and IFNα in cooperation with the cellular transcription factors AP-1 and NF-kB.

Toll-like receptors (TLR) are ancient conserved pathogen receptors that activate signalling pathways leading to expression of antiviral genes and induction of inflammatory cytokines (Akira et al., 2001). The HCMV envelope glycoprotein gB is recognized by TLR 2. The signal is transmitted by several kinases which results in activation of IFNα and IFNβ promoters (Compton, 2003 and 2004). Activation of TLRs is viral replication-independent, indicating a host mechanism for very early detection of virus.

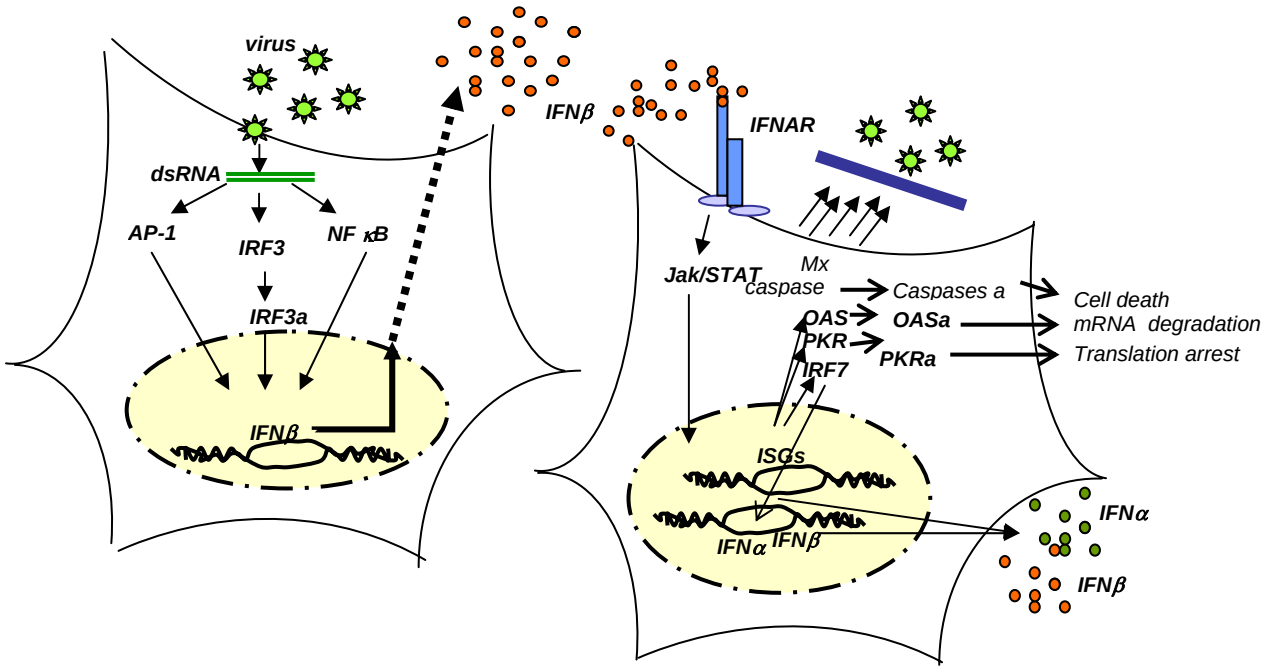


Fig. 5. IFN α/β response upon viral infection.

Produced IFNβ stimulates the neighbouring cells by binding to type I IFN receptors and activates Janus kinase/signal transducer and activator of transcription (Jak/STAT) pathway to stimulate the expression IFN stimulated genes (ISGs). STAT proteins are latent transcription factors, which are activated by phosphorylation and bind IFN regulatory factor 9 (IRF9). The complex is translocated to the nucleus, where binds the promoter of ISGs. The Jak/STAT signalling induces the expression of a number of proteins, including IRF family protein, IRF7. This factor is activated after phosphorylation and forms heterodimers with IRF3 to stimulate the ISGs for

increased expression of IFNβ and IFNα that cannot be induced alone (Sato et al., 2000). The gene products transcribed from ISGs include proteins with antiviral activity: protein kinase R (PKR), oligoadenylate synthase (OAS), and Mx proteins, which establish an antiviral state in the infected cell and define the third phase of the IFN response.

The ISG products are effector proteins with antiviral activity. They have different characteristics and effects on the host. Mx proteins belong to the superfamily of dynamin-like GTPases. They were discovered as factors for genetic resistance to orthomyxoviruses in mice (Pavlovic et al.,1995). Mx proteins are expressed only upon activation of ISGs by IFNα/β through the Jak/STAT pathway (Dupuis et al., 2003). OAS and PKR are constitutively expressed antiviral proteins, maintained in a latent, inactive form. Basal levels are upregulated by IFNα/β or IFNγ, and both enzymes are critically activated by viral dsRNA. The OAS catalyses the synthesis of short oligonucleotides that activate the latent endoribonuclease RNAseL. The activated enzyme degradates both viral and cellular RNA, leading to viral inhibition (Zhou et al., 1997). PKR is a serine-threonine kinase which is activated by the presence of dsRNA. The following dimerization results in autophosphorylation, which leads to phosphorylation of the eukaryotic translation initiation factor eIF2α (Williams, 1999). The eukaryotic translational factor eIF2α is responsible for recruitment of the small ribosomal subunits during initiation of translation. The active form is non-phopshorylated. In presence of dsRNA, eIF2α is phosphorylated and translation initiation of cellular and viral mRNA is blocked. Additional proteins with antiviral activity are ISG20 (Espert et al., 2003), promyeloleukemia protein (Regad et al., 2001), P56 (Guo et al., 2000), RNA-specific adenosine deaminase 1 (ADAR1) (Samuel, 2001) and guanylate-binding protein 1 (GBP1) (Anderson et al., 1999). P56 binds eIF3, thereby inhibiting viral and cellular protein synthesis (Hui et al., 2003). The others have not yet studied functions

1.3.3 Virus escapes the host immune response

Viral infection in immunocompetent organism is characterized by persistence and release of virus for long periods in the face of the host immune system. During evolution, CMV has developed various survival strategies to complete its replication successfully. A large part of the genome encodes proteins that modulate and mimic

the immune response at different times of the replication cycle. Both human and murine cytomegaloviruses have evolved mutliple mechanisms to escape different host responses including the IFN system, cytotoxic lymphocytes, cytokine activation and migration, susceptibility to apoptosis and antibody-mediated defence. A crucial step for the invading virus is to overcome innate immunity as it is the earliest response of the host to viral infection.

CMV interference mechanisms target different stages of IFN production, signalling, and expression of antiviral proteins. HCMV was shown to block IFNβ induction by the phosphorylated tegument protein pp71 (Abate et al., 2004, Browne et al., 2003). Others suggested that IE2 can cooperate with pp65 and block IFNβ production (Taylor and Bresnahan, 2005). The signal transduction of interferon receptors to specific elements of responsive genes is inhibited by HCMV and MCMV at different points. The HCMV protein p48 and MCMV M27 block the STAT dimerization and subsequent expression of the antiviral proteins PKR, OAS, Mx and IRF7 (Khan et al., 2004). HCMV can inhibit IFNγ induction by degradation of Jak1 (Miller et al., 2002). TRS1/IRS1 have been reported to bind dsRNA and prevent the activation of PKR and subsequent phosphorylation of the eukaryotic translational initiation factor eIF2α, therefore avoiding a global protein synthesis shut off (Hakki and Geballe, 2005). On the other hand, HCMV ensures its own translation by encoding a kinase mTOR which supports protein synthesis in the host cell (Kudchodkar et al., 2004).

1.3.4 The MCMV US22 gene family members m142 and m143

Immune evasion is an important for the virus to complete successful replication. The large genome of herpesviruses contains many genes devoted to counteracting the immune response. It is assumed that the conserved blocks preserve essential for the virus functions, one of which is immune evasion.

The US22 gene family is unique for the betaherpesviruses. The family was first described in HCMV, it consists of hypothetical proteins that are characterized by the presence of three or four conserved motifs (Kouzarides et al., 1988; Nicholas and Martin, 1994). Consensus sequences for motifs I and II have been identified, they contain short stretches of hydrophobic and charged residues. Motif I differs between HCMV family members in the unique short (US) and unique long (UL) region

(Nicholas et al., 1996). M139 – m143 share the same motif as the HCMV US family members. Motifs III and IV are less well defined but have stretches of nonpolar residues (Kouzarides et al., 1988). Genes encoded by ORFs m139 to m141 contain all four of these motifs, whereas m142 and m143 lack motif II. In addition, m139, m140, m142 and m143 each have an acidic domain common to herpesvirus transcriptional activators and specifically to MCMV immediate early proteins 1 and 2 (Cardin et al., 1995).

The US22 gene family members are clustered at the either end of the genome. The family consists of 12 members in each murine and human CMV, and 11 in rat CMV. Most of the MCMV US22 genes do not have HCMV sequence homolgoues, suggesting that conserved motifs determine important functions for the virus, resulting in a possible functional homology of HCMV and MCMV family members. The US22 gene family members are transcribed with immediate early or early kinetics of expression. Although the function of many US22 family genes is unknown, their functions have clearly diverged during evolution. Some of the gene products function as transcriptional transactivators, others regulate cell tropism or inhibit apoptosis.

HCMV	MCMV
US22	m128
US23	M23
US24	M24
US26	m25.2
UL23	m139
UL24	m140
UL28	m141
UL29	m25.1
UL36	M36
UL43	M43
TRS1	m142
IRS1	m143

Table.1. US22 members

The HCMV US22 gene is expressed at early times and specifies a nuclear/cytoplasmic protein of unknown function, secreted into the extracellular space (Mocarski, 1988). The UL36 and its homologue in MCMV, M36, encode a potent inhibitor of Fas-mediated apoptosis that involves caspase 8 activation (Skaletskaya et al., 2001, Menard et al., 2003). Other members of the family are important for optimal viral replication. The MCMV gene M43 is necessary for efficient replication in several cell types in vitro (Menard et al., 2003) and in salivary glands in vivo (Xiao et al., 2000). M139, M140 and M141 mediate efficient replication in macrophages (Cavanaugh et al., 1996; Hanson et al., 1999b, 2001; Menard et al., 2003) and are required for viral replication in the spleen, but not in the liver in vivo (Hanson et al., 1999b, 2001).

Deletion of HCMV US22 family genes UL28, UL29, US23, US24 and US26 results in attenuated growth in human fibroblasts (Dunn et al., 2003; Yu et al., 2003),

while UL24 deletion results in impairment of replication in human microvascular endothelial cell (Dunn et al., 2003). However TRS1/IRS1 and m142/m143 are the only members that lack the same conserved sequence, motif II, and are expressed at immediate-early times. Moreover, these four genes are the only members of US22 gene family known to be essential for the virus replication. The common features between the human CMV TRS1/IRS1 and mouse CMV m142/m143 genes distinguish them from the other US22 family members and suggest a special function for the encoded proteins.

The human CMV genes TRS1 and IRS1 include sequence from both repeated and unique segments of the genome. The N-terminal two thirds of pTRS1 is encoded in the c repeat region, and the remainder of the protein is coded within the unique short region. The related protein, pIRS1, is encoded in the internal c‘ region together with the adjacent unique short region (Fig 6).

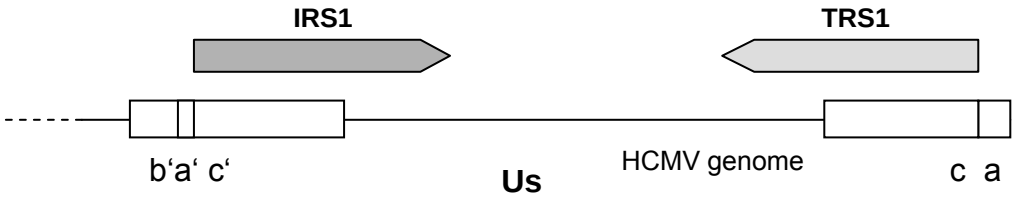


Fig. 6. Schematic presentaion of TRS1 and IRS1 location in the HCMV genome.

Consequently, the N-terminal domains of pTRS1 and pIRS1 are nearly identical, and the two proteins have different C-terminal domains (Wetso and Barrell, 1986). Because their amino-terminal domains are encoded in the repeat region, the transcription of these genes is controlled by identical immediate early promoters. Both TRS1 and IRS1 are packaged into the virion, and therefore are delivered to the cell immediately upon infection (Romanowski et al., 1997). The first function ascribed to pTRS1 and pIRS1 was transcriptional activation. Both proteins were found to act in conjunction with the immediate early transcriptional regulatory proteins, IE1 and IE2, but not on their own, to increase expression from the UL44 promoter in transient transfection assays (Stasiak and Mocarski, 1992; Romanowski and Shenk 1997). Subsequent analyses identified TRS1/IRS1 as 1 of 11 loci that are required for transient complementation of HCMV DNA replication (Pari et al., 1993). In this assay, pTRS1 and pIRS1 likely facilitate the accumulation of the proteins that function directly in the replication process (Iskenderian et al., 1996). In addition, the products

of TRS1, but not IRS1 acts late during infection to facilitate the production of virions (Blankenship and Shenk, 2002, Adamo et al., 2004). Moreover, recently investigators reported that the US22 gene family members TRS1 and IRS1 can reverse the PKR-mediated shut-off of protein synthesis, induced by a recombinant herpes simplex virus type 1 (HSV-1) lacking the $\gamma_1$34.5 gene (Cassady, 2005) and that TRS1 possess dsRNA binding activity (Hakki and Geballe, 2005). Earlier studies have shown that TRS1 and IRS1 can rescue the replication of a vaccinia virus lacking the dsRNA-binding protein E3L (Child et al., 2004).

The MCMV genes m142 and m143 were first identified as immediate early genes by Hanson and coworkers when analyzing transcripts from the HindIII-I region of the MCMV genome. It was before suggested that this region contains genes important for viral replication, since mutants with deleted genome from m137 to m141 and m139 to m143 cannot grow on macrophages. Transcript mapping of the m142 to m144 region revealed that the transcripts derived from this region use a common polyadenylation signal downstream of m142 (Hanson et al., 1999). Further studies showed that disruption of m143 or ATG deletion of m142 impairs virus growth so that virus cannot be detected (Menard et al., 2003), indicating that m142 and m143 have an essential role for viral replication.

1.4 Aim and tasks of the study

The aim of the present work was to analyze the function of the murine cytomegalovirus genes m142 and m143. The basic approach applied in this work was the deletion of the viral genes m142 and m143 by targeted mutagenesis and functional analysis of the mutant viruses. The characterization of mutant viruses, lacking the genes m142 and m143 should reveal their role for virus replication. Analysis of viral gene expression at the protein level would demonstrate the importance of m142 and m143 for viral gene transcription. In an attempt to understand their role in the context of viral infection, the virus-host cell interaction and particularly the involvement of m142 and m143 in counteracting the antiviral immune response was investigated. In the light of existing knowledge, m142 and m143 were tested for functional homology with genes from other viruses. The MCMV genes m142 and m143 were compared with sequence homologues of HCMV, TRS1 and IRS1, which had previously been studied in more detail. The construction of mutant

MCMV genomes, in which m142 or m143 were deleted and TRS1 was inserted should answer the question whether these genes possess similar functions.

The data obtained in the present work provides new insights into the functional role of the essential MCMV genes m142 and m143.

2. MATERIALS AND METHODS

2.1. MATERIALS

Table 2. Antibiotics

Antibiotic	Short name	Producer	Stock (mg/ml)	Working dilution
Ampicillin	*Amp*	*Roth*	100	1:1000
Chloramphenicol	*Cam*	*Roth*	37.5	1:2500
Carbencillin	*Carb*	*Roth*	60	1:1000
Geneticin G418	*G418*	*Invitrogen*	100	0.7%
Kanamycin	*Kan*	*Roth*	100	1:2000
Zeocin	*Zeo*	*Invitrogen*	100	1:4000

Table 3. Antibodies

Antibody		Working dilution	*Supplier*
	Primary antibodies		
Mouse αIE1 (croma101)	Monoclonal antibody	1:1000	***S. Jonjic; Univ. of Rijeka***
Mouse αE1 (croma103)	Monoclonal antibody	1:1000	***S. Jonjic; Univ. of Rijeka***
Mouse αgB (2E8.21A)	Ascites fluid	1:1000	***L. Loh; Univ. of Saskatchewan***
Mouse αM44 (3B9.22A)	Ascites fluid	1:2000	***L. Loh; Univ. of Saskatchewan***
Mouse αHA (16B12)	Monoclonal antibody	1:1000 WB 1:500 IF	***Hiss Diagnostic***
Mouse αPKR (B-10)	Monoclonal antibody	1:200	***Santa Cruz***
Rabbit αphospho eIF2α	Polyclonal antibody	1:750	***Cell signaling***
Rabbit αtotal eIF2α	Polyclonal antibody	1:750	***Cell signaling***
Rat αHA (3F10)	Monoclonal antibody	1:200 IF	***Roche Applied Science***
	Secondary antibodies		
Goat αmouse HRP coupled	Polyclonal antibody	1:1000	***Dako cytomation***
Goat αrabbit HRP coupled	Polyclonal antibody	1:3000	***Cell signaling***
Goat αrat Alexa Fluor 488	Polyclonal antibody	1:1000	***Molecular probes***
Goat αmouse Alexa Fluor 594	Polyclonal antibody	1:250	***Molecular probes***

Table 4. Bacteria and viruses

Strain	Description
***E. Coli* DH10B**	Preparation of plasmid DNA.
***E. Coli* DY380**	Original strain DH10B with defective λ prophage. The prophage encodes *gam*, *exo* and *bet* recombination genes, under control of temperature sensitive repressor (Lee et al., 2001).
MCMV GFP	MCMV Smith strain (U68299) genome and GFP cloned in BAC (Brune et al., 2000, 2001).
HCMV AD169	HCMV lab strain AD169 (X17403) cloned in BAC (Borst et al., 1999).

Table 5. Cell lines

NIH-3T3	Mouse embryonic fibroblasts (ATCC CRL-1658)
10.1	Immortalized mouse embryo fibroblasts (Harvey and Levin, 1991)
Phoenix	293T-derived packaging cell line for MoMuLV (Kinsella and Nolan,1996)

Table 6. Enzymes

DNAaseI	***Qiagen***
Pfu DNA polymerase	***Fermentas***
Klenow fragment	***Fermentas***
Proteinase K (stock 20 mg/ml)	***Fermentas***
Restriction endonucleases	***Fermentas, New England Biolabs***
RNAaseA (stock 10 mg/ml)	***Roth***
Shrimp alkaline phosphatase	***Fermentas***
Taq DNA polymerase	***Fermentas***
T4 DNA ligase	***Fermentas***

Table 7. Plasmids

Plasmid	*Supplier*	Application
pcDNA3	***Invitrogen***	Cloning and expression of viral genes
pLXSN	***Clontech***	Introducing viral genes into the cellular genome
pLXRN	***Clontech***	Introducing viral genes into the cellular genome
pBluescript II KS$^+$	***Stratagene***	Base for constructing different helper plasmids
pReplacer (pBS kan-m0206-P$_{PGK}$)	***I.Jurak and W.Brune***	Helper plasmid for homologous recombination
pCP20	***P. Cherepanov***	Removal of FRT flanked sequence by FLP recombination
pcDNA-M45HA	***W.Brune***	M45-EX probe
pCI-E3L	***M.A. Garcia***	Donor for E3L gene
pZEO4	***W. Bresnahan***	Amplification of Zeo resistance gene

Table 8. Oligonucleotides and primers

Primer / oligo	*Sequence*
	A. Primers for cloning in pcDNA 3
m142HA fw	**5'- AAA *GAA TTC* CAC CAT GGA CGC CCT GTG CGC GGC – 3'**
m142HA r	**5'- AAA AA *CTCGAG* T CAA GCG TAG TCT GGG ACG TCG TAT GGG TA gtc gtc atc gtc ggc gtc cgc – 3'**
m143HA fw	**5' – AAA *GGA TCC* ACC ATG TCT TGG GTG ACC GGA GAT – 3'**
m143HA r	**5'–AAA *GAA TTC* AAG CGT AGT CTG GGA CGT CGT ATG GGT A cgc gtc ggt cgc tct ctc gtc–3'**
TRS1HA fw	**5' – AAA *GAA TTC* CAC CAT GGC CCA GCG CAA CGG CAT GTC G – 3'**
TRS1HA r	**5' – AAA *CTC GAG* TCA AGC GTA GTC TGG GAC GTC GTA TGG GTA TTG AGC ATT GTA ATG GTA GT – 3'**
IRS1HA fw	**5' – AAA *GAA TTC* CAC CAT GGC CCA GCG CAA CGG CAT GTC G – 3'**
IRS1HA r	**5' – AAA *CTC GAG* TCA AGC GTA GTC TGG GAC GTC GTA TGG GTA ATG ATG AAC GTG GTG AGG GG – 3'**
	B. Oligonucleotides for cloning in pBlueSript II KS$^+$
o142 fw	**5' – AAA AAT CTA GAG CG CCA CCC TTC TCC ACC CGT GTT CCC GCT GCC GCC CGT CGC CCT CGC CGA ATT CGA TAT CCT CGA GGT TAA C – 3'**
o142 r	**5' – AAA ATA TCG AAT TCA AGG GCC CCG GGG AGG GGA GGG GTT TAT GTG ATG GCG AGG CGA TGT ACC GTC CGT CCG GTT AAC CTC GAG – 3'**
o143 fw	**5' – TGA ATG *CGG CCG C*GA GGT GGT TGC CTC GGC TCC GCT CCG CTT CGT CCG CCC GTC TCG TGC GC*G GAT CCG TTA ACG AAT TC*G – 3'**
o143 r	**5' – TTT GT*G ATA TC*C ATG TCG TCA CAG GGG AAA ACC GCC CCG TCG TGG ACC TCG ACG AGG CGG C*GA ATT CGT TAA CGG ATC C*G – 3'**
	C. Primers for deleting m142 and m143
Zeo-m142 fw	**5' – GCG ACC ACC CTT CTC CAC CCG TGT TCC CGC TGC CGC CCGTCG CCC TCG CC*G AAT TC*A AGT CCT GCT CCT CCT CGG CCA – 3'**
Zeo-m142 r	**5' – CTC GTC GAA CCG ACC TTC TCT CAT CAG CCA CCC CAG CTG GGA CGC GAA GTT GTT GAC AAT TAA TCA TCG GCA – 3'**
Zeo-m143 fw	**5' – GAG GTG GTT GCC TCG GCT CCG CTC CGC TTC GTC CGC CCG TCT CGT GCG CG*G AAT TC*A AGT CCT GCT CCT CCT CGG CCA – 3'**
Zeo-m143 r	**5' – GGA GGC ATT CGT GAC AAT CTC CCT CCG CCT CGG AGC GCA GGG AGC GCG GCT GTT GAC AAT TAA TCA TCG GCA – 3'**
	D. Primers for RT-PCR *(designed by Dr.Marcus Picard Maureau)*
18s rRNA	**PM024/25-TTATGGTTCCTTTGGTCGCTCG-CACCGGGTTGGTTTTGATCTGA**
gB	**PMLC001/002-GCGATGTCCGAGTGTGTCAAG-CGACCAGCGGTCTCGAATAAC**
gM	**PMLC003/004-TGCTTCGTGAACATCGTGGTG-GATCGCGTTGTACATCGTCAGG**
M44	**PMLC007/008-TGCACCAGGCGCTCTGTAAC-CGCTGAGGAAGTTCTCGATGG**
MCK-2	**PMLC011/012-GTGTCTGGTCAGATCTCGGTC-CATCGGCCACGTACATCATG**
	E. Additional primers for sequencing
m142 rev-i	**3' – ATCGT GCCGCGGTCC AGACGC – 5'**
m142 fw-i	**5' – ACCG AGG AGCTGAGATGGTT – 3'**
m143 F1	**5' – AAG CAG GAG ACC AAC CCC CTG – 3'**
TRS1 F1	**5' – CGG ACC TGC GTC AAC TGT – 3'**
TRS1 F2	**5' – GTG CGT CGG CAC CTG AAC – 3'**
TRS1 F3	**5' – TGC TGG TGG TGC TGC TGG – 3'**

**The letters in italic indicate restriction sites, HA-sequence is underlined.*

Table 9. Kit sets

NucleoBond PC100	***Macherey Nagel***	DNA Midi prep (plasmid, BAC)
NucleoBond PC500	***Macherey Nagel***	DNA Maxi prep (plasmid)
NucleoSpin Plasmid	***Macherey Nagel***	DNA Mini (plasmid)
NucleoSpinExtract II	***Macherey Nagel***	Agarose gel extraction; enzyme, salt or
NucleoTrap	***Macherey Nagel***	unincorporated dd NTPs (sequencing, SB) removal
RNeasy Mini kit (50 preps)	***Qiagen***	RNA column purification
RT Superscript II kit	***Invitrogen***	Reverse transcription of RNA
SYBER Green Fast Master mix	***Roche***	cDNA amplification for real time PCR
BigDye Terminator ver 3.1	***Applied Biosystems***	Cycle sequencing reaction
DIG High Prime Labeling kit	***Roche***	Southern blot

Table 10. Size markers

DNA ladder 1kb	***Fermentas***
Genomic DNA marker	***Fermentas***
RNP800 Rainbow marker	***Amersham***

Table 11. Solutions and buffers

A. Plasmid DNA prep	
Solution 1 (S1)	50 mM Tris/ HCl, 10 mM EDTA
Solution 2 (S2)	200 mM NaOH, 1% SDS
Solution 3 (S3)	2.8 M Potassium Acetate pH 5.1 (CH_3COOH)
TE buffer	10 mM Tris/ HCl, 1mM EDTA pH 7.8 (NaOH)
B. Total DNA preparation	
PK buffer	100 mM Tris, pH 8.0; 5 mM EDTA; 0.2% SDS; Sodium chloride 200 mM
50x TAE	242 g Tris, 57.1 ml conc. Acetic acid, 100 ml 0.5 M EDTA in 1L H_2O
10x TBE	108 g TRIS, 55 g Boric Acid, 40 ml EDTA (0,5M) in 1L water
C. RNA preparation	
10x MOPS	0.2M MOPS, 50mM Sodium acetate, 10mM EDTA, DEPC water
1x Running buffer	100 ml 1xMOPS, 20ml formaldehyde (37%), 880ml DEPC water
5x loading dye	16μl saturated bromphenolblue, 8 μl 0.5M EDTA, 72 μl formaldehyde (37%), 200 μl glycerol, 308 μl formamide, 400 μl 10xMOPS, 4 μl DEPC water

D. Protein analysis	
RIPA lysis buffer	20mM Tris/HCl, pH 7.5; 300mM NaCl; 1% Na-Deoxycholat; 1% Titon X-100; 0.1% SDS
Triton lysis buffer	10mM Tris/HCL, pH 8.0; 140mM NaCl; 0.025 NaN_3; 1% Triton X-100
TRICINE buffer	3M Tris/HCL pH 8.5; 0.3% SDS
Loading buffer (2xPPP)	125mM Tris/HCl, pH6.8; 4% SDS; 20% Glycerol; 10% 2-mercaptoethanol; 0.05% bromphenol blue
5x Cathode buffer	0.5M Tris; 0.5M Tricine; 0.5% SDS
10x Anode buffer	2M Tris/HCl pH 8.9
Transfer buffer	3.0g Glycine; 6.0g Tris; 400 µL SDS; 200ml Methanol
10xTBS	100mM Tris-HCl, pH 8.0; 1.5M NaCl
Blocking reagent	5% Milk in 1x PBS; 3% Milk in 1xTBS; 4% BSA in 1xTBS
Antibody incubation	5% Milk in 1x PBS; 4% BSA in 1xTBS and 0.1 Tween20, 1,5% Milk in 1xTBS and 0.5%Tween20
Washing buffers	1xPBS-0.5% Tween20; 1XTBS-0.5% Tween20

E. Southern blot	
Fragmentation solution	0.25N HCL
Denaturing buffer	0.5M NaOH; 1.5M NaCl
Neutralising buffer	0.5M Tris pH 7.0; 1.5M NaCl
20x sodium chloride/sodium citrate buffer (SSC)	3M NaCl; 0.3M Na Acetate
Maleic Acid buffer	0.1M Maleic Acid; 0.15M NaCl, pH 7.5 (solid NaOH)
Washing buffer	0.1M Maleic Acid; 0.15M NaCl, pH 7.5 (solid NaOH); 0.3% Tween 20
Washing buffer 1	2xSSC, 0.1% SDS
Washing buffer 2	0,5xSSC, 0.1 % SDS
Detection buffer	0.1M Tris/HCL; 0.1M NaCl, pH 9.5

F. Transfection of Phoenix cells (Calcium phosphate coprecipitation)	
Na_2HPO_4 dibasic stock solution	5.2g in 500ml water
2xHBS	8.0g NaCl, 6.5g HEPES, 10ml Na_2HPO_4 stock, pH to 7.0 (NaOH), up to 500 ml water
$CaCl_2$	2M
Chloroquine	50mM

G. Immunofluorescence	
Fixative solution	For 100ml solution: 4g paraformaldehyde; 1M NaOH; 10ml 10xPBS
Ammonium chloride	Dissolve 267mg NH_4Cl in 100ml 1xPBS
Permeabilizing solution	0.3% Triton X-100
Blocking solution	0.2% gelatin in 1xPBS
Washing buffer	0.1% Tween 20; 1x PBS
DAPI staining	250 μg/ml final concentration
Propidium iodide staining	1.0 μg/ml final concentration

Table 12. Reagents and chemicals

A. Color reagents	
Bromphenolblue Na Salt	*Roth*
4'-6-Diamidino-2-phenylindole (DAPI)	*Roth*
Ethidium bromide	*Roth*
Orange G	*Roth*
Propidium iodide	*Roth*
B. Medium and serum	
LB – Broth (Lennox)	*Roth*
Dulbecco's Modified Eagle Medium (DMEM)	*PAN Biotech*
L-Glutamine 200mM (100x)	*Gibco (Invitrogen)*
Fetal Calf Serum (FCS)	*PAN Biotech*
Newborn Calf Serum (NCS)	*PAN Biotech*
Penicillin/Streptomycin (100x)	*PAN Biotech*
Trypsin – EDTA 1x	*PAN Biotech*
Redivue PRO – MIX – L(^{35}S) in vitro Labelling Mix	*Amersham, Biosciences*
RPMI without L – Cys, L – Glu, L – Met	*PAN Biotech*
C. Reagents	
Aqua polymount	*Polyscience Inc.*
Diethylpyrocarbonat (DEPC)	*Roth*
Hexadimethrine bromide (Polybrene)	*Sigma*
phenol/ chlorophorm/ isoamyl alcohol (PCI)	*Roth*
Protease inhibitor cocktail `Complete Mini`	*Roche Diagnostics*
Polyfect	*Qiagen*
Superfect	*Qiagen*
TRIzol	*Invitrogen*

D. Chemicals	
Absolute ethanol	*Roth*
Agar-agar	*Roth*
Agarose	*Roth*
Ammonium persulphate	*Roth*
Ammonium chloride	*Roth*
2- mercaptoethanol	*Roth*
Boric acid	*Roth*
Calcium chloride	
Chlorophorm	*Roth*
Dimethylsulfoxide	*Roth*
Dithiothreitol (DTT)	*PAN Biotech*
Phosphate-buffered saline (PBS)	*Roth*
Ethilenediaminetetraacetic acid (EDTA)	*Roth*
Ethanol	*Roth*
Formaldehyde 37%	*Roth*
Formamide	*Roth*
Gelatine	*Sigma*
Glacial acetic acid	*Roth*
Glycin	*Roth*
Glycerol 86%	*Roth*
HEPES (sodium salt)	*Roth*
Isopropanol	*Roth*
Maleic acid	*AppliChem*
Magnesiumchloride - hexahydrate	*Merck*
Milk powder	*(Market)*
3-(N-morpholino)propanesulfonic acid (MOPS)	*AppliChem*
Na Deoxycholate	*Roth*
Na acetate	*Roth*
N - (trishydroxymethil) methilglycine (TRICINE)	*Roth*
N,N,N',N', Thetramethylendiamine (TEMED)	*Roth*
p-formaldehyde	*AppliChem*
Salt acid (HCl) 37%	*Roth*
Sodium acetate anhydrus	*Roth*
Sodium dodecyl sulfate (SDS)	*Roth*
Sodium hydroxide	*Roth*
Sodium chloride	*Roth*
Sodium hydrogen phosphate dibasic	*Roth*
Triton X-100	*Roth*
Tris base	*Roth*
Tween 20	*Roth*

Table 13. Instruments and support equipment

Agarose gel chamber	*Amersham Bioscience, Biometra, Peque Lab*
Blotting device for proteins	*Fast blot B34, Biometra*
Blotting set for DNA	*TurboBlotter, Schleicher&Schuell*
	Slot blot device, Roth
Centrifuge rotors	*5415 R, 5415D, 5810 R; Eppendorf*
	Avanti J20XP, JA25-15, JA25-50, JLA16-250 , Beckmann Coulter
Cover slips	*1.5 mm, round, Cubre-objectos, Hartenstein*
Confocal microscope	*Zeiss Axioplan, model LSM 510, Oberkochen, Germany*
Developing X-ray films	*X-Ray Retina, Kodak*
Electroporator	*MicroPulser Electroporator Apparatus, Biorad*
Electroporation cuvettes	*2 mm, Biorad*
Fluorescent light microscope	*Zeiss*
Gel dryer	*Bio Rad*
Quasishredder columns	*Qiagen*
Hybridization chamber	*HB-100 Hybridizer, Biometra*
Inverted light microscope	*Zeiss*
Incubators	*Bacterial incubator M100, Memmert*
	CO_2 Incubator, HERA Cell240
Phaselock tubes Heavy (PLG)	*Eppendorf*
Photodocumentation system	*E.A.S.Y. Win32, Herolab*
Pipettes	*Gilson, Labsystem*
Plastic for cell culture	*Greiner, Nunc, Sarstedt*
Polyacrilamide gel system	*Hoefer HF 99X (15x20 cm), Amersham Bioscience*
	Mini Protean 3 Cell (7x8 cm), BIO RAD
Radiographic cassette	*Dr.GoosSuprema*
Shakers	*Incubator shaker ISF1, Kuehner*
	Thermomixer comfort, Eppendorf
	Shaker 3013, GFL Burgwedel
Spectrophotometer	*Eppendorf; NanoDrop, Peque Lab*
Sterile bench	*Laminar-Flow, HeraSafe, BDK Sonnenbuehl-Genkingen*
Thermal cycler	*Gene Amp 9700, Applied Biosystems*
Transfer membrane for proteins	*Hybond ECL Nitrocellulose, Amersham Bioscience*
Transfer membrane for DNA	*NytranSuperCharge Nylon, Schleicher&Schuell*
	Roti-Nylon, Roth
Vortex	*Vortex-genie 2, Scientific Ind.*
UV crosslinker	*Stratagene*

2.2. METHODS

2.2.1. Molecular biology methods

2.2.1.1. Cloning

Gene amplification for cloning in expression vectors and homologous recombination

The genes m142 and m143 were amplified from the MCMV-GFP BAC, and TRS1 and IRS1 from the HCMV AD169 laboratory strain. Primers have included HA tag sequence at the 3´end, they are listed in Table 8A.

The following master mixtures were prepared (1x):

A. for cloning of m142 and m143, final volume 50 μl:

1.0 μl Template DNA (MCMV GFP BAC) 1.6 μg/μl
2.0 μl dNTP 10 mM
2 x 1.0 μl primers 10 pmol/ μl (stock solution 100 pmol/ μl)
5.0 μl 10x Buffer (with $MgSO_4$)
1.0 μl Pfu polymerase (5U/ μl)
39.0 μl dH_2O

B. for cloning TRS1 and IRS1:

1.0 μl Template DNA (HCMV GFP BAC) 1.6 μg/μl
2.0 μl dNTP 10 mM
2 x 2.0 μl primers 10 pmol/ μl (stock solution 100 pmol/ μl)
5.0 μl 10x Buffer (w/o Mg)
3.0 μl 25mM Mg^{++}
Taq + Pfu 1:1

Up to 50 μl dH_2O

C. Amplification of the zeocin resistance gene with different homologous arms (zeo – m142, zeo – m143, zeo – m142/143):

10.0 µl	Template (pZEO4)	25.0 µl	Buffer
10.0 µl	10mM dNTPs	1.0 µl	Taq polymerase
2x1.0 µl	10pmol/µl Primers	194.0 µl	dH_2O

D. Hybridisation reaction of synthetic oligonucleotides o142 and o143

Components:	
25mM Mg^{++}	10.0 µl
Buffer	10.0 µl
Taq polymerase	2.0 µl (2 units)
dNTPs	4.0 µl
Oligos	2x1.0 µl
dH_2O	72.0 µl
Total volume	100.0 µl

Amplification programs

Initial denaturation	95°C		1 min	
Touch down	95°C /65°C /72°C	1 min/30 sec/90 min	10 cycles, annealing step -1° (at 72°C: 150 min for TRS1 and IRS1, 30 sec for zeo cassette)	
Amplification	95°C /55°C /72°C	1 min/30 sec/90 min		35 cycles

Hybridization reaction for synthetic oligos:

Denaturation		94°C	2 min
Annealing	(oligo m143)	54°C	30 sec
	(oligo m142)	50°C	30 sec
Synthesis		72°C	5 min

Digestions

DpnI digestion

The PCR fragments zeo-m142, zeo-m143, zeo-m142/m143, m142HA, m143HA and TRS1HA were purified from the bacterial DANN by digestion with *DpnI*, which recognizes methylated bacterial DNA. The following mixture was set:

PCR product	45 µl	
DpnI	3 µl	
Buffer Y^+	20 µl	
dH_2O	132 µl	(up to 200µl)

The reaction was incubated for 2 hours at 37°C.

Endonuclease restriction digestion

A. For cloning in an expression plasmid, amplified fragments m142 HA, m143 HA, TRS1 HA, IRS1 HA and vector molecules were digested with endonucleases as follows:

EcoRI	2.0 µl	EcoRI	2.0 µl	EcoRI	2.0 µl	EcoRI	2.0 µl
XhoI	2.0 µl	XhoI	2.0 µl	BamHI	2.0 µl	BamHI	2.0 µl
m142 HA **TRS1 HA** **IRS1 HA**	25.0 µl	**pcDNA**	2.0 µl	**m143HA**	25.0 µl	**pcDNA**	2.0 µl
dH_2O	7.0 µl	dH_2O	30.0 µl	dH_2O	7.0 µl	dH_2O	30.0 µl
Buffer	4.0 µl	Buffer	4.0 µl	Buffer	4.0 µl	Buffer	4.0 µl

Reactions were incubated at 37°C for 2 hours.

Ligation products: pcDNAm142 HA, pcDNAm143HA, pcDNATRS1HA and pcDNA IRS1HA.

B. Subsequently the HA genes m142, m143 and TRS1 were subcloned into retroviral plasmids pLXSN or pLXRN. pcDNA constructs containing the corresponding genes and retroviral vectors were digested as follows:

m142	2.0 µl	**m143**	2.0 µl	**TRS1**	2.0 µl	**pLXSN**	10.0 µl	**pLXRN**	10.0 µl
EcoRI	0.2 µl	EcoRI	0.2 µl	EcoRI	2.0 µl	EcoRI	2.0 µl	BamHI	0.2 µl
XhoI	0.2 µl	BamHI	0.2 µl	XhoI	2.0 µl	XhoI	2.0 µl	HpaI	0.2 µl
Buff	2.0 µl	Buff	4.0 µl	Buff	4.0 µl	buff	4.0 µl	2x buff	4.0 µl
dH_2O	15.6 µl	dH_2O	13.6 µl	dH_2O	15.6µl	dH_2O	22.0 µl	dH_2O	5.6 µl

Ligation products: pLXSN TRS1HA, pLXSN m142HA and pLXRN m143HA.

C. For generating the helper plasmids, hybridized synthetic oligos were cloned into pBluescriptII KS^+ with two consequent digestions:

oligo m142	10.0 µL			**pBS**	3.0		
Apa I	1.0 (10 U)	XbaI	1.0 (10 U)	ApaI	1.0	XbaI	1.0
Buffer B	3.0	Buffer Y	5.0	Buffer B	3.0	Buffer Y	5.0
dH_2O	16.0	dH_2O	14.0	dH_2O	23.0	dH_2O	14.0
vol. 30.0, 2h at 37°C		vol. 50.0, ON at 37°C		vol. 30.0, 2h at 37°C		vol. 50.0, ON at 37°C	

oligo m143	10.0 µL			**pBS**	3.0		
EcoRV	1.0	NotI	1.0	EcoRV	1.0	NotI	1.0
Buffer R	3.0	Buffer O	5.0	Buffer R	3.0	Buffer O	5.0
dH_2O	16.0	dH_2O	14.0	dH_2O	23.0	dH_2O	14.0
vol. 30.0, 2h at 37°C		vol. 50.0, ON at 37°C		vol. 30.0, 2h at 37°C		vol. 50.0, ON at 37°C	

Ligation products: pBSo142 and pBSo143.

Probes for southern and slot blot

pcDNA constructs, containing m142, m143 and M45 were digested to prepare probes for southern and slot blot.

DNA fragment	5.0 µl (5.0µg)	pcDNAm142	pcDNAm143	pcDNA M45 EX
Endonuclease enzyme	2.0 µl (20 U)	EcoRI	BamHI	EcoRI
Buffer	3.0 (1x)	Eco72I	XhoI	XhoI
dH_2O	Up to 30 µl	Buffer 2xY	Buffer G	Buffer 2xY

Ligase reaction

Digested and purified fragments were ligated into corresponding vectors. Insert and vector were added at a ratio of 5:1. Reactions were performed with 1U T4 ligase at 16°C overnight or 4 hours at RT.

DNA-end modifications

In order to facilitate the cloning, different DNA end modifications were made. Blunt end cloning was applied when the multiple cloning sites did not allow another opportunity. Klenow reaction was performed to complete the sticky ends. To prevent re-ligation of the blunt-ended vector, dephosphorylation of the fragments was done with Shrimp Alkaline Phosphatase (SAP). After enzyme inactivation, the fragments were separated in 0.8% agarose gel and purified with *NucleoSpin Extract II* or *Nucleo Trap* kit, depending on the fragment size.

Klenow reaction

Digested DNA	30.0 µl
Klenow fragment	0.2 µl
10 mM dNTPs	1.0 µl
Buffer O^+	2.0 µl
dH_2O	16.8 µl

time for incubation: 10 min at 37° C
inactivation: 10 min at 70°C

SAP treatment

Digested DNA	30.0 µl
SAP	1.0 µl
Buffer R^+ (red buffer)	2.0 µl
dH_2O	17.0 µl

time for incubation: 30 min at 37°C
inactivation: 15 min at 65°C

Preparative agarose gel electrophoresis

Digested fragments were separated in 0.8% agarose gel 1xTAE. The agarose was melted in a microwave oven and cooled down. Before casting 3.0 µl ethidium bromid was added. The polymerized gel was loaded with samples and a standard molecular weight marker (1kb DNA ladder).

Fragment extraction from agarose gel

After separation, fragments were cut from the gel and extracted depending on the size of the fragment with a *NucleoSpin ExtractII* or a *Nucleo Trap* kit according to the manufacturer's instructions.

Bacteria transformation

Ligation reactions were transformed into electrocompetent bacteria of strain *E. coli* DH10B.

Preparation of standard electrocompetent cells DH10B

Bacteria cultures were grown overnight at 37°C. On the next day they were diluted 1 : 100 and grown untill OD_{600} of 0.4 to 0.6. Bacteria were incubated on ice for 10 min and washed two times with cold water and once with 10% glycerol. Resuspended in glycerol bacteria were used directly for electroporation or frozen in liquid nitrogen and stored at –80°C.

Transformation with ligated constructs

For transformation of electrocompetent bacteria, 2.0 µl ligation reaction was mixed with 38 µl bacteria and pulsed in a 2.0 mm cuvette at 2.5 kV. After pulsing, 600 µl medium was added and cells were recovered by shaking at 37°C for 90 min. Bacteria were plated on agar containing corresponding antibiotic for selection.

Plasmid DNA preparation (buffers Table 11 A)

High copy plasmids were extracted and purified according to the following protocols:

Mini preps, expected yield about 2.5µg total DNA. (DNA for screening)

DNA for screening was prepared from 2 ml overnight culture. Cultures were pelleted and supernatant removed. Bacteria were resuspended in S1 buffer and lyzed in S2 buffer.

DNA was separated from the dirt by incubation with S3 buffer and precipitated with 60% isopropanol. The pellet was washed in 70% ethanol and resolved in TE buffer containing RNAse (0.1μg/μl final concentration).

Maxi preps, expected yield about 500 μg total DNA. (DNA for further analysis)

Larger scale DNA was prepared and column purified with kit *Nucleo bond PC500*, (Maxi prep high copy plasmid protocol) according to the manufacturer's instructions.

Bacterial glycerol stocks

Bacteria containing final constructs were stored frozen as glycerol stocks at –80°C. Sixty percent glycerol was mixed with 1 volume overnight large scale bacterial culture.

2.2.1.2. Sequencing

DNA quality. Primers

The DNA for sequencing was column purified with a *NucleoSpin Plasmid* kit or prepared with *Nucleo Bond PC100* kit. Genes cloned into expression vectors were sequenced using standard T7 and SP6 primers for pcDNA3 or T3 and M13 primers for pBluescriptII KS^+.

Cycle sequencing

Sequencing reactions were performed with *Big Dye Terminator Ready reaction mix ver. 3.1* kit (BDT) and analyzed with an automated sequencer *ABI Prism* (Institute for Virology and Immunology, University of Würzburg).

Sequencing reactions were prepared in 0.2 ml 8-strips. 1/8 th reaction mixture contains:

BDT	1.0 µl
Primer (5 pmol/ µl)	1.0 µl
2.5x buffer (200 mM TrisHCL, pH 9.0, 5mM $MgCl_2$)	1.0 µl
DNA (plasmid DNA 50ng/ µl)	2.0 µl
Total volume	5.0 µl

Cycle sequencing was performed using the thermal cycler *Gene Amp 9600* :

Initial denaturation	96°C	1 min	
cycle sequencing	96°C /55°C/60°C	20 sec/15 sec/4 min	25 cycles

The products were subsequently column purified with Montage SEQ_{96} Sequencing reaction kit and analyzed with automated sequencer *ABI 3100*.

2.2.1.3. Homologous recombination

Homologous recombination was used to delete viral genes or insert fragments into the MCMV genome (Yu et al., 2000).

Recombination in DY380 cells

E.coli strain DY380 contains a λ prophage, which expresses recombinases under control of temperature sensitive repressor and BAC MCMV-GFP carrying chloramphenicol resistance gene. A PCR-generated linear fragment with 50 bp homologous arms was transformed into electrocompetent, induced DY380 bacteria.

This methodology was used to create the deletion (Δm142, Δm143, Δm142/m143) and replacement/insertion mutants (TRS1Δm142, TRS1Δm143, E3L Δm142, E3L Δm143, IRS1 Δm142 and IRS1 Δm143; TRS1/o142, TRS1/o143, E3L/o142, E3L/o143).

Induction of DY380

Bacterial cultures were grown at 30°C overnight under selection with chloramphenicol. On the next day, they were diluted 1:100 and grown at 30°C up to an OD_{600} of 0.4 to 0.6. Cells were induced by incubation at 42°C for 10 min with intensive shaking and cooling on ice. Bacteria were prepared for electroporation by washing in cold water two times and once in 10% glycerol. Bacteria were resuspended in 10% glycerol and transformed immediately or frozen in liquid nitrogen and stored at -80°C.

Transformation of linear fragment for recombination

For transformation of DY380 cells, 5.0 µl purified fragment was mixed with 35 µl of competent cells. Bacteria were electroporated in a 2 mm cuvette with pulse at 2.5 kV. After pulsing, 600 µl fresh medium was added and bacteria were recovered by shaking at 30°C for 90 min. Transformed bacteria were plated on agar containing chloramphenicol and an additional antibiotic for selection.

Arabinose-inducible recombination

DH10B cells containing the MCMV-BAC, were transformed with a plasmid (pKD46) expressing recombinases upon induction with L-arabinose.

For constructing revertant mutants, DH10B cells were transformed with BAC genomes of MCMV deletion mutants. These bacteria were made competent using a quick procedure. 4 ml cultures were pelleted, washed twice in cold water and once in 10% glycerol. The bacteria were immediately transformed with an *amp* resistant plasmid pKD46. After recovery, they were plated on agar with selective antibiotic. Grown cultures were induced with 1mM L-arabinose and used to make standard competent cells (described before), which were transformed with linear DNA fragments generated by synthetic oligonucleotides hybridization. The fragment contained an HA-tagged gene and a *kan* cassette flanked with FRT sites. Transformants were recovered by shaking at permissive for pKD46 temperature and plated on agar with kanamycin for selection. Plates were incubated at the non-permissive temperature for pKD46 to eliminate the

plasmid. Recombinant bacteria were selected for loss of zeocin and ampicillin resistance.

FLP recombination

FLP recombination was used to remove the FRT-flanked *kan* cassette introduced in the revertant mutants (Cherepanov and Wackernagel, 1995). The FLP recombinase, which recognizes FRT sites, was expressed from plasmid pCP20. DH10B containing the BAC revertants were transformed with an *amp* resistant plasmid pCP20, recovered and incubated at a temperature permissive for replication of the plasmid (30°C). Transformants were colony purified at 43°C and selected for loss of kanamycin and ampicillin resistance.

2.2.1.4. DNA analysis

BAC DNA preparation

BAC Mini preps

BAC DNA for screening was prepared from 5 ml overnight cultures. Bacteria were pelleted and resuspended in S1 buffer, then lyzed in S2 buffer. DNA was cleaned from cell debris by incubation with S3 buffer proteins were removed by phenol-chloroform extraction. DNA was precipitated with isopropanol, washed in 70% ethanol, and resuspended in TE containing RNAse A.

BAC Midi prep

For further analysis, column purified BAC DNA was prepared using *Nucleo Bond PC100* kit (Midi prep low copy plasmid protocol). The expected yield was 10 μg of BAC DNA.

Genomic DNA preparation (buffers Table 11B)

Total DNA was extracted from infected cells. Cells were trypsinized and washed in PBS. The pellets were resuspended in PBS and PK buffer, containing Proteinase K with 1mg/ml final concentration. Digestion was incubated at 55°C overnight. Proteins were removed by a phenol-chloroform extraction. The DNA was precipitated with 1/10 volume 3M sodium acetate and 2.5 volume absolute ethanol by incubation at -20°C for 30 min. Precipitated DNA was pelleted by centrifugation at full speed for 30 min at 4°C. The pellet was washed with 70% ethanol, dried, and dissolved in TE buffer containing RNAse A (0.1μg/μl). High molecular weight DNA was left overnight at 4°C for rehydratation.

Analytical agarose gel electrophoresis

Mutated BACs and derived mutant viruses were characterized by specific restriction digestions and separated in 0.6% agarose gel.

0.6 % agarose gel, 0.5x TBE

running buffer: 1.6 L 0.5x TBE
(80 ml 10x TBE)
5.0 μl Etidium Bromide

gel: 1.8g agarose in 300 ml dH_2O
10 μl Etidium Bromide
Run O.N. at 70V

samples: mix each sample with loading dye, load the gel.

Digestion

	MINI prep.	MIDI prep.
BAC DNA	25.0 μl	5.0 μl
Enzyme	2.0 μl	1.0 μl
Buff	3.0 μl	3.0 μl
dH_2O	0	21.0 μl

Total vol. 30 μl

Incubation 2h, 37° C

Probes for southern and slot blot

Fragments from m142, m143 and M45, cloned in pcDNA, were digested, gel purified (*Nucleo Spin Extract II kit*) and labeled with *DIG High Prime DNA labelling kit*, Roche. Before labelling, the DNA fragments were denaturated at 100°C for 10 min and chilled on ice.

Labelling reaction:

Purified fragment	25 μl
DIG labelling (vial 1)	5 μl
Incubate ON at 37°C	

After labelling the reaction was inactivated at 65°C for 10 min and purified with *Nucleo Spin Extract kit II* to remove the unincorporated DIG – ddNTPs.

Southern blot (buffers Table 11E)

Digested viral DNA was separated in 0.6% agarose gel for 4 hours at 120 V in a cool room or ON at 70 V RT. After separation, the gel was denaturated, neutralised and equilibrated in appropriate buffers. Gel was blotted as it is described in the *Turbo blotter* manual by *Schleicher&Schuell*. Blotting was performed at room temperature for 4 hours with 20x SSC transfer buffer. The blotting membrane was washed in 2xSSC for 5 min at room temperature and fixed at 120 mJ/cm^2 in a UV crosslinker *(Stratagene)*. The membrane was preincubated with 10 ml hybridization buffer from a *DIG High Prime kit* (*Roche*) for 30 min at 43°C. Hybridization was done overnight at the same temperature with m142 or m143 denaturated probes, following the manufacturer's instructions. On the next day, the membrane was washed 2x 5min with 2xSSC, 0.1% SDS and 2x 15min 0,5xSSC, 0.1 % SDS at 68°C. Probed DNA was detected as it is described in the *DIG High Prime kit (Roche)* manual.

Slot blot

Total DNA extracted from infected cells was loaded on a positively charged nylon membrane (*ROTI-Nylon Plus*) under vacuum with a slot blot device. Samples were denaturated in 1 volume 6xSSC buffer at 100°C for 10 min and cooled in an ice/water bath. Before loading, slots were washed with 300µl 6xSSC. Samples were loaded under vacuum, and slots were again washed with the 6xSSC buffer. The membrane was fixed, hybridyzed (probe – M45EX), and detected using the same protocol from *DIG High Prime kit, Roche.*

2.2.1.5. RNA analysis

General considerations

In order to prevent RNA from degradation during work, certain rules should be followed:

- Bake the glassware 4 h at 240° C to destroy the RNAses.
- Treat the water and solutions with DEPC. Subsequent autoclaving is needed to inactivate the chemical, because DEPC destroys the RNA. Note that chemicals with primary amines, like TRIS cannot be treated with DEPC.
- Clean the electrophoresis chambers and bench with RNAase-Off reagent (*AppliChem*) and rinse them with DEPC water.
- Use RNAse free plasticware, plugged tips and single – use pipettes.
- It is important to keep RNA always on ice and avoid repeated freezing and thawing.

Preparation of total RNA

Infected cells were lyzed on the plate with TRIzol reagent, containing phenol and guanidinium thiocyanate. Phases were separated by centrifugation in phase – lock gel tubes (PLG heavy, Eppendorf). The PLG tubes allow better separation of organic and inorganic phases and more efficient removal of proteins and high molecular weight DNA. RNA was precipitated with isopropanol, washed with 75% ethanol, and dissolved in DEPC water. Furthermore, the RNA was column purified (*RNeasy kit, Qiagen*) and DNAse I (Roche) digested to remove any traces of viral and genomic DNA. RNA was further washed as described in the manual and eluted from the column in 80 μl DEPC-treated water. After measuring the RNA concentration, aliquots were stored at – 80°C for further analysis.

RNA gel electrophoresis (buffers Table 11C)

RNA quality was tested on a 1.2 % agarose gel, with 0.5 μg of total RNA per sample. Samples were mixed with loading dye, denatured for 10 min at 65°C and cool on ice. Denaturated samples were loaded on a 1.2% agarose gel, containing 3.7% formaldehyde and 1 μl ethidium bromide (10 mg/ml). The gel was run at 50 V.

2.2.1.6. Protein expression and detection (buffers Table 11D)

Expression of different viral and cellular proteins was detected in western blot and immunofluorescence.

Cell lyzates preparation

$2.0x10^5$ cells were washed in PBS and lyzed in 100μl lysis buffer, containing protease inhibitors (*Roche*). Cells were incubated on ice for 20 min. After incubation cells were collected by scratching and centrifugated to pellet the cell debris. When the cells were lyzed in RIPA buffer, columns (*Qiashredder*, *Qiagen*) were used to shear the high

molecular weight DNA. Cell lyzates were boiled with 1 volume loading buffer at 100°C for 7 min, cooled on ice and separated by PAGE or stored frozen at -80°C.

Western blot

Cell lyzates were separated in different percentage PA gels, depending on the size of the proteins. 5% PA gels are suitable for separation of large proteins (> 120 kD) as well as glycoproteins, 7.5 % for > 25 kD, and 10% for small proteins more than 10 kD.

Table 14. SDS PAAG

SDS PAAG	5 %		7.5 %		10 %		Staking gel, 4%	
	Mini	large	Mini	large	Mini	large	Mini	large
AA/BA (37.5:1)	1.7	5.1 ml	2.5	7.5 ml	3.4	10.2 ml	0.7	2.1 ml
Tricine buffer	3.3	9.9 ml	3.3	9.9 ml	3.3	9.9 ml	1.3	3.9 ml
Glycerol 86%	1.2	3.6 ml	1.2	3.6 ml	1.2	3.6 ml	0	0
H_2O	3.8	11.4 ml	3.0	9.0 ml	2.1	6.3 ml	3.0	9.0 ml
Mini gel	APS 100µl		TEMED 10 µl					
Large gel	APS 300µl		TEMED 30 µl					

After separation, the gel was blotted onto a nitrocellulose membrane (*Hybond ECL, Amersham Bioscience*) in a semi-dry blotting chamber (*Biometra*). Membrane and papers were immersed in water and soaked in transfer buffer. The blotting sandwich was assembled as follows, from bottom to the top: 5 sheets of Whatman paper soaked in transfer buffer, membrane, gel, cover the free membrane areas with parafilm, put another 5 sheets of Whatman papers on top. The blot was run at 1 mA/cm^2 for 90 min.

The blotted membrane was blocked with 5% non-fat milk/PBS or 3% milk/TBS (for *Cell Signalling* antibodies) for 1 h at RT or at 4°C overnight. The membrane was incubated with diluted primary antibody (see Table 3). After washing with 0.5% PBS – Tween (or TBS-Tween, respectively) buffer, the secondary antibody solution was added and incubated at room temperature for 1 hour. The membrane was washed 4 times with washing buffer (PBS or TBS-Tween). Signals were detected with detection kit *ECL*, *Amersham Bioscience*.

Immunofluorescence (buffers Table 11G)

Infected or transduced cells were grown on 1.5 mm cover slips, flamed with ethanol. On the day of detection, cells were washed twice with PBS and fixed in 4% paraformaldehyde for 20 min at RT. After washing with PBS, cells were incubated with ammonium chloride to adjust the pH. The cells were washed with PBS, permeabilized in 0.3% Triton X-100 / PBS for 10 min at RT and blocked with 0.2% gelatin / PBS for 10 min at RT. For the counterstaining with propidium iodide, RNAse was added to the blocking solution. Cells were incubated with solution containing diluted primary antibody for 2 h at 37°C. The unbound antibody was removed by washing 3 times with 1xPBS and once with 0.1% Tween 20/PBS. The secondary antibody was diluted as recommended (see Table 3) and incubated for 1h at 37°C light-protected. The cover slips were washed 4 times with 1xPBS and once with millipore water. The nucleus was counterstained with DAPI at 250 μg/ml final concentration or propidium iodide at 1 μg/ml. The colour reagent was incubated with the cells for 10 min at RT. Cover slips were washed 3 times with millipore water and sealed with *Aqua Polymount medium.* Samples were stored light-protected and analyzed with a confocal microscope.

2.2.2. Cell culture and virus propagation

2.2.2.1. Cell lines maintaining

Cell culture propagation

Mouse NIH-3T3 fibroblasts were cultured in DMEM supplemented with 10% NCS and 1% Penicillin/Streptomycin (P/S). Cells were split every 3 days at a ratio of 1:5. Stably transduced NIH-3T3 (stable cell lines 3T3-m142 HA, 3T3-m143 HA and 3T3 -TRS1 HA) were maintained in DMEM containing 5% NCS and 1% P/S. They were split in ration of 1:5 every 3 days and selected with 0.7% G418 once per month for one week. Phoenix cells were grown in DMEM/10% FCS with 1% P/S. They were split 1:5 every 2 – 3 days and selected with Hygromycin (300 μg/ml)/Diphteria toxin (1 μg/ml) once monthly for one week.

Freezing and defreezing of cells

Cells were stored frozen in liquid nitrogen in freezing medium. For freezing cultured subconfluent cells were trypsinized and resuspended in DMEM. Cells were pelleted by centrifugation in a precooled rotor at 1200 rpm for 10 min and resuspended in freezing medium, which consists of 10% DMSO and 90% FCS.

Frozen cells were recovered by fast thawing in a 37°C water bath and washing once in PBS. Pelleted cells were resuspended in DMEM, supplemented with the appropriate serum and antibiotics.

2.2.2.2. Stable cell lines

Stable cell lines were created by transduction with retroviral vectors. Phoenix cells were seeded 1.5 – 2.0 x 10^6 in a 6 cm plate and transfected with 5-10 μg DNA retroviral vector plasmid containing the gene of interest, using the calcium phosphate precipitation method (buffers in Table 11F). The collected supernatant from Phoenix cells (3 ml from a plate) was filtered trough a 0.45 μm sterile filter to remove the cell debris. The retrovirus containing supernatants were supplied with 5 μg/μl Polybrene (1 μl per 1 ml virus) to improve the receptor binding capacity and stored at –80C° or used immediately. Target NIH3T3 cells were seeded in a 6cm plate (5x10^5 NIH3T3) and incubated with the supernatant, containing the retrovirus for 4-8 hours. After two days the transduced cells were cultivated with medium, containing the G418 to select for positive clones. Selection was done for two to three weeks. Cells were tested for expression of the viral proteins, expanded and stored frozen in liquid nitrogen.

2.2.2.3. Virus propagation

Recombinant and wild-type virus was reconstituted by transfection of the BAC DNA into murine embryonic fibroblasts NIH-3T3, or the complementing cell lines using transfection reagents *Polyfect* or *Superfect (Qiagen)*. Protocol was according to the

producer's instructions. BAC DNA for transfection was column-purified (*NucleoBond PC100, Low-copy plasmid protocol, Macherey Nagel*) and checked with specific digestions. Transfections were performed in 2.5 cm or 6 cm dishes.

2.2.2.4. Virus stocks

Subconfluent NIH-3T3 cells were infected with wild-type or revertant viruses. Stable cell lines, expressing the corresponding genes were infected with deletion or replacement mutant viruses. When all cells showed a cytophathic effect (CPE), the virus was harvested. Harvested virus was pelleted at 2000 rpm for 10 min at 4°C to remove the cell debris. Virus was centrifuged at 16 000g for up to 4 hours at 4°C and resuspended in fresh DMEM, containing 10% NCS. Aliquots were frozen and stored at -80°C.

2.2.2.5. Analysis of virus properties

NIH-3T3 cells were infected at different conditions to study the properties of the virus. The multiplicity of infection (MOI) defines the number infectious units per cell.

$$\text{MOI} = \frac{\text{virus titer x volume (ml)}}{\text{Cell number}}$$

Multistep growth curves

NIH-3T3 cells, wild type or transduced, were infected with a low MOI to show the ability of different mutants for spreading. 1.5 x 10^5 cells per 2.5 cm well were infected with an MOI of 0.1. After 2 hours, fresh medium was added (2 ml DMEM/10% NCS). Supernatant at the day of infection was collected as a control (day 0). At different time points after infection the supernatants of three separate cultures were harvested (2 x 0.5

ml) and 1ml fresh media was added until the day 7. Samples were stored frozen at -80°C.

Single step growth curves

$2.5x10^5$ NIH-3T3 cells were infected at an MOI of 5. After 2 hours the cells were washed once with PBS, and 1 ml fresh media was added. Every following day samples from three separate cultures were collected (2x250 μl) for 5 days, and 500 μl fresh media was added.

RNA transcript analysis

$1.2x10^6$ NIH-3T3 cells were plated in 10cm plates and infected with at an MOI of 1. RNA was harvested at 24 and 72 hours after infection.

DNA replication and construct analysis

2 x $2.5.0x10^5$ NIH-3T3 cells were seeded in 6-well plate, they were infected at an MOI of 3 with centrifugal enhancement. Virus was calculated for infection at an MOI of 0.3, diluted in 2 ml/well DMEM/10% NCS. Cells were centrifuged at 1000g in a centrifuge prewarmed to 37°C for 30 min. Twelve hours after infection the medium was changed. DNA was harvested 24 and 72 hours post infection.

Viral protein expression

1.0x 10^6 NIH-3T3 cells were plated in 6 cm plate and infected with MOI 0.5 to study viral protein expression at different times. Proteins were harvested at 24 and 72 hpi. $2.0x10^5$ NIH-3T3 cells were seeded in 6-well plates and infected at an MOI of 1 to analyze the cell host antiviral response. Cell lysates were analyzed in western blot. To study the intracellular distribution of viral proteins, $1.0x10^5$ cells per well were seeded on cover slips in 12-well plate, infected with MOI 0.5 and fixed at 14 and 24 hpi. Proteins were detected by immunofluorescence.

Metabolic labeling

Labeling:

3.0 x 10^5 NIH-3T3 cells per well were seeded in 6-well plates and infected at an MOI of 3. Twenty four hours postinfection, the cells were washed with PBS and medium was exchanged with RPMI without methionine and cystein (1ml/well), containing 10% FCS and 2mM Glutamine (*Gibco*). Cells were incubated for 1 hour with L – ^{35}S cell labelling mix (*Amersham*), containing 143 μCi total radioactivity per well. Samples were harvested as described for western blot, using RIPA buffer. The high molecular DNA was removed by centrifugating for 30 min at 13 000 rpm at 4°C. Protein was measured according to BCA method and 30 μg of each lyzates were loaded on a 10% PAAG and run overnight.

Detection:

The gel was fixed for 1 hour in solution containing 250ml ddH_2O, 50ml 100% acetic acid and 200 ml methanol. The gel was washed 3 times with cold Millipore water and dried under a vacuum for 1 hour at 80°C. The dried gel was exposed to X – ray film (*Kodak*) for 1 to 3 hours.

2.2.3. Quantification methods

2.2.3.1. Cell number determination

Cells number was determined by counting in a *Neubauer chamber*, 0.1mm depth and 0.0025 cm^2 area. The number of counted cells in 1 big square corresponds to 10^4 per 1 ml cell suspension.

2.2.3.2. Spectrophotometric measurements

Nucleic acid concentration

DNA and RNA concentrations were determined with a spectrophotometer *Nano Drop, (PeqLab)*. The instrument was initialized with 2.0 μl water. Samples (2.0 μl) were measured against TE buffer, at optical density A_{260} and A_{280}. The calculated ratio $A_{260/280}$

indicated unpurity of the sample. A ratio of 1.8 is generally accepted as pure for DNA; a ratio of about 2.0 is generally accepted as pure for RNA. Lower ratio in either case may indicate the presence of protein, phenol or other contaminants that absorb strongly at A_{280}.

Protein concentration

Equal amounts (2 μl) of the cell lyzates were diluted 1:5 in PBS and incubated with BCA protein reagents A+B, mixed in ratio 1:50 (*Pierce*). The reaction was incubated at 37°C for 30 min. In parallel, protein standards were incubated with the BCA reagents for 30 min at 37°C. After incubation, absorption was measured at λ 492. Protein concentration was determined by extrapolation to a standard curve. All samples and standards were measured in duplicates.

Bacteria density

The density of bacterial cultures was determined with an *Eppendorf* spectrophotometer. One ml sample was measured at λ_{600} using LB medium as a reference.

2.2.3.3. Viral titer determination

Median Tissue Culture Infectious Dose method ($TCID_{50}$)

Virus titers were determined with the Median Tissue Culture Infectious Dose method ($TCID_{50}$). Cells were seeded $4x10^5$ in a 96 well plate with 100μl DMEM per well. They were infected with 100μl per well increasing 10-fold virus dilutions (Table 15). Titers were read after 7 days.

Cells infected with MCMV-GFP-based viruses show green fluorescence, which allows a faster detection and easier titer determination. The following formula was used to calculate the virus titer:

$$\text{Titer } (TCID_{50}) = \frac{\text{wells with CPE in one row}}{\text{0.1 ml virus suspension}} \times \text{highest dilution with CPE}$$

Table 15. Ten – fold dilutions for TCID50/mL

Row in the plate (x 100 μl / well)	Dilution fold	DMEM / ml	Virus
A	10^{-1}	1 350	150 μl undiluted
B	10^{-2}	1 350	150 μl virus 10^{-1}
C	10^{-3}	1 350	150 μl virus 10^{-2}
D	10^{-4}	1 350	150 μl virus 10^{-3}
E	10^{-5}	1 350	150 μl virus 10^{-4}
F	10^{-6}	1 350	150 μl virus 10^{-5}
G	10^{-7}	1 350	150 μl virus 10^{-6}

Statistical significance

For the virus growth analysis and stocks, each titration was done in triplicate. For the growth curves, the average and the standard deviation were calculated.

3. RESULTS

Previous studies have provided indirect evidence for essential role of m142 and m143. To perform a functional analysis and investigate the role of m142 and m143 for MCMV replication, the genes were deleted and mutant viruses were analyzed for their growth properties and viral gene expression. Additionally, the MCMV genes were compared to the US22 gene family members of HCMV, TRS1 and IRS1, to find possible functional homologies.

3.1. Construction and propagation of mutant viruses

For construction of mutant viruses, the MCMV genome cloned as infectious bacterial artificial chromosome (BAC), was modified by targeted mutagenesis. Deletions and insertions into the viral genome were performed by homologous recombination of MCMV-GFP, cloned as a BAC, and a fragment containing the corresponding gene and sequence homologous to the target sequence. The fragments for homologous recombination were made by cloning of m142, m143 and TRS1 in different vectors.

3.1.1. Cloning and expression of m142 and m143

The genes m142 and m143 were amplified from the MCMV genome and cloned in expression vectors to verify the sequences. The HCMV genes TRS1 and IRS1 were amplified from the HCMV AD169 laboratory strain, cloned in expression vectors and sequenced. The m142, m143 and TRS1/IRS1 were amplified (Fig. 7) as epitope tagged genes with primers that include a hemaglutinin (HA) tag sequence at the 3’ end of the coding sequence as described in Materials and methods, table 8.

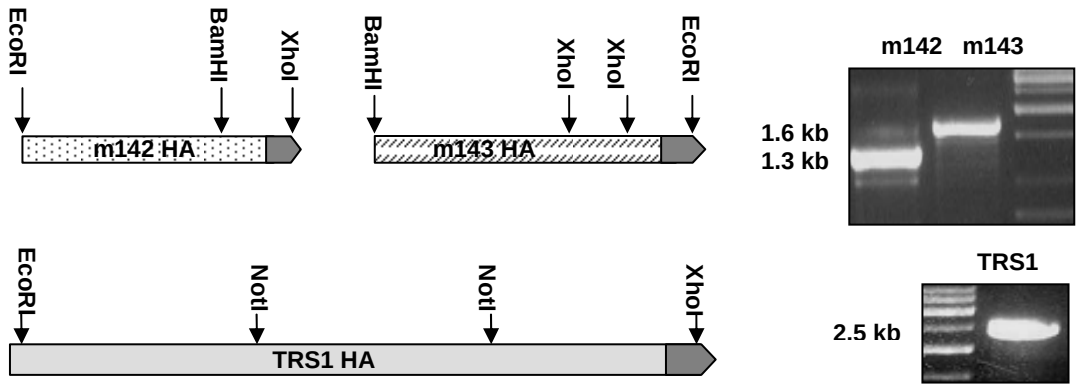


Fig.7 Amplification of m142, m143 and TRS1 as HA tagged genes.

The amplified fragments were cloned into expression vector pcDNA3 to generate the plasmids pcDNA-m142HA, pcDNA-m143HA, pcDNA-TRS1HA and pcDNA-IRS1HA (see Appendix 1 A). The constructs were digested with specific enzymes to confirm the right inserts (Fig.8).

Cloned genes were sequenced with standard T7 and SP6 primers, and the data was compared with published sequences at Genbank. BLAST was performed using reference sequences with accession numbers U68299 for the MCMV Smith strain and X17403 for the HCMV AD169 laboratory strain. Data analysis showed several single nucleotide mismatches found in ORFs m143, TRS1 and IRS1 regarding published MCMV and HCMV sequences, the changes are shown in appendix 2.

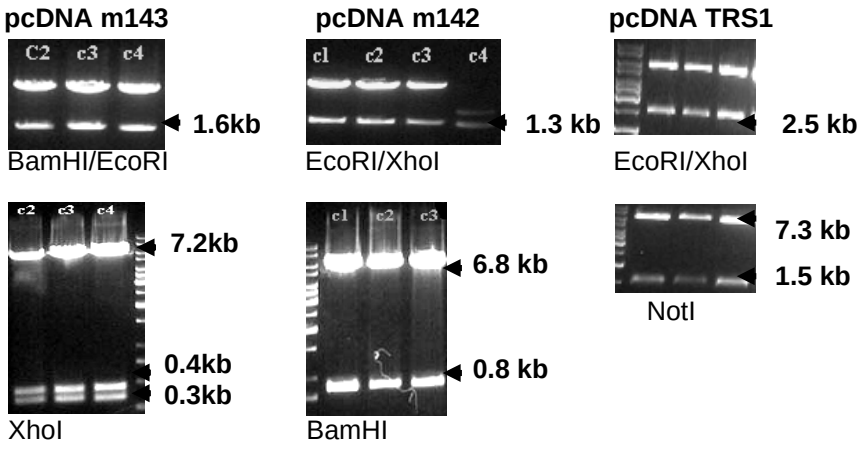


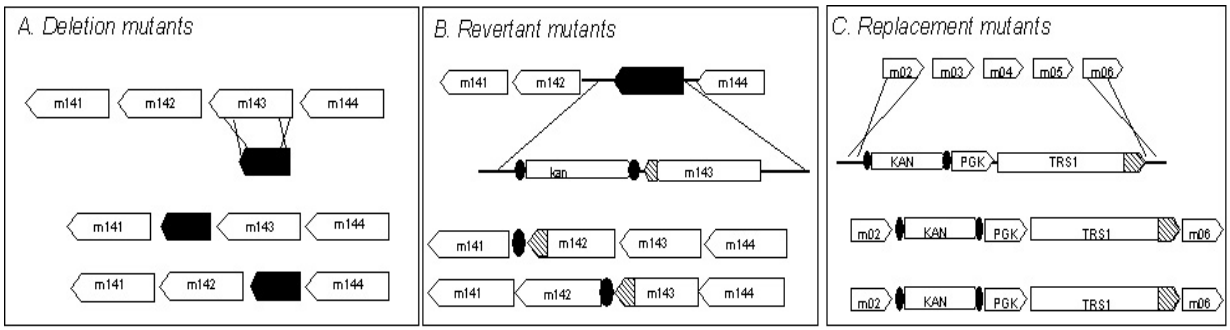
Fig. 8 Cloning in pcDNA 3. Cloned genes were confirmed with specific digestions.

3.1.2. BAC mutagenesis

The homologous recombination was performed in *E.coli* strain DY380, which contains a prophague, expressing recombinases under control of a temperature senstive promoter (see Table 4).

Deletion mutants (Δ142, Δ143) were created by replacing ORFs m142 and m143 with a zeocin resistance gene. The zeocin cassette was amplified from pZEO4, flanked with sequences homologous to adjacent regions of m142, m143 or m142/m143. The purified fragment was transformed into *E.coli* DY380 electrocompetent bacteria containing the MCMV-GFP BAC genome (Fig 9A, Table 17). Both genes are encoded on the complementary strand. They were deleted, respectively, for m142 ORF from 200 798 to 199 671 nt and for ORF m143 from 202 563 to 201 121 nt. A double knockout mutant (Δ142/143) was created, spanning deletion from 202 563 to 199 621 nt.

In order to demonstrate that the phenotypes of the deletion mutants were due to the lacking genes, m142 and m143 were reinserted into the MCMV genome. The revertant mutants (r142, r143) were based on deletion mutant BACs, where m142 or m143 were re-introduced as HA-tagged genes. Fragments for recombination were derived from helper vectors pBSo142/m142 HA kan and pBSo143/m143 HA kan. The helper vectors were created by cloning of hybridized synthetic oligonucleotides, including restriction sites and 50bp homologous regions flanking ORFs m142 or m143. The sequences of synthesized oligonucleotides are presented in Table 8.



Fg.9 Targeted mutagenesis. A. The black arrow indicates zeocine cassete; B. Corresponding HA tagged genes are shown with hatched box. C HA tagged genes were inserted at the place of m02 – 06 ORFs with kan FRT cassette and PGK promoter.

Resulting plasmids (pBS o142 and pBS o143, Fig. 10) served as vectors for cloning the HA tagged genes m142 and m143 derived from pcDNA constructs.

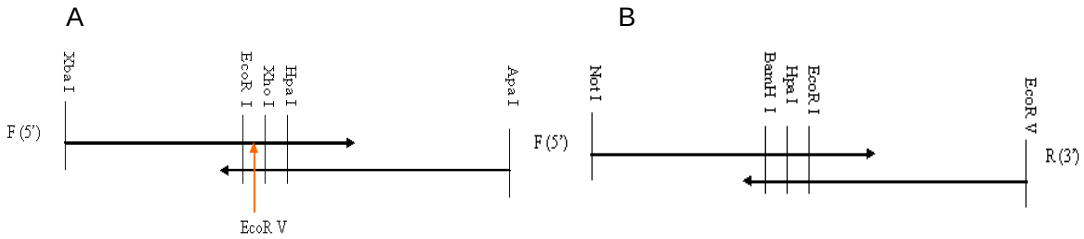


Fig. 10 Helper vectors for recombination, containing oligonucleotides homologous to m142 (A) or m143 (B) and carrying specific restriction sites.

Additionally, to select the mutants conatining re-inserted genes, a kanamycin cassette flanked by FRT sites (kanFRT) was cloned into the described plasmid (see App1 and Table 16).

Insert cut from:	***Vector molecule:***	***Resulted plasmid***
pcDNA m142HA	pBSo142	pBSo142-m142HA
pcDNA m143HA	pBSo143	pBSo143-m143HA
pcDNATRS1HA	pBSo142	pBSo142-TRS1HA
pcDNATRS1HA	pBSo143	pBSo143-TRS1HA
pCI E3L	pBSo142	pBSo142-E3L
pCI E3L	pBSo143	pBSo143-E3L
pcDNATRS1HA	pBSm0206PGK-kan	pBSm0206PGK-TRS1HA
pcDNAIRS1HA	pBSm0206PGK-kan	pBSm0206PGK-IRS1HA
pCI E3L	pBSm0206PGK-kan	pBSm0206PGK-E3L

Cut helper plasmid:	***Cut pSLFRTkan:***	***Resulted helper plasmid***
pBSo142-m142HA HpaI	EcoRI, blunted	pBSo142-m142HA/kan
pBSo143-m143HA EcoRI	EcoRI	pBSo143-m143HA/kan
pBSo142-E3L	EcoRI, blunted	pBSo142-E3L/kan
pBSo143-E3L	EcoRI	pBSo143-E3L/kan

Table. 16. Description of constructed helper vectors.

The fragments including oligonucleotides, HA-tagged genes and kanFRT cassette, were excised from the helper plasmid and transformed into electrocompetent DY380 cells, which carried ∆142 MCMV-GFP BAC or ∆143 MCMV-GFP BAC genome (Fig 9B, Table17).

Mutant BAC	*Fragment for recombination*	*Target sequence BAC MCMV-GFP*	*Kan cassette*	*Resulting resistance*
∆ m142	Zeo-m142	Wild type	None	Cam + Zeo
∆ m143	Zeo-m143	Wild type	None	Cam + Zeo
∆ m142/m143	Zeo-m142/m143	Wild type	None	Cam + Zeo
Revertant m142 HA	m142 HA-o142	∆ m142	+	Cam + Kan
Revertant m143 HA	m143 HA-o143	∆ m143	+	Cam + Kan
Revertant m142 HA K$^-$	Kan cassette (to cut)	FRT sites	None	Cam
Revertant m143 HA K$^-$	Kan cassette (to cut)	FRT sites	None	Cam
Replacement PGK – TRS1 delta m142	PGK m0206 TRS1 HA	∆ m142	+	Kan + Cam + Zeo
Replacement PGK – TRS1 delta m143	PGK m0206 TRS1 HA	∆ m143	+	Kan + Cam + Zeo
Replacement TRS1/o142	Kan-TRS1HA-o142	∆ m142	None	Cam + kan
Replacement TRS1/o143	Kan-TRS1HA-o143	∆ m143	None	Cam + kan

Table. 17. Description of the recombinant BAC genomes.

The recombination resulted in replacing the target sequence containing zeocin in the deletion mutants with a fragment flanked by homologous arms, including the corresponding gene, and a kanamycin cassette for selection. Mutants were selected for kanamycin resistance and loss of zeocin resistance. Because the insertion mutants were made to have a phenotype comparable to the wild type MCMV, the *kan* cassette was subsequently removed by FLP recombination, leaving behind one short FRT sites.

3.1.3. Construct characterization

Targeted mutagenesis of the MCMV genome resulted in insertions of heterologous genes, or at least restriction site changes which determined specific digestion patterns. Recombinant BAC genomes were characterized by EcoRI digestions.

Digested BACs were separated in 0.6% agarose gel and stained with ethidium bromide (Fig. 11).

Homologous recombination of the wild-type MCMV-GFP genome with the zeocin cassette resulted in an additional EcoRI site and disruption of the 9 kb fragment containing the ORFs m142 and m143. The ∆m142 has additional bands at 1.8 and 6.6 kb, whereas ∆m143 is characterized by the appearance of new fragment at 3.4 kb (Fig 11 A and B).

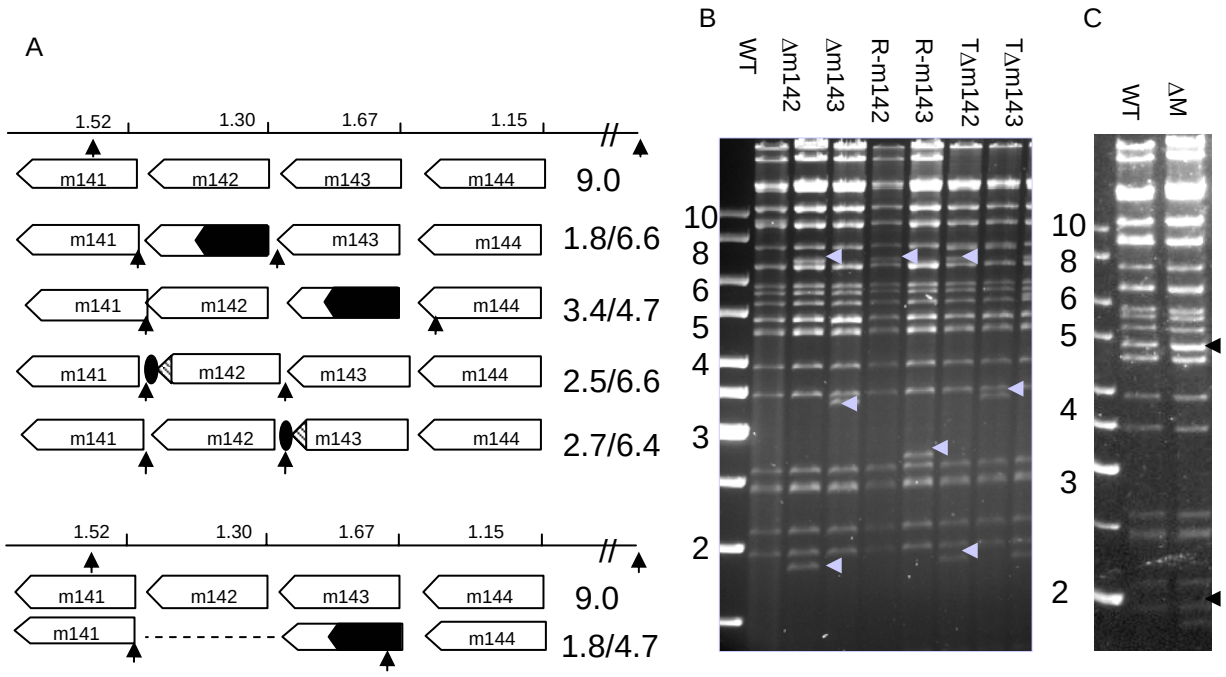
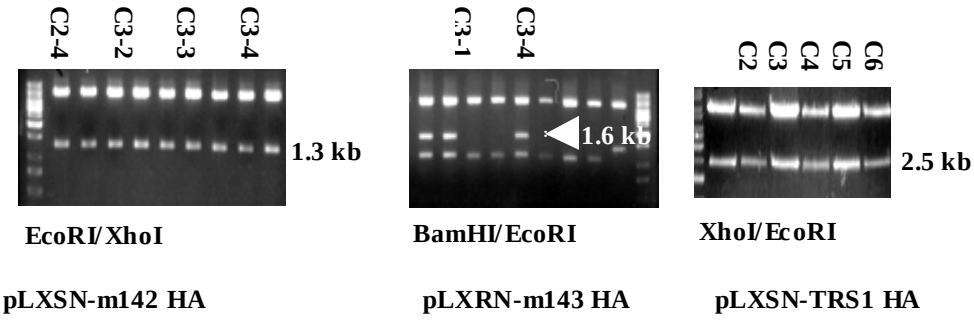


Fig.11 Charcterisation of recombinant BAC genomes by EcoRI digestion. A. Schematic drawing of the BAC mutants, the open arrows depict an ORF and the small ones indicate EcoRI restriction sites. Large black arrow represent the zeocine cassette. B. Ethidium bromide stained gel of digested BAC mutants: wild type (lane1), ∆ m142 (lane2), ∆ m143 (lane3), revertant m142 (lane4), revertant m143 (lane5), TRS1 ∆m142 (lane6) and TRS1 ∆m143 (lane7). C. Characterisation of the double deletion mutant (∆M) with EcoRI digestion. The construct is shown at lower the part in panel A.

The replacement of both ORFs m142 and m143 with zeocin cassette, resulted in appearance of a 1.8 kb band and disruption of the 9 kb fragment (Fig. 11 A and C). Reinsertion of the HA-tagged genes m142 and m143, resulted in three new EcoRI fragments. The revertant m142 is distinguished by the presence of a 6.6 kb band and revertant m143, has additional bands at 2.7 and 6.4 kb.the reinsertion of HA-tagged m142 or m143 resulted in disruption of the 9 kb EcoRI fragment which contains the entire m142 and m143 ORFs in the MCMV genome (Fig. 11 A and B).

3.1.4. Virus propagation

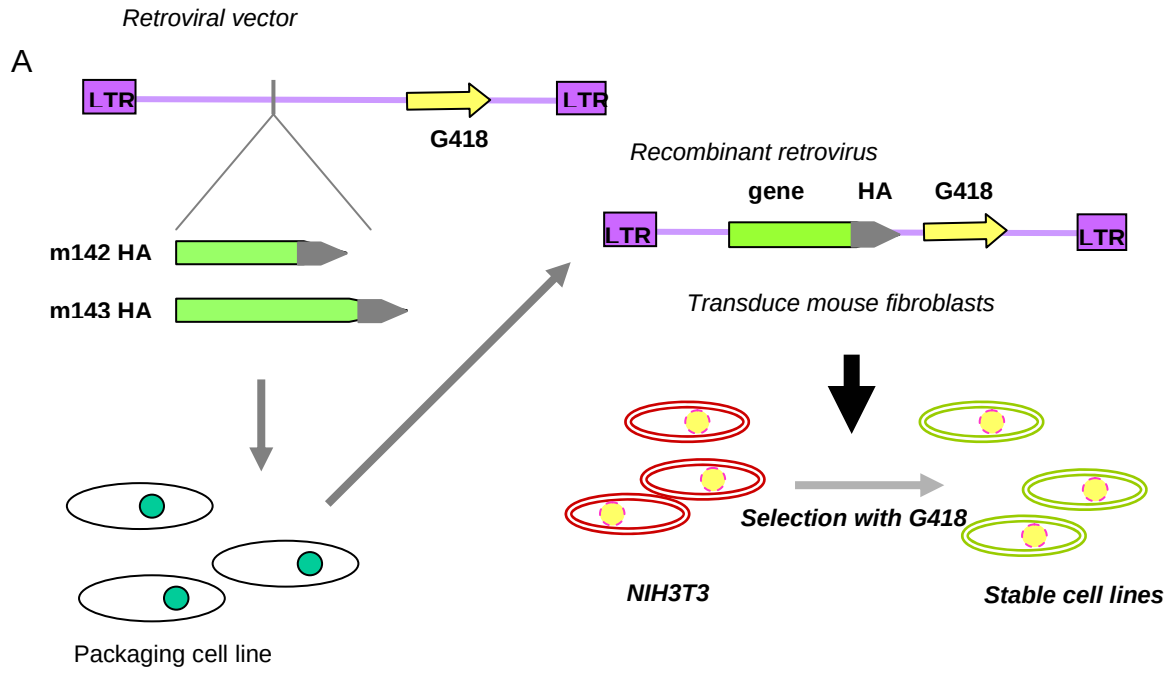
Mutant BAC genomes were transfected into NIH3T3 cells. The wild type and revertant BAC genomes produced infectious virus, whereas the deletion mutants did not grow. To overcome this problem, the viral genes m142 and m143 were expressed from NIH3T3 cells. To create stable cell lines, expressing m142 or m143, the corresponding genes were subcloned into retroviral vectors. The HA-tagged genes m142 and m143, were excised from pcDNA constructs and subcloned into plasmids containing the retroviral backbones pLXSN or pLXRN to obtain the constructs pLXSN m142HA, pLXRN m143HA and pLXSN TRS1HA (see Appendix 1B). The genes m142 and TRS1 were cut from pcDNAm142HA or pcDNATRS1HA with EcoRI/XhoI and cloned into pLXSN at EcoRI/XhoI; m143 was cut from pcDNAm143HA with BamHI/EcoRV and cloned into pLXRN into BamHI/HpaI. Vector constructs were verified with specific digestions (Fig



12).

Fig. 12 Retroviral vector characterisation. Constructs were checked with specific digestions. Different clones (C2....C6) were anlysed.

The retroviral vectors contain elements derived from the Moloney murine leukaemia virus (MoMuLV) and Moloney murine sarcoma virus. They encode the extended viral packaging signal (psi) and a Geneticin (G418) resistence gene, which is used for selection in eukaryotic cells. The expression of inserted genes is controlled by the retroviral promoter, located at the 5' long terminal repeat (5' LTR), whereas the G418 is expressed from an independent simian virus 40 (in pLXSN) or a Rous sarcoma virus (in pLXRN) promoter. To produce an infectious, replication-competent virus, the vector was transfected into a packaging cell line, which provides the structural viral genes (*gag*, *pol* and *env*) necessary for particle formation and replication. Thus, the retroviral constructs, containing the corresponding gene and a G418 resistance gene were transfected into Phoenix packaging cells (Table 5) (Fig 13 A). The packaged virus is released from the cells into the supernatant. Harvested recombinant retrovirus was used to transduce target NIH3T3 cells. MoMuLV is a retrovirus, which can stably integrate into the cellular genome and thereby deliver the inserted heterologous gene.



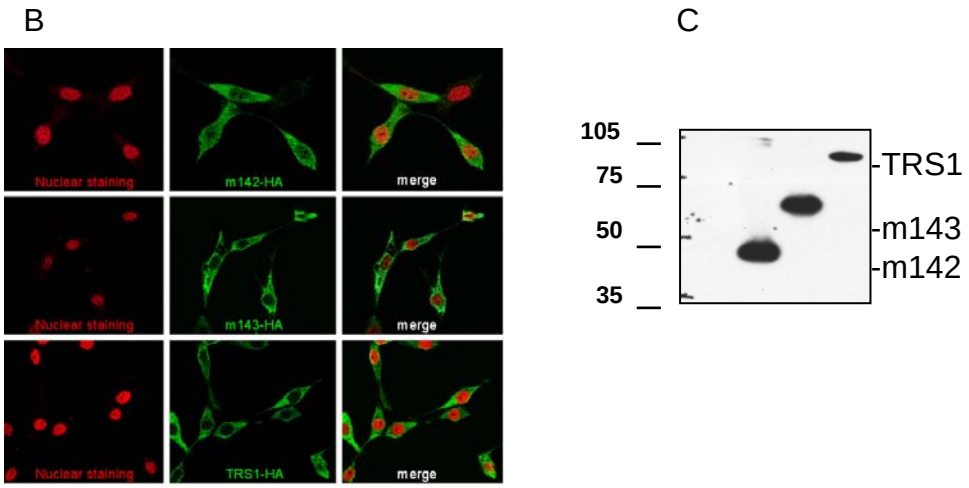


Fig.13. Stable cell lines, protein expression. A. Original NIH3T3 cells were transduced with recombinant retrovirus, containing m142, m143 or TRS1 HA tagged genes and G418 resistance gene. Protein expression was verified in B. Immunofluorescence (IF) and C. western blot of stable cell lines. Anti – HA tag antibodies were used to detect the proteins: 3F10 for IF and 16B12 for western blot.

The transduced cells were cultivated with G418 to select for positive clones. Successful integration and expression of the inserted genes was controlled in immunofluorescence assay and expected size of the proteins was confirmed in western blot. Further the expression was maintained by temporary cultivation of the transduced cells with G418 to ensure a long term optimal expression of m142 and m143 (Fig. 13 B and C). Additionally, a cell line expressing TRS1 was created to test deletion mutants for complementation (Fig. 13 B and C).

3.1.5. Virus complementation

The NIH3T3 cells expressing m142 or m143 were transfected with the deletion mutant BAC genomes. Virus was successfully regrown, indicating that the expression of viral genes, m142 and m143 from NIH3T3 cells can complement the deletion mutants (Fig 14).

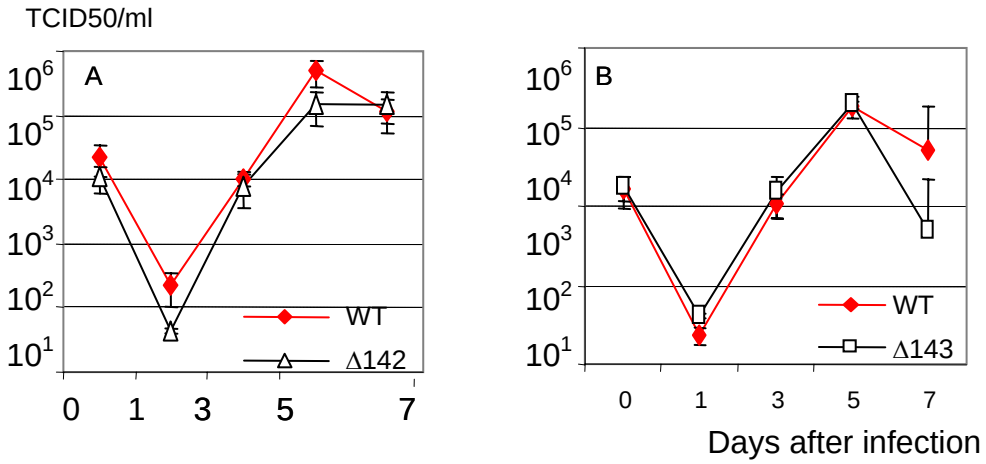


Fig. 14. Deletion mutants were regrown on complementing cells. A. Δ m142 grows on NIH3T3 - m142 and B. Δ m143 grows on NIH3T3 - m143.

In several attempts it was not possible to regrow the double deletion mutant on original or complementing cells. This fact supports the hypothesis that m142 and m143 are required together for the virus to complete successful replication.

3.1.6. Virus growth on original NIH3T3 cells

The reconstituted viruses were used to infect non-complementing NIH3T3 cells. The deletion mutants failed to produce a virus at detectable levels (Fig 15 A). However, the revertant viruses containing reinserted m142 or m143 grew to similar titers as the wild-type MCMV (Fig. 15 C), indicating that the defect of the deletion mutants is due to the lacking genes. Obviously, the insertion of the missing genes restored the wild type-like phenotype, showing that both, m142 and m143, are required for the virus to grow. In some cases the effect of a missing viral gene can be compensated by high MOI infection. For instance, deletion of the HCMV gene IE1 results in replication-deficient virus, but infection at a high MOI can rescue the replication (Greaves and Mocarski, 1998; Mocarski et al., 1996). The deletion mutants Δm142 and Δm143 did not grow on non complementing NIH3T3 cells even at high MOI infection (Fig.15 B).

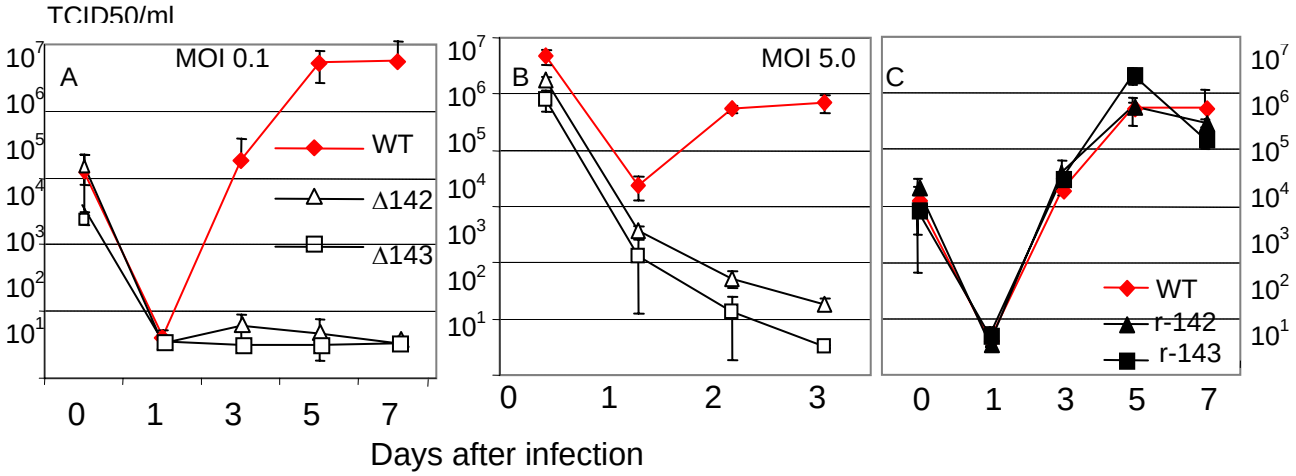


Fig. 15. Deletion mutants cannot grow on original NIH3T3 cells at low (A) or high (B) MOI infection. Reinsertion of m142 and m143 into the MCMV genome restores the wild type phenotype (C).

This result clearly shows that m142 and m143 are essential for virus replication and corresponds to the published data, that disruption of m142 and m143 results in impaired virus growth (Menard et al., 2003). The presence of one of these genes is not sufficient to restore the wild type-like phenotype, only when both m142 and m143 are present the virus can grow. This fact, together with the observation that double deletion mutant BAC (Δm142/m143) could not be reconstituted, suggests that m142 and m143 are required together in the context of viral infection.

3.1.7. Mutant virus genome

The confirmation of the essential role of m142 and m143 for virus replication was an important starting point for the followed experiments. Therefore, the virus preparations were verified to confirm the constructs and exclude contamination with the wild type MCMV-GFP. Viruses derived from mutant BAC genomes were grown on original or complementing cells. Total DNA from infected cells was digested with EcoRI and separated in agarose gel. The DNA was blotted onto a nylon membrane and probed for m142 and m143 (Fig. 17). To verify the deletions and insertions that resulted from the targeted mutagenesis, expected bands were calculated on the basis of mutated BAC

genomes. The detected bands corresponded to the band size from the EcoRI digestion of the BAC mutants. All mutant viruses lack the 9 kb fragment as a result from disruption due to the insertions, excluding possible contamination with the wild type MCMV-GFP. Probes were made from DIG labeled fragments, derived from pcDNA constructs containing m142 or m143. Fragments were purified and labeled as described in Materials and methods.

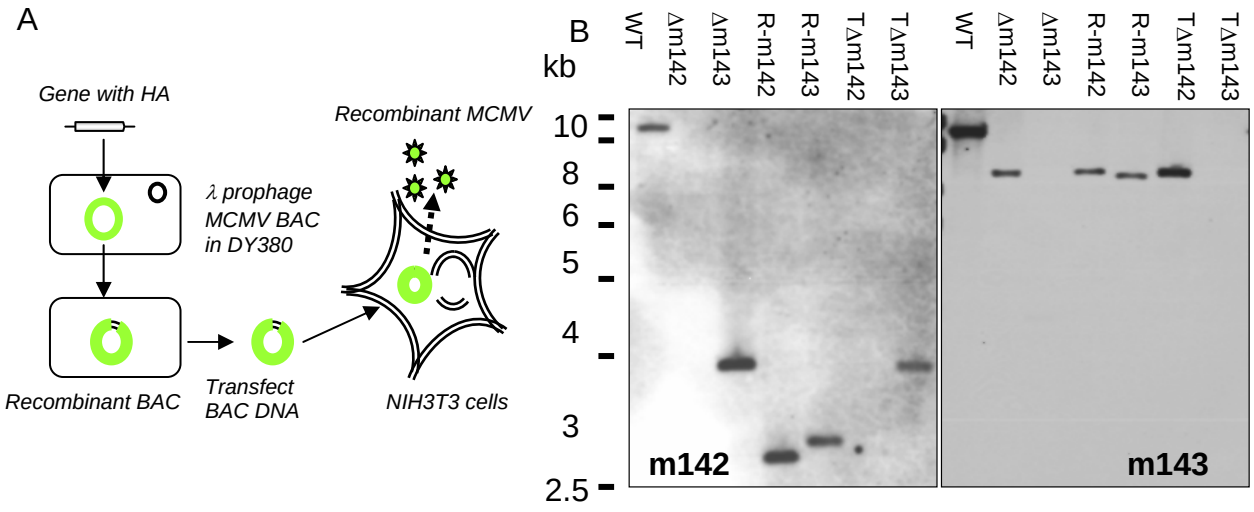


Fig.16 The MCMV BAC based viruses were confirmed by Southern blot. A. Recombinant BAC genomes were transfected into NIH3T3 cells. B. Virus genomes were confirmed with probes for m142 and m143.

The mutants containing reinserted HA-tagged genes, m142 or m143, were further characterized to demonstrated their functional capability. The expression of the inserted gene is controlled by the corresponding promoters, of m142 or m143, respectively, and expected to be expressed at immediate-early times. Non-complementing NIH3T3 cells were infected with revertant mutants and fixed after 24 hours postinfection.

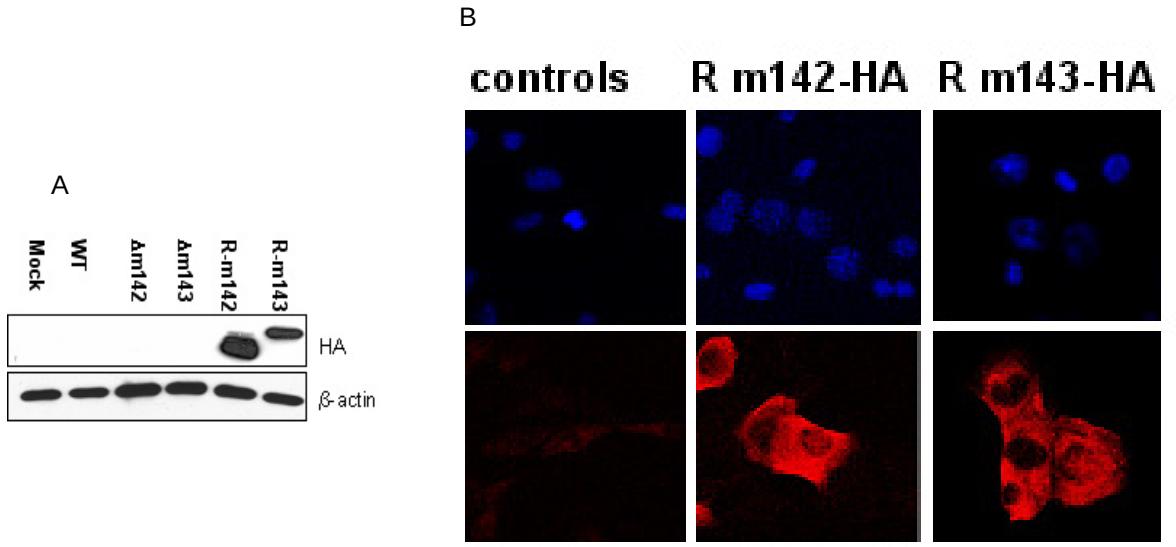


Fig. 17 Expression of HA tagged m142 and m143 in NIH3T3 cells infected with revertant viruses. The HA-tagged proteins were detected with a specific ant-HA antibody 16B12 in western blot (A) or 3F10 in immunofluorescence (B).

The expression of m142 and m143 was detected with an anti-HA tag antibody (Fig.17 B). Laser scanning image analysis showed a predominantly cytoplasmic localization of the MCMV proteins m142 and m143. The protein size was confirmed in western blot of infected cells with an anti-HA tag antibody (Fig17 A).

These results confirmed that the reinserted m142 and m143 are expressed and appropriately localized in the context of viral infection. The size and intracellular distribution of the studied proteins was in complete agreement with recently reported data by Hanson and co workers (2005).

3.2. Functional analysis of the deletion mutants

The core aim of the present work was to study the function of m142 and m143. To analyse the role of m142 and m143 for virus replication, deletion mutants were used to infect non-complementing cells and study viral gene expression in the context of viral infection.

3.2.1. Viral gene expression and replication

CMV replication is a synchronized process governed by viral and host factors, which results in accumulation of viral DNA. Analysis of DNA extracted from infected cells showed that deletion mutants synthesize reduced amounts of DNA at 72 hours postinfection (Fig 18). NIH3T3 cells were infected with mutant viruses and DNA was extracted at 24 and 72 hours postinfection. Total DNA was blotted and probed with DIG labelled M45 fragment. The probe was generated from a pcDNA-M45 construct, cut with EcoRI and XhoI restriction enzymes. The 1.9 kb fragment was purified and labelled as described in Materials and methods. Thus, deletion mutants failed to accumulate viral DNA, indicating that m142 and m143 are both required for successful replication.

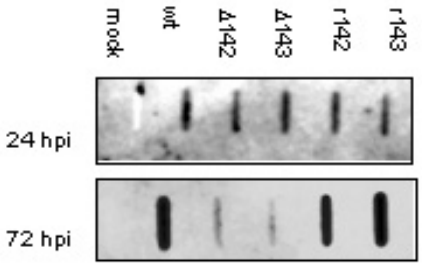


Fig. 18 Viral DNA replication

This result suggested that the replication defect could be a consequence of a block in the gene cascade expression. To investigate this, the expression of viral genes representing different kinetic classes was analysed. Expression of viral proteins was detected at 24 hours postinfection. In addition, samples were harvested also at 72 hours postinfection to show a possible delay of expression by the mutant viruses. The experiment demonstrated that expression of viral proteins with immediate-early, early, or early-late kinetic was not changed dramatically. The corresponding proteins, IE1, E1 and M44, were detected at 24 and 72 hours postinfection in all mutants, although the IE1 was less expressed by the deletion mutants (Fig. 19). In contrast, the late protein gB was not detected at 72 hours postinfection, indicating that m142 and m143 are required for late gene expression. Since earlier studies have reported a transcriptional transactivating function for the US22 gene family members TRS1 and IRS1 (Stasiak and

Mokarski; Romanowski and Shenk, 1997), it was considered that m142 and m143 might have a transactivating function.

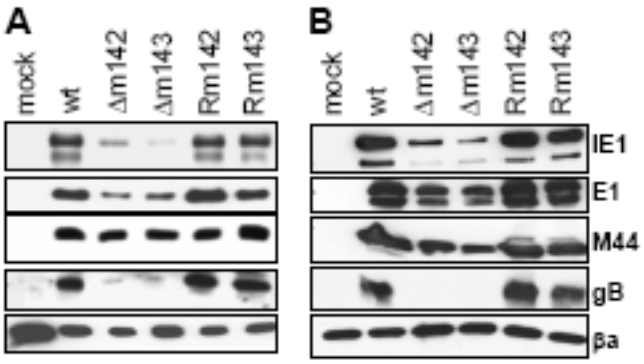


Fig. 19 Viral genes expression at 24 (A) and 72 hpi (B).

Although a more recent report (Hanson et al., 2005) showed that m142 and m143 failed to transactivate genes in transient transfection assays, it was tested whether these genes are required for the late gene transcription in the context of viral infection. Late gene transcripts, coding for the early-late gene M44 and late genes M55(gB) and M100(gM) were evaluated by real-time PCR (data provided by Marcus Picard-Maureau).

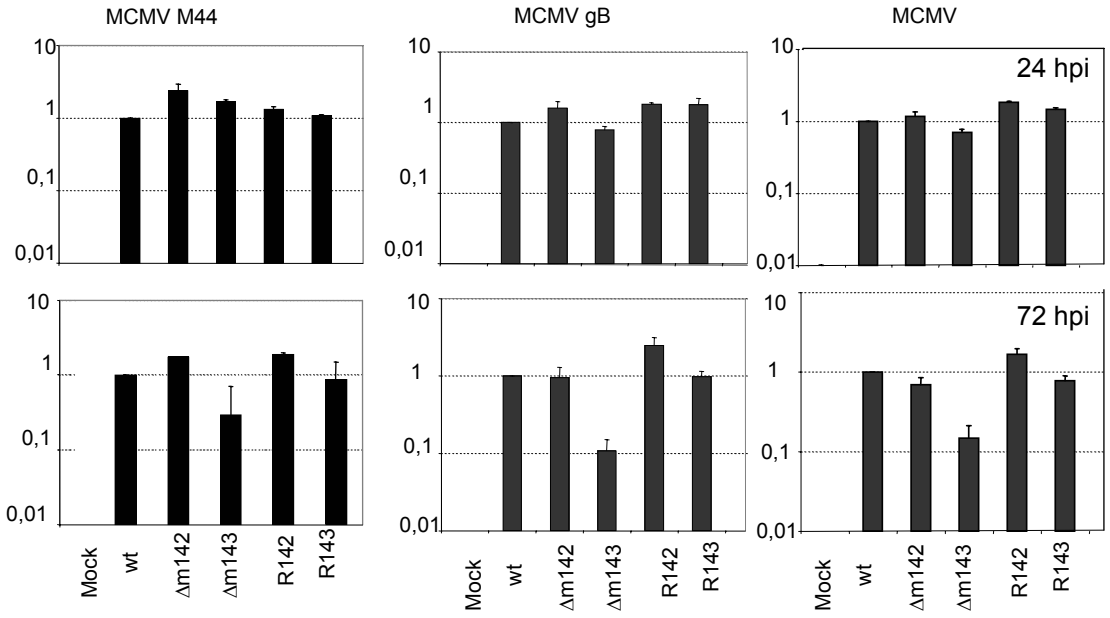


Fig 20. RNA transcript analysis. A. Early late proteins M44. B Late proteins gB and gM. Data kindly provided by Marcus Picard-Maureau.

Analysis of RNA transcripts did not show a significant decrease in RNA levels for gB at 24 and 72 hpi. The only observed difference was for ∆m143, where RNA was 10-fold decreased. The same result was shown for the other transcripts. RNA was analyzed also for the early late gene product M44 and that corresponds to the protein expression pattern, where no difference to the wild-type virus was observed (Fig. 20).

The analyzed data showed that m142 and m143 are not involved in regulation of the late gene transcription. However, the late protein gB was not detected, suggesting that protein synthesis is inhibited at the posttranslational level. This observation was confirmed by an experiment, which demonstrated a global protein synthesis shut down in cells, infected with deletion mutants. The cells were infected with mutant viruses and protein synthesis was analyzed by metabolic labeling with (^{35}S)-methionine and -cysteine. The proteins were separated by SDS-PAGE and visualized by exposing to an X-ray film (Fig 21).

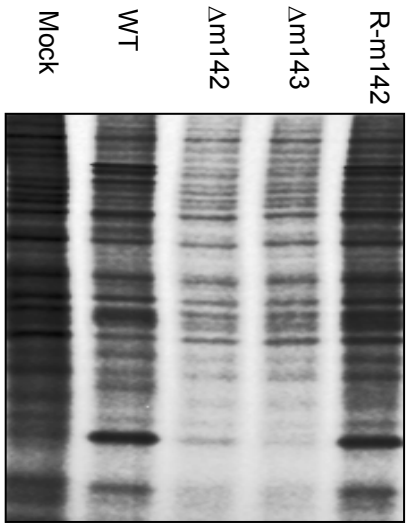


Fig. 21. Protein synthesis shut down. Metabolic labeling of infected cells.

It was clearly visible that deletion mutant-infected cells showed reduced protein synthesis, suggesting that m142 and m143 are required to prevent the global shut down of protein synthesis.

3.2.2. Protein synthesis inhibition

The global protein synthesis shut down is one of the mechanisms that results from the activated antiviral response and leads to limiting the viral infection. It represents the earliest defense of the infected cell, which involves IFNα/β induction (Fig 22). The pathway is triggered by dsRNA, resulting from the virus replication. MCMV is a double stranded DNA virus which carries RNA molecules. Moreover viral gene transcription may result in dsRNA structures, formed by opposing transcripts. Viral dsRNA is a strong inducer of IFNα/β genes and therefore plays an important role in antiviral defense during innate immune response. Double-stranded RNA triggers the expression of IFN-stimulated genes or upregulates and activates effector proteins with antiviral activity. Some viral genes encode proteins that counteract the effector antiviral proteins and prevent consequent translation arrest. Since the RNA analses showed that late transcripts were present but protein synthesis was impaired in cells infected with the deletion mutants, it was suggested that the products of m142 and m143 are involved in preventing the protein synthesis shut down during the antiviral response.

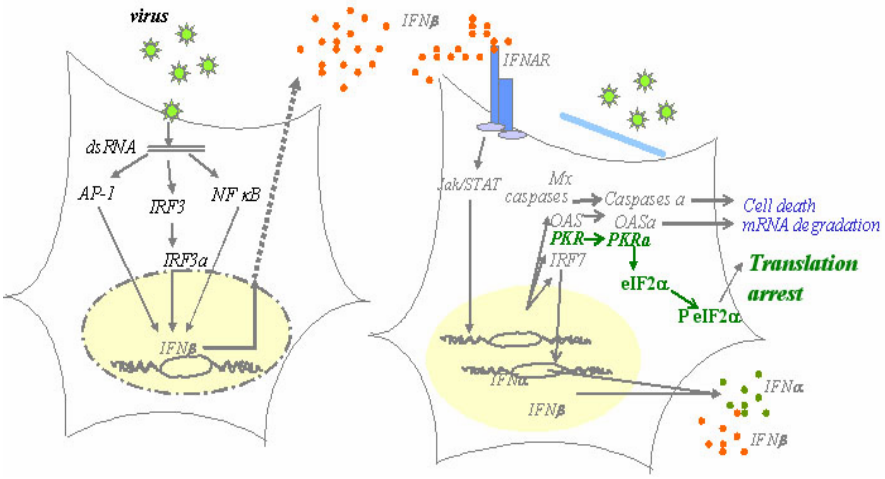


Fig.22. Antiviral response and immune evasion mechanisms.

To test this hypothesis, the pathway leading to translational arrest upon IFN induction was investigated. This signalling involves the cellular protein dsRNA-dependent

proteinase K (PKR), which phosphorylates the α subunit of the translational factor eIF2. The phosphorylated eIF2α is inactive and the protein synthesis is blocked. Western blot analysis of infected cells showed that the PKR and eIF2α are phosphorylated when m142 or m143 were deleted (Fig 23). Western blot analysis of infected cells showed that infection with deletion mutants resuts in a mobility shift of the bands detected with an anti-PKR antibody. Moreover the initiation factor of translation, eIF2α was phosphorylated in cells infected with the deletion mutants (fig 23 A). The appearance of additional bands for PKR was associated with activation, e.g. phosphoralytion since cells infected with deletion mutants showed reduced protein synthesis (Fig 21). This assumption was confirmed by detection of the phosphorylated form of PKR by an anti-phopsho PKR antibody (pT451), shown in figure 23B. Another experiment demonstrated that treatment of NIH3T3 cells with the dsRNA homolog poly I:poly C results in a similar mobility shift of the PKR bands (Fig 23B).

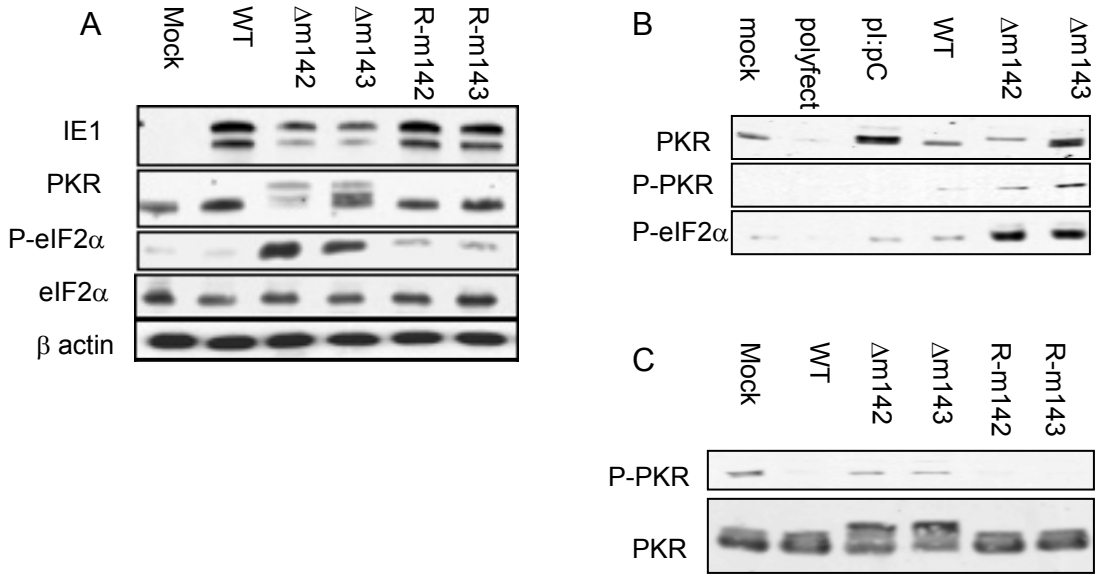


Fig. 23 Phosphorylation of PKR and eIF2α. A. Infection with deletion mutants results in phosphorylation of eIF2α and mobility shift of PR. B. PKR is phosphorylated when m142 or m143 are deleted. C. Detection of phoshop-PKR in cells infected with deletion mutants, compared to the wild type and revertant viruses.

PKR is normally inactive, but is activated by binding to dsRNA. The activation leads to conformational changes and unmasking of a catalytic domain. The active form is a dimer, with two PKR molecules binding one dsRNA molecule. The two PKR molecules phosphorylate one an other at several serines and threonines. Activated PKR phosphorylates the α subunit of the eukaryotic translation initiation factor eIF2 (eIF2α) and prevents recycling of initiation factors (Clemens and Elia, 1997). The eIF2 consists of three subunits (α, β and γ), and is responsible for recruitment of the small ribosomal unit. GTP-bound eIF2 forms a complex with the initiator *Met*-tRNA in the initial step of translation. The complex interacts with mRNA, the large ribosomal subunit and additional initiation factors to form a pre-initiation complex. Subsequently the GTP bound to the eIF2 is hydrolyzed. The GDP-eIF2 (hydrolyzed form) must be exchanged for GTP to be able to participate in next round of translation initiation. The exchange reaction is catalyzed by the guanine exchange factor eIF2B. The binding of phopshorylated eIF2α with eIF2B, is irreversible and the recycling of eIF2 is blocked (Clemens and Elia, 1997). Since the cellular levels of eIF2B are limited, the translation is inhibited.

The described experiments showed that deletion of m142 and m143 results in phopshorylation of the eIF2α and activation of PKR, reinforcing the conclusion that protein synthesis of infected cells with deletion mutants is inhibited as a result of PKR-mediated translational arrest. This suggests that m142 and m143 encode proteins that are necessary to prevent PKR and eIF2α phosphorylation.

3.2.3. Functional homologues of m142 and m143

The MCMV genes m142 and m143 are the closest sequence homologues of the HCMV genes TRS1 and IRS1. This fact was supported by the published data that TRS1 can rescue the replication of the vaccinia virus mutant lacking the E3L gene (Child et al., 2004) and a herpes simplex virus mutant lacking the $\gamma_1$34.5 gene (Cassady, 2005). The products of E3L and $\gamma_1$34.5 have been reported to have role in preventing the PKR mediated protein synthesis shut down. Since the present work showed that m142 and m143 deletion results in PKR mediated protein synthesis inhibition, a possible functional homology of m142 and m143 with the HCMV genes TRS1 and IRS1 was considered.

To test whether TRS1 can substitute for either m143 or m142, several replacement mutants were created. The heterologous gene was inserted at different positions, under control of different promoters (Fig.24).

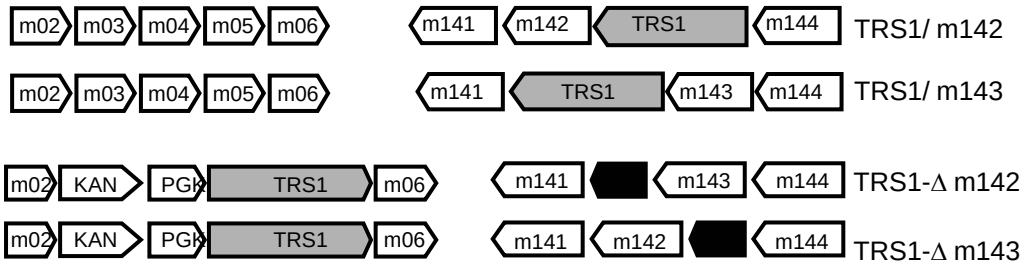


Fig. 24. TRS1 replacement mutants. PGK-phosphoglycerate kinase promoter, KAN-kanamycin cassette, black arrow shows the zeocin resistance gene.

TRS1 was cloned into the pBS o142 and pBS o143 plasmids, which contain homologous sequences to m142 or m143 (see Appendix 1C). The resulting helper vectors are described in table 17. The fragment containing TRS1, flanked by m142 or m143 homologous sequences was inserted at the deletion BAC genome. The recombination resulted in replacing the zeocine cassette with an HA-tagged TRS1, where the expression was controlled by m142 or m143 promoters. The mutated genomes were reconstituted on complementing cells. The derived viruses, were used to infect non-complementing NIH3T3 cells. However, neither of the mutants yielded a replication-competent virus. Since it has been already shown by Hanson and co-workers (2005) that m142 and m143 have relatively weak promoters it was not an unexpected result. Further, it was considered preparing a construct where TRS1 expression is driven by another promoter. For this, a helper plasmid was used, which contains 50bp sequences homologous to the region upstream of m02 and downstream of m06 ORFs, a kanFRT cassette, and a phosphoglycerate kinase (PGK) promoter (Jurak and Brune, 2006). The HA-tagged TRS1 was cloned into the described plasmid and derived a fragment, including PGK promoter, HA tagged TRS1 and m02 – m06 homologous arms and *kan* cassette (Fig 8C). The fragment was excised from the vector and transformed into bacteria carrying Δ142 MCMV GFP BAC or Δ143 MCMV GFP BAC genome.

Recombination resulted in insertion of TRS1 into the MCMV deletion mutant genomes. Construction of the recombinant BAC is presented in table 17.

Recombinant BAC genomes were characterized by EcoRI digestion. The insertion of fragment including the PGK promoter, HA-tagged TRS1 and a kanFRT cassette, resulted in disruption of the 22 kb EcoRI band. Additionally, the insertion mutants have the bands showing the replacement of m142 or m143 ORFs by a zeocin gene (Fig. 10). The verified mutant BAC genomes were transfected into complementing cells and NIH3T3 cells were infected with the derived virus. The correct insertion of TRS1 into the viral genome was confirmed by southern blot (Fig. 15). Total viral DNA extracted from infected cells was digested with EcoRI and probed for m142 or m143. Southern blot showed that the 9.0 kb EcoRI fragment, containing the entire m142 and m143 ORFs is not present, excluding possible contamination with wild type MCMV. Deletion of m142 and m143 was verified by the absence of the corresponding bands. The HCMV gene TRS1 was introduced into the MCMV genome as an HA-tagged gene. Expression of TRS1 in the context of viral infection was shown in western blot. The confocal microscope analysis of NIH3T3 cells infected with MCMV-TRS1 mutants, showed that the protein, encoded by the inserted gene is distributed in the cell predominantly in the cytoplasm (Fig. 25). This corresponds with previously published data on TRS1 in HCMV infected cells (Romanowski and Shenk, 1997).The mutants TRS1-Δm142 and TRS1-Δm143 could grow on non-complementing NIH3T3 cells (Fig 26 A). Although the titers were about 100-fold lower than the wild type, these data suggested that TRS1 can substitute for m142 and m143. This finding was confirmed by complementation of deletion mutant viruses. NIH3T3 cells were transduced with a recombinant retrovirus, containing HA-tagged TRS1.

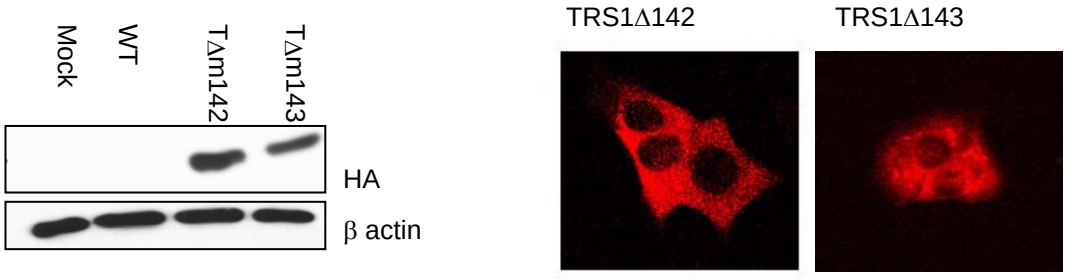


Fig. 25. Characterisation of the TRS1 mutants. The HA tagged TRS1 is expressed in NIH3T3 cells infected with TRS1 mutants. A. Wetsren blot B. Immunofluorescence.

Cells were selected and characterized. The size of the protein was confirmed in western blot. Confocal image analysis showed the expected intracellular distribution. Infection of the complementing cells demonstrated that TRS1 can complement for m142 and m143, when provided in *trans*-position (Fig 26 B). This observation supports the idea that TRS1 is a functional homologue of m142 and m143. Moreover, the insertion of TRS1 into a deletion mutant genome restores the expression of late proteins (Fig 27). Although IE1 and gB have reduced levels compared to the wild-type virus, which is relevant to the lower titers that replacement mutants can reach. This suggested partial functional homology of TRS1 with the MCMV genes m142 and m143.

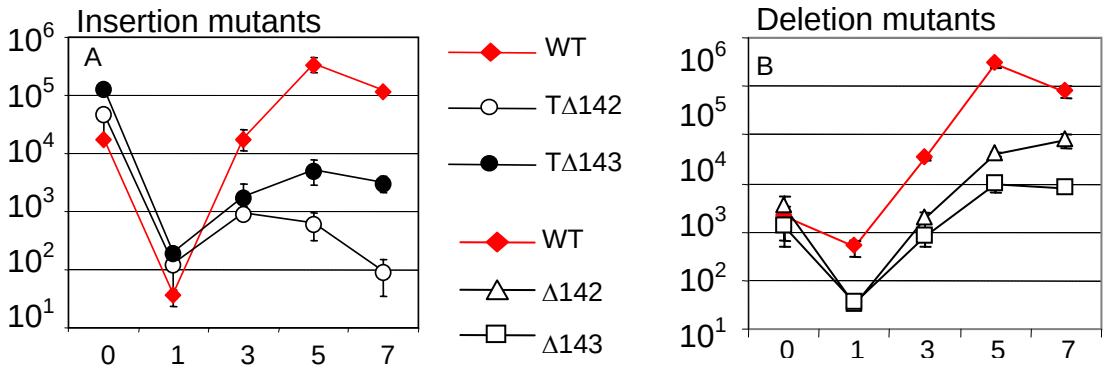


Fig. 26 TRS1 can substitute for m142 and m143. A. Insertion mutants grow on NIH3T3 cells. B. Deletion mutants grow on TRS1 expressing NIH3T3 cells.

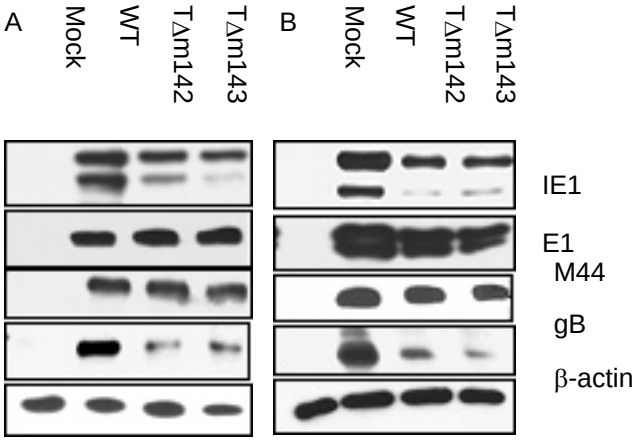


Fig. 27 The late protein expression is restored by insertion of TRS1. Infected NIH3T3 cells were harvested at 24 (A) and 72 hpi (B).

The successful complementation of m142 and m143 with TRS1 suggested that other genes closely related to TRS1 could also substitute for m142 and m143. The most appreciated candidates were the sequence homolog IRS1 and the vaccina virus E3L gene, which was recently shown to be complemented by TRS1 (Child et al, 2004). Several different mutants were created as described above (Fig.28). Mutated BAC genomes were reconstituted on complementing cells and the derived vrius was used to infect NIH3T3 cells.

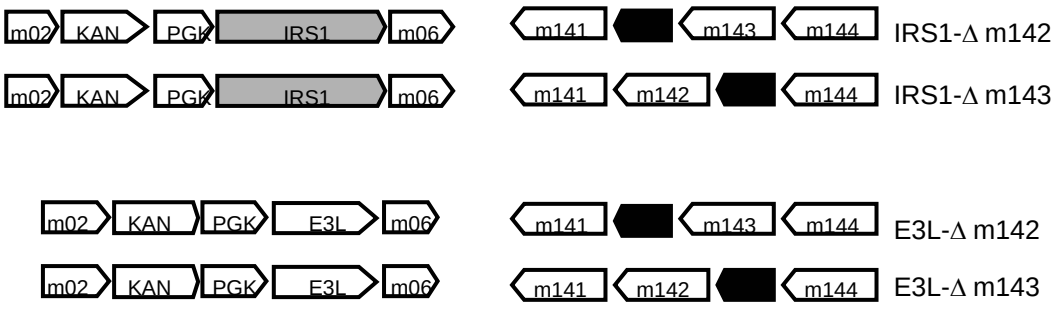


Fig. 28. Other insertion mutants. The HCMV gene IRS1 and vaccinia virus E3L were inserted at the m02-m06 position under control of a PGK promoter.IRS1-Δ m142

However, insertion of IRS1 under control of PGK promoter did not complement the m142 and m143. This suggested that IRS1 is more distant from m142 and m143 than TRS1 in functional respect. The attempt to substitute m142 and m143 with the vaccinia virus gene E3L was unsuccessful even when the gene was expressed from the PGK promoter. In this case, a possible explanation might be the fact that poxviruses are more distant biological species.

3.3. Conclusion

The presented data showed that the MCMV genes m142 and m143 are essential for the virus replication. They are not involved in viral gene transcription, but required for late protein expression. The m142 and m143 proteins were found to prevent PKR and eIF2α phosphorylation during infection, thereby avoiding the protein synthesis shut-down as part of the antiviral response. Comparison of the MCMV genes m142 and m143

with the closely related HCMV gene TRS1 showed partial functional homology. These results demonstrated that m142 and m143 are essential for the MCMV replication because they encode proteins that counteract the host antiviral response.

4. DISCUSSION

4.1. The MCMV genes m142 and m143 are essential US22 gene family members

Cytomegaloviruses devoted a large part of their genome to encode genes, which modulate the host cell behaviour and response to infection. Data from sequencing and functional genomic analyses revealed that about 45 to 57 out of 160 predicted viral genes are dedicated to essential tasks of replication (Mocarski and Courcelle, 2001; Dunn et al., 2003a). This involves a number of herpesvirus-common genes, identified in all mammalian and avian herpesviruses (Davison et al., 2003). The herpesvirus-common genes form seven conserved blocks. Arrangement of the conserved blocks is a typical characteristic of the three herpesvirus subfamilies. The β-herpesviruses form the most divergent subgroup, as they have unique genome organisation. During co-evolution of the virus with its host, duplications of viral genes occurred and resulted in gene families. The β-herpesviruses are distinguished by the presence of additional gene families; one of them is US22 gene family.

The US22 gene family is characterised by the presence of four conserved sequence motifs. It has been speculated that the conserved motifs preserve essential functions for the virus. However, functional analyses have shown that the members of this family have quite diverse functions. Some of them were shown to be transcription activators (Cardin et al., 1995; Flebbe-Rehwaldt et al., 2000; Romanowski and Shenk, 1997), others were found to optimize viral growth in certain cell types (Menard et al., 2003; Cavanagh et al., 1996) or have an antiapoptotic function (Skalletskaya et al., 2001, Menard et al., 2003). Only four members of the family have so far been reported to be essential for the virus replication. These are the HCMV genes TRS1 and IRS1 and m142 and m143 of MCMV. It was previously shown that deletion of both TRS1 and IRS1 results in a replication-deficient virus (Brune et al., unpublished). At the beginning of the project, investigators reported that disruption of m142 and m143 results in a replication-incompetent virus (Menard et al., 2003), but no one had successfully regrown MCMV mutants lacking the genes m142

or m143. This work presents the first data on analysis of MCMV mutants where the genes m142 or m143 were deleted. The mutants were reconstituted on complementing NIH3T3 cells, stably expressing the viral genes m142 or m143. Growth analyses confirmed the expectation that m142 and m143 are essential for virus replication. The defect of the deletion mutants could not be compensated by high MOI infection. However, reinsertion of the corresponding genes into the MCMV genome completely restored the wild-type phenotype. Moreover, from the present experiments it is clear that m142 and m143 are both required for efficient virus replication. An analysis of viral DNA replication showed that the deletion mutant-infected cells have reduced levels of DNA. Only when the missing gene is reinserted and both, m142 and m143 are present, the DNA levels are comparable to the wild-type virus. Additionally, the inability to reconstitute the double deletion mutant on NIH3T3 cells expressing either m142 or m143 confirmed the hypothesis that they cannot compensate for each other and most likely have different roles for MCMV replication.

To investigate further the role of m142 and m143 in the MCMV replication cycle, gene expression pattern was analyzed. Since it was already reported that TRS1 and IRS1 have transcriptional activity in transient transfection assays (Stasiak and Mockarski, 1992), it was assumed that m142 and m143 also might be involved in regulation of gene transcription. It was shown that the viral proteins, expressed at different times in cells infected with deletion mutants are detectable with exception of the late proteins. However RNA analyses revealed that late viral transcript levels are not changed when m142 or m143 are deleted, excluding a role of these proteins in gene transcription. Further characterisation of the proteins in the context of viral infection showed that m142 and m143 are predominantly localized in the cytoplasm. This finding is in agreement with independently obtained data by Hanson and co-workers (2005). Although some of the US22 gene products reported to be transcriptional transactivators were also detected in the cytoplasm (Mori et al., 1998; Romanowski and Shenk, 1997).

4.2. The HCMV genes TRS1/IRS1 and MCMV m142 and m143 encode proteins with homologous functions

Sequence comparison of HCMV ORFs to m142 and m143 showed that the closest sequence homologues are US26 and US23, respectively (Rawlinson et al., 1996). Both genes were found to influence the virus growth, as the deletion of US26 or US23 results in attenuated phenotype (Dunn et al., 2003; Yu et al., 2003). However neither of them is essential. Screening of the MCMV genome for homologues to IRS1 and TRS1 showed that m142 and m143 are the closest. Additionally, TRS1 and IRS1 as m142 and m143 are the only members of the family which lack the conserved sequence motif II. It was previously shown that deletion of both TRS1 and IRS1 results in a growth-deficient virus (Brune et al., unpublished). Moreover, the present study demonstrated that TRS1 can substitute for m142 and m143, when provided in *cis*- or *trans*-position. The deletion mutants could be complemented by TRS1, expressed from NIH3T3 cells. As a confirmation, it was shown that insertion of TRS1 into the MCMV genome, lacking m142 or m143 resulted in a replication-competent virus.

The IRS1 gene, which has 75% sequence homology with TRS1 and share sequence from both repeated and unique segments of the genome, was also tested for complementation. The N-terminal two thirds of pTRS1 is encoded in the c repeat region, and the remainder of the protein is coded within the unique short region. The related protein, pIRS1, is encoded in the internal c‘ region together with the adjacent unique short region. Consequently, the N-terminal domains of pTRS1 and pIRS1 are nearly identical, and the two proteins have different C-terminal domains (Wetso and Barrell, 1986). However, insertion of IRS1 into the MCMV lacking m142 or m143 did not yield a detectable virus. This result correlates with the reported data that deletion of IRS1 has no impact on HCMV replication (Blankenship and Shenk, 2002; Jones and Muzithras, 1992), whereas deletion of TRS1 results in attenuated growth (Blankenship and Shenk, 2002). The defect of the HCMV∆TRS1 mutant could be due to a viral assembly failure (Adamo et al., 2004). The accumulation of viral transcripts in infected cells with the TRS1 mutant is comparable to those found in the nonattenuated IRS1 mutant (Blankenship and Shenk, 2002). The MCMV genes m142 and m143 are both essential for virus replication, whereas TRS1 and IRS1 are essential only when deleted together. The present data showed that only TRS1 can

rescue the replication of m142 and m143. Moreover the expression of late proteins was partially restored by reinsertion of TRS1 into the MCMV deletion mutants, suggesting a functional homology.

Recently it was reported that TRS1 and IRS1 can rescue the replication of a vaccinia virus lacking the ds-RNA-binding protein E3L (Child et al., 2002, 2004). Later, Hakki and Geballe (2005) proved that TRS1/IRS1 have dsRNA binding activity. Other investigators have reported that TRS1 and IRS1 can restore the dsRNA-dependent-proteinkinase R (PKR) mediated protein synthesis shutoff, induced by a recombinant herpes simplex virus type 1 (HSV-1) lacking the $\gamma_1$34.5 gene (Cassady 2005). The finding that TRS1 is a functional homolog to m142 and m143, suggested that the MCMV genes are also involved in prevention of protein synthesis shutoff. This possibility was analysed by metabolic labelling of infected cells which showed that deletion of m142 or m143 results in reduced protein synthesis. This result led to the conclusion that m142 and m143 encode proteins which prevent the shut-down of protein synthesis.

4.3. Protein synthesis inhibition by the host cell

The protein synthesis shutoff is one of the mechanisms employed by the host cell for limiting viral replication. It is triggered by dsRNA. Virus replication of mammalian cells involves the following events, which start with entry, continues with RNA expression and processing, polypeptide synthesis and modification, genome replication, and maturation. As intracellular parasites, viruses are dependent on the cellular machinery and resource to complete their life cycle. The dsRNA structures are formed as annealed bi-directional overlapping transcripts from DNA viruses such as herpesviruses (Schneider and Mohr, 2003). Additionally, short interfering RNAs and micro RNAs are processed from longer dsRNAs or RNAs with hairpins, which can mimic dsRNA and contribute to the antiviral response (Li and Ding. 2005; Matzke et al., 2002).

Double-stranded dsRNA is a strong inducer of interferon beta (IFNβ), which represents the first defence line of infected cells in response to viral infection. The induction of IFNβ is regulated at the level of transcription initiation. The cytomegalovirus virion is recognized by Toll-like receptors, and the following signal transduction results in activation of IFNβ genes. Produced IFNβ stimulates the

neighbouring cells by activating type I IFN receptors. This triggers activation of the Jak/STAT pathway, which induces the expression of a broad spectrum of cellular genes, named interferon-stimulated genes (ISGs). These genes encode proteins that are constitutively expressed at low levels but are upregulated in presence of dsRNA. ISGs encode proteins with antiviral activities, one of the best characterized is the dsRNA-dependent protein kinase R (PKR).

PKR is a major player in the innate antiviral immune response. It is a multifunctional protein that also regulates apoptosis, cell proliferation, signal transduction, and differentiation (Proud, 1995). Overexpression of PKR has been suggested to inhibit cell proliferation in yeast, insect, and mammalian cells (Chong et al., 1992). In contrast, expression of catalytically inactive mutants of PKR in NIH3T3 cells results in tumorigenicity in nude mice, which is attributed to a dominant-negative effect of mutant PKR (Koromilas et al.,1992). Mouse embryo fibroblasts derived from PKR-deficient mice are resistant to cell death induced by dsRNA and lipopolysaccharides (Der et al., 1997). PKR has been suggested to be involved in dsRNA transduction pathways leading to NF-κB activation and the p38 mitogen-activated kinase pathway (MAPK) (Goh et al., 2000). In PKR deficient, but not wild-type cells, dsRNA fails to induce NF-κB activation, which correlates with the lack of interferon-β production.

PKR is one of the key factors in the innate immune response. It is subject to a fine regulation. PKR activity is positively regulated by a cellular protein, named PKR-activating protein (PACT)/RAX (Patel and Sen, 1998). This protein was identified as a PKR interacting protein, which activates PKR in absence of dsRNA. PACT is expressed under stress conditions, treatment of cells with arsenite, and interleukine-3 deprivation. This results in phosphorylation of PACT/RAX and association with PKR, which is followed by PKR activation and phosphorylation of eIF2α. PKR is also subject to a negative regulation by P58. P58 was initially identified as an influenza virus-activated protein that interacts with the kinase domain of PKR and inhibits its activity. In normal cells, the P58 associates with a heat shock protein 40 and forms an inhibitory complex. Cellular stress or virus infection induces dissociation of P58 from heat shock protein 40. Therefore, the released P58 can bind PKR and disrupt its activity. Overexpression of P58 reduces eIF2α phopshorylation, mediated by the PKR-like endoplasmatic reticulum kinase (PERK) in mouse embryonic stem cells. In general PKR and PERK are activated in response to different stimuli. However, a

crosslink exists between the PKR and PERK pathways (Baltzis et al., 2004). In response to vesicular stomatitis virus infection, phosphorylation of PKR is reduced in PERK deficient MEFs as compared to the wild type cells and the virus replicates efficiently. Chemical induction results of PKR phosphorylation in PERK expressing MEFs but not in PERK-negative, showing that antiviral action of PERK is mediated by PKR. The mechanism of PKR activation by PERK is unknown. Activated PKR phosphorylates the translation factor eIF2α, which is responsible for recruitment of the small ribosomal subunit during the initial step of protein synthesis. The phosphorylated eIF2α cannot be utilized anymore, and therefore protein synthesis is inhibited. Translational arrest is an important immune evasion mechanism. To date only two genes from human cytomegalovirus have been reported to prevent the PKR-mediated protein synthesis shutoff: TRS1 and IRS1. Since the experiments described in this work have shown that TRS1 can restore late protein synthesis of the MCMV mutants lacking the genes m142 or m143, it was obvious to assume that the genes have a similar function. Analysis of infected cells by western blot showed that eIF2α is phosphorylated when m142 or m143 are deleted. Moreover detection of PKR in deletion-mutant-infected cells resulted in additional bands with shifted mobility. The obtained data contributed to the conclusion that m142 and m143 are required to prevent the PKR mediated protein synthesis shut-down. The same result was observed when cells were treated with the dsRNA homolog poly I:poly C. However, whether activation of PKR is due to direct effect of deleted genes or a consequence and how PERK is involved is currently unknown.

This is the first report on US22 gene family members of MCMV involved in preventing the PKR-mediated translational arrest. These results correlate with unpublished data from Child and co-workers (2006), that m142 and m143 have dsRNA binding activity, providing knowledge about the mechanism of action. As it was already demonstrated in this study, the MCMV genes m142 and m143 are both required for virus replication, it is speculated that they prevent PKR activation in a cooperative manner. Up to now the exact mechanism remains unknown. It is possible that at least one of the proteins has a dsRNA-binding domain, capable of sequestering the dsRNA and preventing PKR from being activated. Alternatively, the other protein might prevent autophosphorylation of PKR by direct binding to the effector protein or suppressing the upregulation by unknown mechanism, involving virus or cellular transcription factors. In this respect the analysis of interaction

partners of m142 and m143 should be of great interest for future studies. The potential interaction partners could be viral or cellular proteins.

The prevention of PKR-mediated protein synthesis shut-down was demonstrated in this work for m142 and m143, which correlated with previously reported results about TRS1. However, the complementation of the deletion mutants with TRS1 was only partial, suggesting that these proteins have a multifunctional nature and not all functions are shared. For instance, the TRS1 and IRS1 proteins, unlike m142 and m143 function as transcriptional transactivators in transient transfection assays (Romanowski and Shenk.,1997; Stasiak and Mocarski,1992). Other investigations showed that TRS1 but not IRS1 is required for efficient virion assembly (Adamo et al., 2004). Up to now it is not known if m142 or m143 are involved in this process.

Considering the multiple function of TRS1, it may appear surprising that it could complement for m142 and m143 only partially. The insertion of TRS1 into MCMV deletion mutants resulted in replication competent virus only when the HCMV gene was expressed from the heterologous PGK promoter. This finding may be explained by the fact that m142 and m143 have unusually weak promoters (Hanson et al., 2005). Thus, the observed partial functional homology of TRS1 with m142 and m143 is most likely due to conservation of herpesvirus common genes and differences arising from the adaptation of the specific virus to its host.

4.4. Evasion of the host antiviral response

PKR activation and subsequent translational inhibition is part of the antiviral response. To establish productive infection, viruses have evolved mechanisms to overcome the deleterious effect of PKR. The inhibition of PKR pathway is immune evasion mechanism, exploited by many viruses. The herpes simplex virus $\gamma_1$34.5 protein directs the cellular protein phosphatase 1 to dephosphorylate eIF2α, whereas poliovirus employs a cellular proteinase to degrade PKR. In addition, viruses employ counter measures to inhibit interferon production or signal transduction initiated by interferons. Recent studies have shown that cytomegalovirus can perturb the PERK pathway (Isler et al., 2005, Tirosh et al., 2005). The phosphorylation of PERK was detected at late times during infection. However, eIF2α was phopshorylated only to a limited extent and translation of proteins was not impaired, suggesting that the virus

affects the downstream eIF2α signalling. Currently no CMV gene product has been identified as an inhibitor of PERK activation.

A number of viruses encode dsRNA binding proteins. For example, the NS1 protein from Influenza virus (Tan and Katze, 1998) and E3L from vaccinia virus (Sharp et al., 1998) bind directly to PKR and inhibit its function. Although the influenza virus NS1 protein is critical for its ability to overcome the IFN response, it was also reported to induce the activation the cellular inhibitor of PKR, P58 (Lee et al., 1990, 1992; Melville et al., 1997). NS1 also regulates the nuclear export of cellular mRNAs (Fortes et al., 1994) and affects pre-mRNA maturation by inhibiting splicing and polyadenylation-site cleavage (Chen et al., 2002). Another virus that regulates the activity of PKR is vaccinia virus. Vaccinia encodes two genes, E3L and K3L, both of which determine resistance to interferon (Langland and Jacobs, 2002). The E3L protein, synthesized early during infection, contains an amino-terminal Z-DNA-binding domain and a carboxyl-terminal domain with a dsRNA binding motif (Chang et al., 1995). The carboxyl-terminal domain of E3L sequesters the dsRNA and prevents the activation of PKR and phopshorylation of eIF2α. E3L deletion mutant is highly sensitive to interferon, and the gene is essential for the virus growth in mice (Brandt and Jakobs, 2001). The K3L protein has homology to eIF2α and acts as a pseudo substrate for PKR in competition with eIF2α. Both proteins act together to prevent the phosphorylation of eIF2α and block the host cell protein synthesis shutoff.

E3L was reported to have functional homology with the HCMV genes TRS1 and IRS1. Each of these genes could rescue the replication of vaccinia virus lacking E3L (Child et al., 2004). Other studies have shown that E3L and TRS1 can prevent the phosohorylation of eIF2α (Child et al., 2004, Cassady, 2005). Since the present work demonstrated that TRS1 can substitute for m142 and m143, it was expected that the vaccinia virus E3L gene product could complement the deletion mutants as well. However the insertion of E3L into the MCMV deletion mutant genome did not result in a replication competent virus. The expression of E3L under control of PGK promoter could not rescue the virus replication as well. The inability of the vaccinia virus gene to substitute for the MCMV genes could be due to the different nature of poxviruses and that E3L and m142/m143 are more distant than TRS1/IRS1. However, the MCMV genes m142 and m143 could rescue the replication of a mutant vaccinia virus, lacking the E3L gene (Hanson et al., 2006).

4.5. New insights into the MCMV immune evasion mechanisms

Immune evasion mechanisms play a crucial role in virus replication. The innate immune response is the first defence line that the virus has to overcome in order to complete successfully its replication and assure efficient transmission to a new host. CMVs have developed various strategies to modulate the environment in the host and facilitate efficient infection. The impacts of immunomodulatory functions, that counteract the host immune response, become better understood because many herpesviruses, adenoviruses and poxviruses encode similar functions. Human and murine cytomegalovirus-encoded proteins modulate many categories of host defence and inflammation: classical as well as non-classical major histocompatibility complex (MHC) protein function, leukocyte migration, activation and cytokine responses, host cell susceptibility to apoptosis, induction and activity of cytokines and interferons, and antibody defence mechanisms (reviewed in Mocarski, 2004).

The present study reports that the MCMV genes m142 and m143 are both required to prevent the PKR-mediated protein synthesis shut-down. This represents an important mechanism for MCMV to escape the innate immune response and executes the essential task for m142 and m143. This is the first data presenting the importance of the antiviral protein PKR for murine cytomegalovirus replication. All US22 gene family members studied up to now are involved in the innate or adaptive immune response. The present work extends the existing knowledge and provides new insights into MCMV immune evasion and survival strategies. Table 18 summarizes the current data about innate immune evasion genes encoded by CMVs and the new knowledge obtained from the presented study (Table 18).

Immunity type	***Immune response***	***HCMV***	***MCMV***
Innate	***IFN induction***	*-*	*-*
	IFN signalling	*UL27, IE1*	*M27*
	IFN effectors (PKR)	*TRS1, IRS1*	***m142, m143***
	NK cells	*UL16, UL40, UL18*	*m144,m152, m155, m157*
	Cytokine	*UL111a, UL146, US28, UL21.5, UL128, UL147, UL33, US27*	*m131-m129*

Table. 18. Immune evasion genes encoded by HCMV and MCMV. The genes indicated in bold are discussed in the text, the others are reviewed in Mocarski, 2002.

In the light of previous data, obtained by other investigators, the results of the present work were used to generate a model. The proposed model suggests that the MCMV genes m142 and m143 and TRS1/IRS1 of HCMV, can bind dsRNA and thereby prevent the autophopshorylation of PKR and subsequent inhibition of protein synthesis. As discussed above, the same was shown by other investigators for the vaccinia virus protein E3L. Moreover, vaccinia virus possesses an additional gene K3L, which binds to PKR and prevents its dimerization, which would result in eIF2α activation and subsequent protein synthesis inhibition. Although E3L failed to substitute for m142 and m143, it is possible that m142 or m143 act in a similar manner. As it was shown by Child and co-workers (unpublished), both proteins m142 and m143 are required for dsRNA binding. The present data demonstrates that each of them is necessary to prevent PKR and eIF2α phopshorylation. This suggests that either m142 or m143 may cooperate with PKR to prevent its dimerization and subsequent phosphorylation of eIF2α. The other protein might be necessary to sequester dsRNA and block the induction of IFNβ. To date, the effect of m142 and m143 on IFNβ induction has not been analysed.

In this respect, testing for possible interaction partners of m142 and m143, especially PKR is a promising research perspective. Alternatively, it is possible that either m142 or m143 cooperate with factors regulating the PKR activity: P58, heat shock protein 40 (HSP40) or PACT. This would explain a potential indirect effect of m142 and/or m143 on activation of PKR. The protein P58 forms a complex with HSP40, but is released upon stress or infection. The released P58 can bind to PKR and thereby inhibit its activation. It was shown that influenza virus can activate P58 (Lee et al., 1990, 1992). Whether m142 or m143 have similar function remains to be determined. PACT is phosphorylated upon infection, which results in association with PKR and subsequent activation of the enzyme. Eventual binding of virus-encoded proteins to PACT would prevent the activation of PKR. Thus, analysis of possible interaction of m142 or m143 with PACT would contribute to understanding the mechanism of immune evasion. The present work demonstrated that the MCMV genes m142 and m143 are not involved in regulation of viral gene transcription. However, it is unclear whether they can influence the transcription of the cellular genes in the context of viral infection. As it was shown in the described experiments, deletion of m142 and m143 results in activation of PKR. It is known that PKR is an effector protein, which expression levels are regulated by activation of ISGs. The

activation of PKR is associated with upregulation of the cellular level. Therefore it is considered that m142 or m143 can suppress the upregulation of the PKR expression by interacting with transcriptional factors, most likely virus-encoded. Potential candidate is the MCMV gene product IE1, which is known to have regulatory function (Mocarski et al., 1996, Paulus et al., 2006). Further, analysis of the conserved domains responsible for binding with certain cellular or virus-encoded factors would reveal the underlying mechanism of immune evasion.

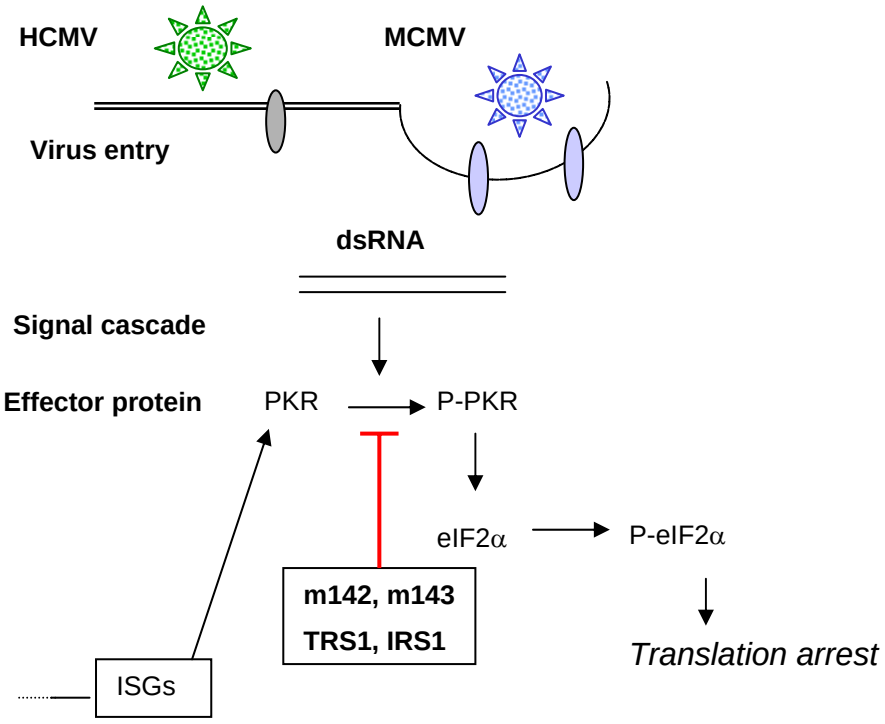


Fig. 29. Proposed model for new immune evasion mechanism of CMV. The HCMV genes TRS1 and IRS1 as well as the MCMV genes m142 and m143 bind the dsRNA to prevent the activation PKR. The products of m142 and m143 interact directly to prevent the activation of PKR. It is considered also indirect effect of m142 and m143 on PKR activation. These genes may interact with the factors, regulating the PKR activity. PACT is a positive regulator and P58 can inhibit the PKR activation after its dissociation from the heat shock protein 40 (HSP40).

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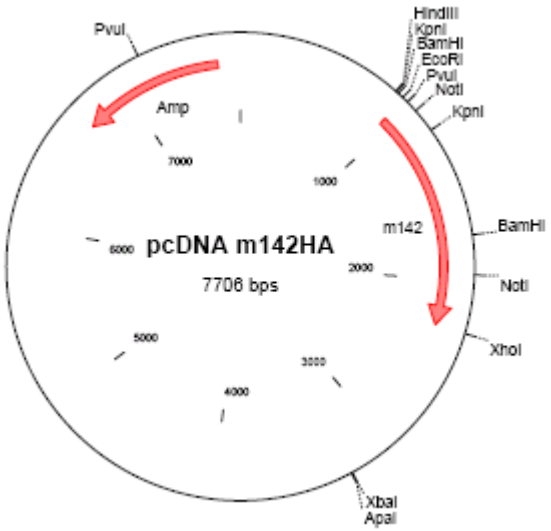
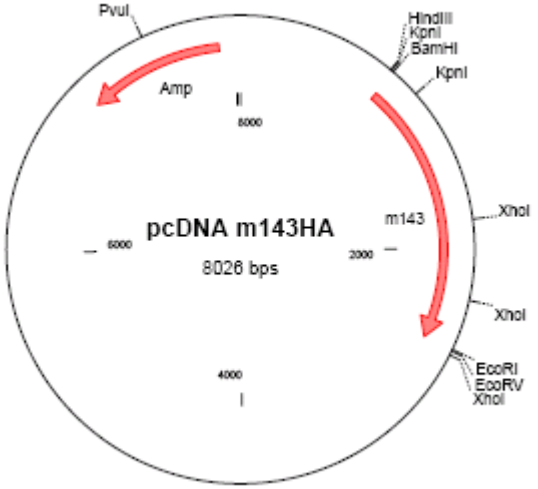
6.1. Abbreviations

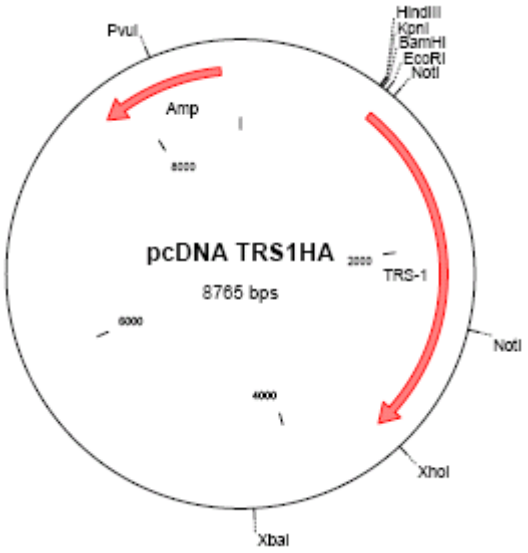
α-	Anti - (indicating antibody against a protein)
μ-	Mikro - (μg, μL)
Δ-	Delta - (indicating deleted sequence)
Alexa Fluor 488/594	Antibody conjugated fluorescent dye emitting at 488/594 nm
AA/BA	Acrylamide/ bisacrylamide
bp	Base pairs
BAC	Bacterial artificial chromosome
CPE	Cytopathic effect
DIG High Prime	Digogexigenin labeling kit
ECL	Enhanced chemiluminiscence
E.Coli	Echerichia Coli
GFP	Green fluorescent protein
gB	Glycoprotein B
HA-tag	Hemaglutinin tag
HRP	Horse raddish peroxidase
HCMV	Human cytomegalovirus
IF	Immunofluorescence
INF	Interferon
ORF	Open reading frame
kD	Kilo Daltons
LC	Light Cycler
MCMV	Murine cytomegalovirus
MOI	Multiplicity of infection
MMLV	Moloney Murine Leukemia virus
Nt	Nucleotides
PAAGE	Polyacril amide gel electrophoresis
PAAG	Polyacrylamide gel
P_{PGK}	Phosphoglukokinase promoter
PCR	Polymerase chain reaction
RT PCR	Real time PCR
SAP	Shrimp alkaline phosphatase
SB	Southern blot
WB	Western blot

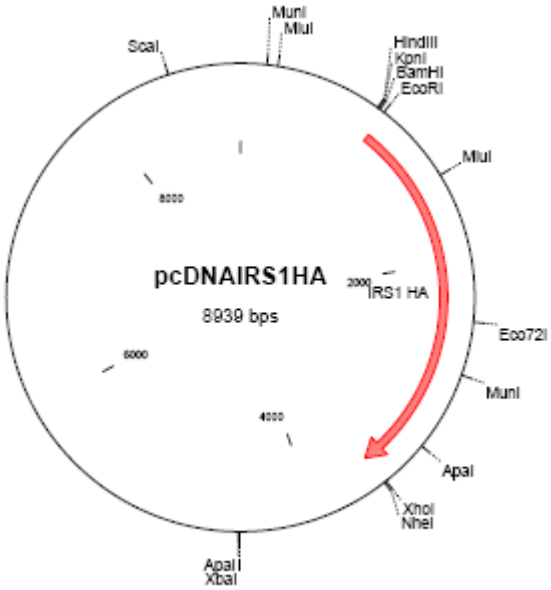
6.4. Appendix 1. Maps of constructed plasmids

A. Expression plasmids

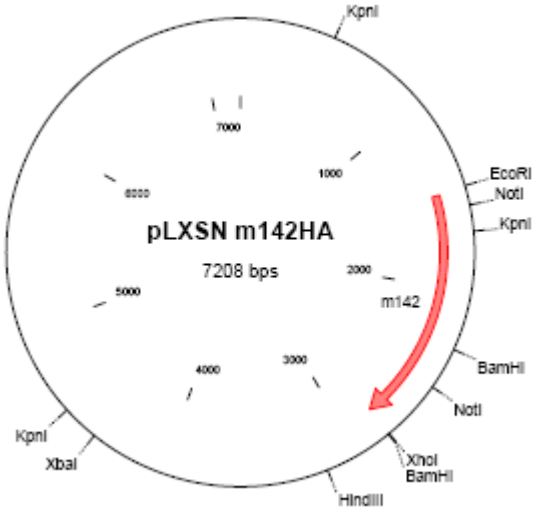


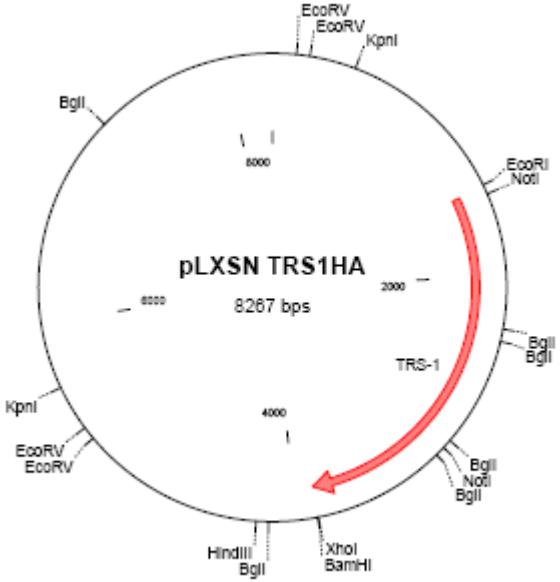




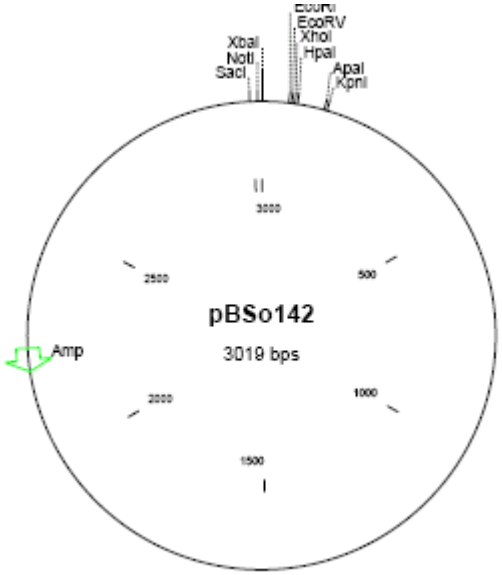


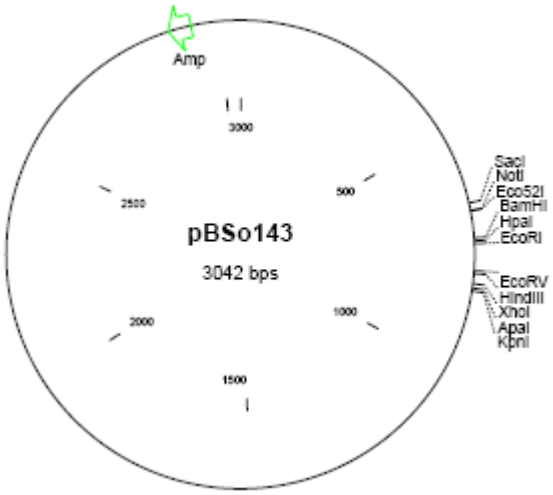
B. Retroviral vectors

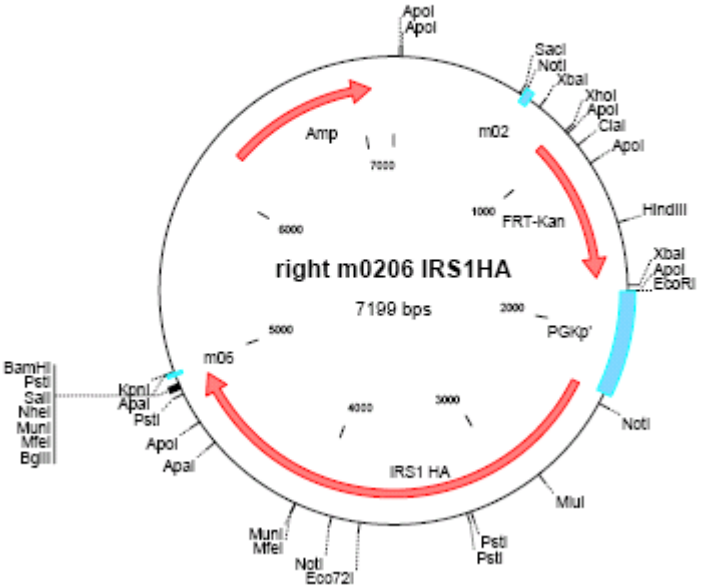


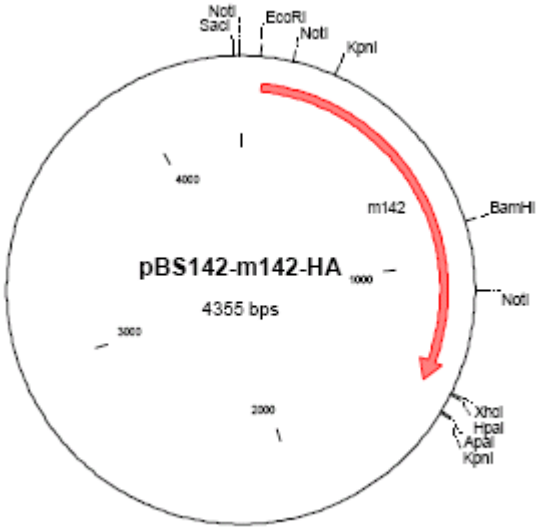


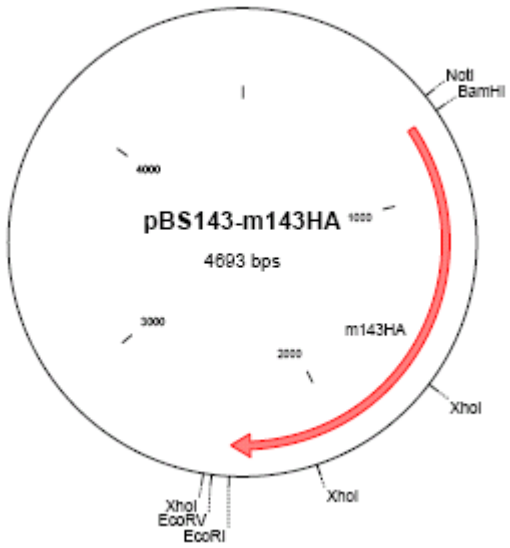
C. Replacement vectors

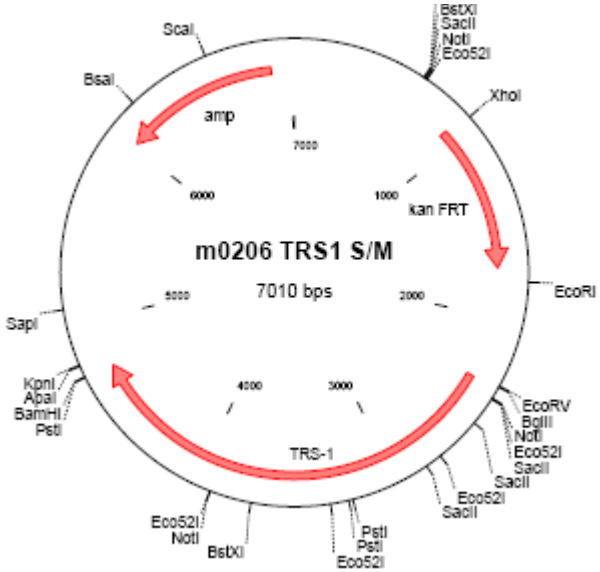












Appendix 2

ORF	*Position (bp)*	*Published sequence*	*Working sequence*	*Effect*
m143	201 403	C – C	CGC	13 AA[1] shorter protein
TRS1	227 911	A	C	Silent
	228 247	T	C	Gly – Ser
	228 437	A	G	Val – Leu
	226 119	G	C	
	226 388	G	A	
	228 437	C	T	Silent
	228 247	G	A	Gly – Ser
	227 911	G	T	Val – Leu
IRS1	189 806	T	C	Silent
	189 887	A	C	Silent
	189 996	A	G	Gly - Ser

Identified mismatches. Sequences were aligned against HCMV genome lab strain AD169, accession number gi 59591 or MCMV Smith strain u68299.

6.5. Statement / Erklärungen

Hiermit erkläre ich ehrenwörtlich, dass ich die Dissertation „Functional analysis of the murine cytomegalovirus genes m142 and m143“ selbständig angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

Zudem erkläre ich, dass diese Dissertation weder in gleicher noch in anderer Form bereits in einem Prüfungsverfahren vorgelegen hat.

Ich habe früher außer den mit dem Zulassungsgesuch urkundlich vorgelegten Graden keine weiteren akademischen Grade erworben oder zu erwerben versucht.

Würzburg den,

Ralitsa Stamatova Valchanova

6.6. Curriculum vitae

Personal data

Ralitsa Stamatova Valchanova

Born on 27th January, 1979 in Varna, Bulgaria

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Education

2002 - 2006 PhD student
Faculty of Biology, University of Wuerzburg
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2001 Degree Master of Science
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1996-2001 Undergraduate student of Molecular Biology
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6.7. Publication list

Ralitsa Valchanova, Marcus Picard-Maureau, and Wolfram Brune. 2006. Murine cytomegalovirus m142 and m143 are both required to block protein 2 kinase R-mediated shut-down of protein synthesis. *Submitted to Journal of Virology*.

Poster presentations:

Ralitsa Valchanova, Marcus Picard-Maureau, Wolfram Brune. 2006. Mouse *Cytomegalovirus genes m142 and m143 counteract the PKR mediated host immune response*. (Poster presentation at Young Investigator Symposium on Infection**,** Biology, Wuerzburg – Berlin, Berlin, March 2006).

Ralitsa Valchanova, Wolfram Brune. 2005. *Functional analysis of murine cytomegalovirus genes m142 and m143.* (oral presentation at the meeting of Graduate colleague 'Target proteins' RVZ Wuerzburg, Germany).

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